Eur J Nutr (2014) 53:897–906
DOI 10.1007/s00394-013-0593-8

ORIGINAL CONTRIBUTION

Molecular pathology of acute kidney injury in a choline-deficient model and fish oil protective effect

Valeria Denninghoff · Georgina Ossani · Ana Uceda · Matias Rugnone · Elmer Fernández · Cristóbal Fresno · German González · Maria Luisa Díaz · Alejandra Avagnina · Boris Elsner · Alberto Monserrat

Received: 9 November 2012 / Accepted: 30 September 2013 / Published online: 16 October 2013

Abstract
Purpose The aim of this work was to investigate the potential protective effects of fish oil on the basis of kidney transcriptomic data on a nutritional experimental model.
Methods Male weanling Wistar rats were divided into four groups and fed choline-deficient (CD) and choline-supplemented (CS) diets with vegetable oil (VO) and menhaden oil (MO): CSVO, CDVO, CSMO and CDMO. Animals were killed after receiving the diets for 6 days. Total RNA was purified from the right kidney and hybridized to Affymetrix GeneChip Rat Gene 1.0 ST Array. Differentially expressed genes were analyzed.
Results All CSVO, CSMO and CDMO rats showed no renal alterations, while all CDVO rats showed renal cortical necrosis. A thorough analysis of the differential expression between groups CSMO and CDMO was carried out. There were no differential genes for $p < 0.01$. The analysis of the differential expression between groups CSVO and CSMO revealed 32 genes, 11 were over-expressed and 21 were under-expressed in CSMO rats.
Conclusions This work was part of a large set of experiments and was used in a hypothesis-generating manner. The comprehensive analysis of genetic expression allowed confirming that menhaden oil has a protective effect on this nutritional experimental model and identifying 32 genes that could be responsible for that protection, including Gstp1. These results reveal that gene changes could play a role in renal injury.

Keywords Acute kidney injury · Choline-deficient · Gene expression · Menhaden oil · Protective effect

Valeria Denninghoff, Elmer Fernández and Alberto Monserrat: Member of the Research Career at the National Scientific and Technical Research Council (CONICET), Argentina.

Electronic supplementary material The online version of this article (doi:10.1007/s00394-013-0593-8) contains supplementary material, which is available to authorized users.

V. Denninghoff (✉) · G. Ossani · A. Uceda · A. Monserrat
Departamento de Patología, Facultad de Medicina, Centro de Patología Experimental y Aplicada (CPEA), Universidad de Buenos Aires, J. E. Uriburu 950, 5° piso, (C1114AAD), Ciudad Autónoma de Buenos Aires, Argentina
e-mail: vdenninghoff@cemic.edu.ar

V. Denninghoff · A. Avagnina · B. Elsner
Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno" (CEMIC), Ciudad Autónoma de Buenos Aires, Argentina

M. Rugnone
Laboratorio de Genómica Comparativa del Desarrollo Vegetal, Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas (IIB-BA, CONICET), Buenos Aires, Argentina

E. Fernández · C. Fresno · G. González
Facultad de Ingeniería, Universidad Católica de Córdoba, Córdoba, Argentina

M. L. Díaz
Medicina Nuclear, Laboratorio de Vitaminas, Hospital Británico, Ciudad Autónoma de Buenos Aires, Argentina

Introduction

Choline is a quaternary amine involved in multiple metabolic pathways, which modulates the basic signaling processes within the cell. It is a structural element in membranes and is vital during critical periods in brain development. Choline is widely distributed in food; the requirement varies according to individual variations and to complex interactions between choline, methionine, folic acid and vitamin B_{12} [1]. Although dietary intakes for humans have been established, there is now evidence that current choline

recommendations may be suboptimal for a large percentage of the population [2], mainly for those more vulnerable such as infants or pregnant and lactating women [3]. We have observed that weanling male rats fed a choline-deficient diet with vegetable oils (VOs) as lipids develop acute kidney injury (AKI) with morphological alterations ranging from focal tubular necrosis to massive cortical necrosis, and most of them die of AKI [4, 5]. Ischemia and primary tubular damage have been proposed as cellular and tissue physiological impairments leading to necrosis. Local intravascular coagulation has been suggested as the link between tubular and cortical necrosis [5]. The possible pathogenic role of changes in renal lipids has been repeatedly studied in this experimental model, without clear evidence of a correlation between a particular lipid change and renal histology [6–8]. It is known that the quantity and quality of dietary lipids can modulate renal lesions in rats fed a choline-deficient diet. Coconut oil (rich in saturated fatty acids) has a protective effect that would be associated with its content of myristic acid. Fish oil (menhaden oil: MO) is rich in eicosapentaenoic (20:5), docosahexaenoic (22:6) and myristic (14:0) acids. These acids may influence renal fatty acid composition and arachidonic acid metabolism, which plays a key role in renal physiopathology [9–12]. The aim of this work was to investigate the potential protective effects of fish oil on the basis of kidney transcriptomic data on a nutritional experimental model.

Methods

Since female rats are known to be more resistant than male rats to AKI [13], we used 21-day-old weanling male Wistar rats from the Animal Facility at the Center for Experimental and Applied Pathology. Twenty-four animals were divided into four groups and fed the following diets: (1) choline-supplemented diet with VO (corn and hydrogenated oils) as lipids (CSVO); (2) choline-deficient diet with VO as lipids (CDVO); (3) choline-supplemented diet with MO as lipid (CSMO); and (4) choline-deficient diet with MO as lipid (CDMO), as shown in Table 1. In each group, mean body weights at weanling (standard deviation) were as follows: CDVO = 45.65 (3.73); CSVO = 47.48 (3.72); CDFO = 47.88 (5.26); CSFO = 49.33 (8.33). There were no significant differences among groups. The study was approved by the University of Buenos Aires School of Medicine ethics committee. Authors have adhered to appropriate NIH Guide for the Care and Use of Laboratory Animals [14]. Twenty-four animals were sacrificed after receiving the experimental diets for 6 days. Serum was obtained for biochemical markers analysis. The right kidney was cryopreserved for microarray analysis. The left kidney was fixed in buffered formalin and embedded in paraffin.

Table 1 Dietary groups

Diet components (g/100 g)	CSVO	CDVO	CSMO	CDMO
Soybean protein (1)	20.00	20.00	20.00	20.00
Hydrogenated vegetable oil (2)	14.30	14.30	0.00	0.00
Corn oil (3)	5.70	5.70	0.00	0.00
Menhaden oil (4)	0.00	0.00	20.00	20.00
Saccharose	49.15	49.50	49.15	49.50
Cellulose (5)	4.00	4.00	4.00	4.00
Vitamin mix (without choline) (6)	4.00	4.00	4.00	4.00
Salt mixture (7)	2.00	2.00	2.00	2.00
L-cystine (8)	0.50	0.50	0.50	0.50
Choline chloride	0.35	0.00	0.35	0.00

CSVO choline-supplemented diet with vegetable oils as lipids, *CDVO* choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils), *CSMO* choline-supplemented diet with fish oil as lipid, *g* grams, *ID* identification, *CDMO* choline-deficient diet with fish oil (menhaden oil) as lipid

(1) MPB 902940, Solon, Ohio, USA; (2) Vegetalina Dánica, Buenos Aires, Argentina; (3) Mazola, Córdoba, Argentina; (4) MPB 296012, Solon, Ohio, USA; (5) MPB 191499, Solon, Ohio, USA; (6) MPB 904655, Solon, Ohio, USA; (7) MPB 902851, Solon, Ohio, USA; (8) MPB 101454, Solon, Ohio, USA

Histopathology

Sections were cut and stained with Hematoxylin and Eosin to analyze histopathological alterations and determine the presence of tubular or cortical necrosis, which involved tubules and glomeruli and was characterized by pyknosis, karyolysis and increased eosinophilia.

Serum markers

Homocysteine (ARCHITECT i System, ABBOTT), vitamin B_{12} (Siemens) and folic acid (Siemens) were measured according to the manufacturer's instructions. The ARCHITECT Homocysteine assay is a chemiluminescent microparticle immunoassay for the quantitative determination of total L-homocysteine. Vitamin B_{12} and folic acid were simultaneously measured (Solid Phase No Boil, Dualcount). This kit is a protein competitive radioassay designed for the simultaneous measurement of vitamin B_{12} in serum or plasma, and folic acid in serum, plasma or whole blood.

Gene expression

Cryopreserved kidneys were wrapped in a mortar with liquid nitrogen to keep cryopreservation and were pulverized with a pestle. The tissue was broken up to be completely pulverized. For each condition, CSVO, CDVO,

CSMO and CDMO, 2 pools of 3 animals each were carried out with the powder obtained (24 animals). A mass pooling method was used, and once all samples were wrapped, 30 mg of each was pooled, and the pools were preserved in nitrogen until RNA extraction. Each sample represents a pool of three different animals. Sample subpooling is a strategy that can be used to reduce the biological variation. The design of the test guarantees the standardization of data analysis, since each independent sample will consist of three different kidneys. Total RNA was purified from 30 mg of frozen rat kidney pools, using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The biological concentration, quality (ratios of 260 nm/280 nm > 1.7, and 260 nm/230 nm > 2) and integrity (RNA Integrity Number: RIN > 6) of the RNA obtained were analyzed using NanoDrop 2000c (Thermo Fisher Scientific, DE, USA) and Bioanalyzer Agilent 2100 (Agilent Technologies, CA, USA).

Five hundred nanograms of total RNA was processed and hybridized to Affymetrix GeneChip Rat Gene 1.0 ST Array (Affymetrix Inc., Singapore, Singapore), according to Ambion WT Expression Kit instructions (Ambion Inc., TX, USA). Total RNA obtained during tissue extraction was processed to obtain a double strand cDNA. Then, we performed an in vitro transcription to generate antisense cRNA (aRNA), which was used to generate a single-stranded DNA (ss-DNA) using random primers and the dUTP +dNTP mix. The obtained ss-DNA containing the unnatural uracil base is then treated with uracil-DNA glycosylase, which specifically removes the uracil residue from the ss-DNA molecules. In the same reaction, the APE 1 enzyme then cleaves the phosphodiester backbone where the base is missing, thus leaving a 3′-hydroxyl and a 5′-deoxyribose phosphate terminus. Before performing these processes, short ss-DNA fragments were labeled by terminal deoxynucleotidyl transferase (TdT) that covalently linked the 3′-hydroxyl phosphate terminus with biotin allonamide triphosphate. The GeneChip® Rat Gene 1.0 ST Array enables whole-genome, gene-level expression studies for well-characterized genes. It is a single GeneChip®-brand array comprised of more than 722254 unique 25-mer oligonucleotide features accounting for more than 27342 gene-level probe sets. Gene expression levels are proportional to RNA sample hybridization intensity on the probe sets, which is a competitive process. Arrays were scanned with GeneChip® Scanner 3000 7G (Affymetrix Inc, Tokyo, Japan), and intensity levels normalized using Affymetrix Expression Console Software. In addition, call values were retrieved by MAS5 algorithm, and only genes with a "p" (present) call value were used in the analysis. In order to identify differentially expressed genes, raw data were processed by robust media average (RMA) method [15]. A linear model accounting for main treatment (diets and oils) with interaction (oil by diet) effects was applied on a gene-by-gene basis:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij} \quad i = 1, \ldots, 4$$
$$j = 1, \ldots, 2\, \varepsilon_{ij} \sim N(0, \sigma^2)\, \mathrm{cov}\left(\varepsilon_{ij}, \varepsilon_{i'j'}\right) = 0\, \forall i \neq i' \wedge j \neq j'$$

where y_{ij} is the intensity level for the ith treatment (CSVO, CDVO, CSMO and CDMO); jth is the replicate of the gene in turn analyzed; μ is the mean intensity; τ_i is the treatment contribution; and ε_{ij} is the error term, which is normally distributed, with independent errors. All the intensities are expressed in $\log_2$ normalized a-dimensional values. The model was adjusted using limma R package and pair-wise biological meaningful treatment. Difference hypothesis test was carried out, i.e., H_0: $\tau_i - \tau_j = 0$ $i \neq j$ versus H_1: $\tau_i - \tau_j \neq 0$ [16]. False discovery rate (FDR) p value adjustment has been applied in order to account for multiple test comparison error. Genes achieving an FDR <0.01 and absolute fold change value >1.5 were considered differentially expressed [17].

Statistical analysis

The normality of the variables was studied by graphic (Q–Q Plot, Box-Plot) and analytic methods (Kolmogorov–Smirnov). Comparison between groups of variables with normal distribution (renal weight/100 g body weight, homocysteine, vitamin B_{12} and folic acid) was made by ANOVA followed by Tukey test when $p < 0.05$; on the contrary, variables without normal distribution (final body weight and right kidney) were analyzed by Kruskal–Wallis test followed by Mann–Whitney test (Table 2).

Results

Histopathology

The histopathology of the four groups was studied. All CSVO, CSMO and CDMO rats showed no renal alterations, while all CDVO rats showed renal cortical necrosis (Fig. 1). Renal damage was characterized by a purplish red discoloration, and a higher size and weight of the kidneys. High renal weight expressed as percentage of body weight in CDVO rats was observed due to renal necrosis and lower final body weight. Mean final body weights at day 6 (standard deviation) of each group were as follows: CSVO = 70.88 (4.15); CDVO = 52.72 (3.50); CSMO = 53.30 (3.59); CDMO = 51.55 (2.42). There were significant differences between CSVO versus all the other groups (Table 2). CSVO has a higher final body weight, which statistically differs from the other three groups.

Table 2 Group characteristics and homocysteine, vitamin B_{12} and folic acid serum levels: (A) Results, (B) Statistical analysis

(A)	CSVO (A) ($n = 6$)	CDVO (B) ($n = 6$)	CSMO (C) ($n = 6$)	CDMO (D) ($n = 6$)
Final body weight (grams)[a]	70.88 (4.15)	52.72 (3.50)	54.30 (3.59)	51.55 (2.42)
Right Kidney (grams)[a]	0.42 (0.033)	0.64 (0.064)	0.33 (0.030)	0.30 (0.020)
Homocysteine (μmol/L)[b]	2.68 (0.84)	2.73 (0.37)	7.07 (0.91)	8.25 (3.20)
Vitamin B_{12} (pg/mL)[b]	1,400 (167.33)	3,233.3 (859.46)	1,291.66 (205.9)	1,223.33 (291.3)
Folic acid (ng/mL)[b]	30.16 (2.48)	26.16 (7.25)	21 (7.80)	25.16 (4.44)

(B)	Test ($df = 3$); significance	Post hoc					
		A versus B	A versus C	A versus D	B versus C	B verus D	C versus D
Final body weight (grams)[a]	$\chi^2 = 14.01$; $p = 0.003$	0.004	0.004	0.004	0.33	0.75	0.20
Right Kidney (grams)[a]	$\chi^2 = 19.90$; $p < 0.001$	0.004	0.006	0.004	0.004	0.004	0.092
Homocysteine (μmol/L)[b]	$F = 16.89$; $p < 0.001$	1.00	0.001	<0.001	0.002	<0.001	0.65
Vitamin B_{12} (pg/mL)[b]	$F = 25.10$; $p < 0.001$	<0.001	0.98	0.91	<0.001	<0.001	0.99
Folic acid (ng/mL)[b]	$F = 2.44$; $p = 0.092$	–	–	–	–	–	–

CSVO choline-supplemented diet with vegetable oils as lipids, *CDVO* choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils), *CSMO* choline-supplemented diet with fish oil as lipid, *CDMO* choline-deficient diet with fish oil (menhaden oil) as lipid, *g* grams,

[a] Kruskal-Wallis Test and Mann–Whitney as post hoc

[b] ANOVA and Tukey as post hoc

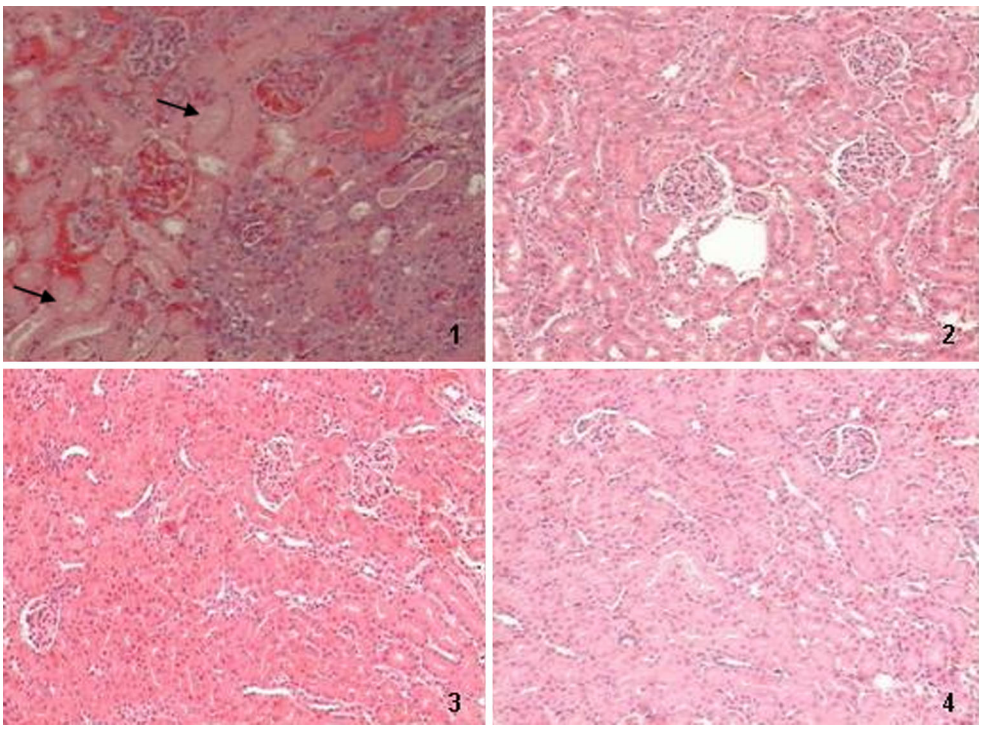

Fig. 1 Histopathological analysis of kidney: Hematoxylin–Eosin staining: **1** CDVO rat kidney (HE 10X); **2** CSVO rat kidney (HE 10X); **3** CDMO rat kidney (HE 10X); **4** CSMO rat kidney (HE 10X). *Arrows* show necrotic tubules

Serum markers

We analyzed biochemically the serum from the four groups of animals. The results expressed as mean ± standard deviation of homocysteine, vitamin B_{12} and folic acid are shown in Table 2. Differences in homocysteine and vitamin B_{12} values were observed among groups ($p < 0.05$).

Gene expression

Adequate spectrophotometric values were found in all RNA samples, with ratios of 260 nm/280 nm and 260 nm/230 nm between 1.7 and 2.1. RIN median was 9.1, with a range between 6.2 and 10. Microarray data were submitted to Gene Expression Omnibus: GSE34139 [NCBI tracking system #16217806]. Supplementary Materials are available at 1 and 2. The differential expression analysis between the four groups is shown in Table 3. In our analysis, a five-set Venn diagram using congruent ellipses in a radially symmetrical arrangement was designed. Venn diagram or set diagram is a drawing that shows all possible logical relations between a finite collection of sets (Fig. 2). Figure 2 and Table 4 reveal that oil effect is marginal on choline supplement diet (with only 33 and 32 genes statistically expressed in groups B1 and B2). This suggests high similarity (almost same behavior) between CDMO and CSVO. On the contrary, choline deficiency has a harmful effect and corn oil produces no improvement, as suggested by the large number of differentially expressed genes under "control CSVO" and "control like" (CDMO and CSMO) diets. As suggested by Table 4 analysis, its main outcome effect could be associated to renal necrosis; since in the comparisons between groups A1-3, we found 542 deregulated genes shared in all of these groups. Figure 2 shows two sets with 203 and 18 genes. These genes are deregulated by choline deficiency, under the presence of corn oil effect, since no differentially expressed genes were found (in those gene sets) between CDVO against CSVO, CDMO and CSMO. The statistical model reveals that the set with 203 genes is affected by the oil effect. Moreover, the set with 18 genes is affected by choline deficiency. On the other hand, there is an additional set with 30 genes, affected by both choline effect and menhaden oil, suggesting treatment interaction.

For a better understanding, differential genes were arranged in descending order. We then evaluated the choline or lipid effects, with (A1-3) and without (B1-3) renal necrosis, as a consequence of the diets, and found the same expression pattern in the lipid effect distribution according to the descending order of the genes (Table 4).

CDVO was the most negative state and was the only group that showed necrosis. If any of the two variables, either CD or VO, was changed—separately or together, the histopathology observed in the kidney for the three possible combinations would be normal, but gene expression in each group would differ. We analyzed the groups in Table 4:

A1-CDVO versus CSMO showed the combined effect of lipids and choline. The CSMO group did not show renal necrosis, and 879 genes were found in the differential expression against CDVO. We could not reach a final conclusion after the comprehensive analysis of the genes found in the comparison between CDVO and CSMO, since the effect of each one of the parameters was overlapped by the simultaneous change in the other.

A2-CDVO versus CDMO showed lipid effect without choline. CDMO group had no necrosis and showed 836 differential genes compared to CDVO group, which presented renal necrosis. The group of differential genes was compared using the multi-reference contrast method (MRCM) proposed by Fresno et al. [18]. The MRCM is used in the database for annotation, visualization and integrated discovery (DAVID) platform, as the basis for the enrichment of biological terms. Those impaired genes arise from this experiment, compared to what is expected in the genome, microarray or a list of genes of interest [19]. Particularly, the analysis was done on the gene ontology (GO), which classifies the knowledge in three directed acyclic graphs (DAGs) in a hierarchical manner, where nodes represent biological terms associated with genes [20]. Once results were obtained for the enrichment of the CDVO versus CDMO comparison, we proceeded to identify those pathways that allow explaining the prevention of renal necrosis with the addition of MO to the diet. In this context, ontology showed: (1) On the Biological Process graph, the following terms were enriched: those related to methionine biosynthetic process (four genes), the cysteine metabolic process (three genes), transsulfuration, tyrosine and L-phenylalanine catabolic process (three genes) and the NAD biosynthetic process (five genes). These processes are associated with the Gspt1 gene. These terms were only enriched by genes upregulated

Table 3 Number of differential genes using cut-off value (p adj <0.01 & |logFC| > 1.5)

		CD		CS	
		VO	MO	VO	MO
CD	VO				
	MO	836			
		(425, 411)			
		2,2–6,5			
CS	VO	724	33		
		(458, 266)	(10, 23)		
		2,2–6,8	2,0–4,5		
	MO	879	0	32	
		(429, 470)		(11, 21)	
		2,2–6,9		2,1–5,2	

Number of differential gene (up regulated number, down regulated number) Mean–Maximum fold changes

CDVO choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils), *CSVO* choline-supplemented diet with vegetable oils as lipids, *CDMO* choline-deficient diet with fish oil (menhaden oil) as lipid, *CSMO* choline-supplemented diet with fish oil as lipid

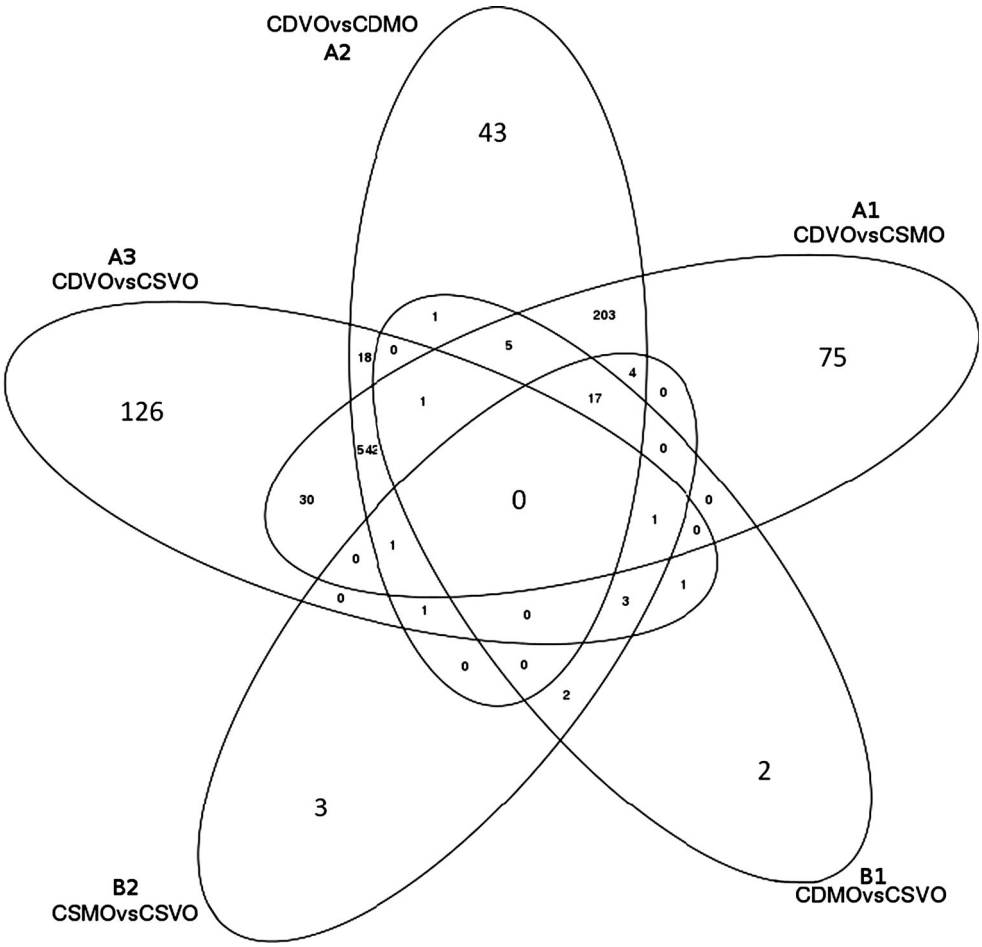

Fig. 2 Venn diagram. Differentially expressed genes for the different biological treatment combination contrasts explored in this analysis. *CDVO* choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils), *CSVO* choline-supplemented diet with vegetable oils as lipids, *CDMO* choline-deficient diet with fish oil (menhaden oil) as lipid, *CSMO* choline-supplemented diet with fish oil as lipid

Table 4 Differential genes were arranged in descending order

Group Comparison	A1 CDVO versus CSMO	A2 CDVO versus CDMO	A3 CDVO versus CSVO	B1 CDMO versus CSVO	B2 CSMO versus CSVO	B3 CDMO versus CSMO
Differential expression genes	879	836	724	33	32	0
Renal necrosis	X	X	X			
Choline deficiency	X		X	X		X
Protective effects of fish oil	X	X		X	X	

CDVO choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils), *CSVO* choline-supplemented diet with vegetable oils as lipids, *CDMO* choline-deficient diet with fish oil (menhaden oil) as lipid, *CSMO* choline-supplemented diet with fish oil as lipid

in MO. On the contrary, the processes associated with inflammatory response were upregulated with VO, though not with MO (positive regulation of type IIa hypersensitivity, nine genes). The branches of the graph associated with the immune response or inflammation were activated only in VO, as phagocytosis (13 genes), positive regulation of endocytosis (13 genes), regulation of macrophage differentiation (three genes), positive regulation of myeloid cell differentiation and trophoblast giant cell differentiation. (2) On the Molecular Function graph, the functions upregulated with VO were those associated with interleukins and cytokines, while those upregulated with MO were acyl-CoA dehydrogenase activity, acid-thiol ligase activity and some functions associated with ions transport and glutathione binding. (3) However, in Cellular Component, there was no relevant information regarding the presence of a predominant location for biological processes and molecular functions of interest.

A3-CDVO versus CSVO showed the choline effect with VO. In the CSVO group, the animals received choline and vegetable fatty acids in their diet; this is the condition closest to the normal one. As it has been previously stated, we did not find necrosis in these kidneys, and 724 genes were found in the differential analysis against CDVO.

B1-CDMO versus CSVO showed the combined effect of lipids and choline. The comparison between CDMO and CSVO showed that none of them showed necrosis, and that there were 33 genes with differential expression, since the effect of each one of the parameters was overlapped by the simultaneous change in the other.

Table 4 describes the combined effect of lipids and choline, A1-3 groups with renal necrosis versus B1-3 groups without necrosis. The number of differentially expressed genes varies from 879 (CDVO vs. CSMO, Group A1) to 33 (CDMO vs. CSVO, Group B1). Differential gene expression for A1-3 groups has a median of 813 genes and for B1-3 groups a median of 21 genes. Since we consider that the main difference of this quantity of genes lies in renal necrosis (CDVO), a Venn diagram was used to understand this significant difference, which depicts that 542 genes are associated to genes deregulated only by the necrosis outcome induced by deficiency in choline (in the intersection of the comparisons of groups A1, A2 and A3). The increase in the amount of differentially expressed genes in these groups above the 542 genes is due to the specific comparison effect, such as fish oil and/or choline supplement in diet. In addition, these 542 genes have enriched 3 KEGG pathways: "complement and coagulation cascades" (15 genes), "cytokine–cytokine receptor interaction" (14 genes) and "focal adhesion" (13 genes), which are strongly related to necrosis.

As the objective of this study was to analyze the mechanism of the protective effect of fish oil on the AKI model induced by choline deficiency, these two relevant groups must be evaluated:

B2-CSVO versus CSMO showed the lipid effect with choline and no necrosis and 32 genes (11 up- and 21 downregulated) with differential expression. A list of selected genes is shown in Table 5. In our opinion, this is the truly informative group, whose genes are crucial for the analysis of the potential protective effect of fish oil; since in this transcriptomes comparison, there was no choline influence overlapping the effect on lipid metabolism. Thus, these 32 genes could be identified as being responsible for the protective effect of the MO administered and could be associated with the pathogenic mechanisms involved in this protective effect in this experimental model. Given the small number of genes involved (32 genes) in the CSVO versus CSMO comparison by using MRCM, no enriched terms/nodes were identified after the addition of MO to the diet. This would reveal functional similarity between both diets. This observation led us to focus on these genes, where the Gstp1 would seem to be an interesting molecular target. We found no network connecting Gstp1 to any of these genes. The exhaustive knowledge of choline metabolism allowed us to discover Gstp1 and to understand the role this enzyme would play in detoxification. Therefore, this enzyme becomes more important.

B3-CDMO versus CSMO showed the choline effect with MO (no difference). These groups showed neither necrosis nor differences between transcriptomes, even when studied under maximum statistical requirement. Thus, we could hypothesize that MO could be offsetting, both morphologically and genetically, choline deficiency in this model. Then, given that the differential expression between CDMO and CSMO showed zero differential genes, we could genetically confirm the protective effect of the MO as it has already been described in the histological analysis. As far as histology and gene expression are concerned, we could infer that the addition of MO in the diet would offset choline absence. This would imply that MO protects kidney against the damage induced by choline deficiency in this animal model, causing both experimental conditions to behave similarly in terms of gene expression. This biological finding could be of clinical importance for AKI patients.

Discussion

The treatment and technical management of AKI have changed dramatically over the last decades. However, mortality rates seem to have remained unchanged at around 50 % [21, 22]. Some patients will never regain full renal function, which will therefore lead to end-stage renal failure requiring lifelong dialysis or kidney transplant [23]. The mechanisms underlying the etiology and progression of kidney diseases are not fully understood. However, integration of dynamic changes at molecular levels with clinical parameters would be crucial for identifying the biological basis and developing biomarkers and therapeutic agents of kidney diseases [24].

Lipid effects

Weanling rats fed a choline-deficient diet develop AKI, with morphological changes varying from focal tubular necrosis to massive cortical necrosis or reparative changes in surviving animals. Changes in the lipid composition of cellular membranes could alter the generation of second messengers and cell signal transduction pathways, making cells more resistant to the renal deleterious effects induced by choline deficiency. Dietary fish oil can modulate different types of diseases, including different renal

Table 5 Differentially expressed genes between CSVO and CSMO (FDR adjusted p value <0.01 and $|\log_2 FC| > 1.5$)

	AffyID	EntrezID	GeneSymbol	GeneDescription	$\log_2$FC	p value	p adj
Up regulated in CSMO versus *CSVO*							
1	10771978	286989	Ugt2b7	UDP-glucuronosyltransferase 2 family, polypeptide B7	5.22	3.04E−11	1.15E−06
2	10810867	24314	Nqo1	NAD(P)H dehydrogenase. quinone 1	3.26	8.96E−07	2.11E−04
3	10789301	364634	Csmd1	CUB and Sushi multiple domains 1	2.78	2.22E−08	3.36E−05
4	10771957	266685	Ugt2b5	UDP-glucuronosyltransferase 2 family. polypeptide B5	2.72	2.52E−05	1.61E−03
		29623	Ugt2b37	UDP-glucuronosyltransferase 2 family. member 37			
5	10937734	363465	Pir	Pirin (iron-binding nuclear protein)	2.29	1.55E−06	2.88E−04
6	10893630	314638	Creb3l3	cAMP responsive element binding protein 3-like 3	2.05	1.52E−06	2.86E−04
7	10901166	362850	Angptl4	Angiopoietin-like 4	1.95	3.15E−06	4.35E−04
8	10727429	24426	Gstp1	Glutathione-S-transferase. pi 1	1.75	2.13E−07	9.63E−05
9	10922151	494500	Yc2	Glutathione-S-transferase Yc2 subunit	1.73	1.14E−04	4.31E−03
10	10840791	296271	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	1.69	1.06E−06	2.34E−04
11	10752941	304125	Cldn17	Claudin 17	1.58	2.19E−05	1.45E−03
Down regulated in CSMO versus *CSVO*							
12	10891880	690195	Prima1	Proline rich membrane anchor 1	−1.51	2.33E−06	3.70E−04
13	10734853	114592	Aurkb	Aurora kinase B	−1.56	6.53E−05	2.97E−03
14	10749762	287884	Sectm1b	Secreted and transmembrane 1B	−1.59	1.16E−06	2.46E−04
15	10822852	114494	Ccna2	Cyclin A2	−1.60	3.84E−04	9.77E−03
16	10710627	25515	Plk1	Polo-like kinase 1 (Drosophila)	−1.74	1.36E−04	4.83E−03
17	10734242	287382	Mfap4	Microfibrillar-associated protein 4	−1.76	9.03E−06	8.30E−03
18	10915751	307056	Anln	Anillin. actin binding protein	−1.82	6.25E−05	2.89E−03
19	10784172	361047	RGD1307201	Similar to chromosome 13 open reading frame 3	−1.84	9.83E−05	3.90E−03
20	10843588	100169711	Lcn11	Lipocalin 11	−1.85	5.30E−05	2.58E−03
21	10799084	307056	Anln	Anillin. actin binding protein	−1.86	7.19E−05	3.17E−03
22	10849737	296137	Bub1	Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	−1.87	2.20E−04	6.67E−03
23	10726408	291234	Mki67	Antigen identified by monoclonal antibody Ki-67	−1.87	8.85E−05	3.64E−03
24	10742194	64193	Pttg1	Pituitary tumor-transforming 1	−1.87	9.21E−05	3.74E−03
25	10829761	54237	Cdc2	Cell division cycle 2. G1 to S and G2 to M	−1.92	1.37E−04	4.86E−03
26	10858707	297594	Cdca3	Cell division cycle associated 3	−1.95	1.83E−04	5.89E−03
27	10746976	360243	Top2a	Topoisomerase (DNA) II alpha	−2.04	2.27E−04	6.80E−03
28	10842239	81687	Mmp9	Matrix metallopeptidase 9	−2.08	8.39E−06	7.93E−04
29	10779638	289993	Cdkn3	Cyclin-dependent kinase inhibitor 3	−2.34	3.91E−04	9.90E−03
30	10810144	362720	Rrm2	Ribonucleotide reductase M2	−2.36	2.33E−05	1.52E−03
31	10883903	362720	Rrm2	Ribonucleotide reductase M2	−2.37	2.01E−05	1.37E−03
32	10730349	246074	Scd1	Stearoyl-Coenzyme A desaturase 1	−2.85	1.24E−06	2.57E−04

pathologies, in experimental animals and human beings [25]. Higher final body weight in CSVO rats was probably associated with a better balanced diet (Table 2).

Choline effects

In a previous work, our group showed that MO added to a choline-deficient diet has a protective effect on AKI in this animal model [12]. Based on these histological findings already defined, we have analyzed massive gene expression in this same model. This is an exploratory study, since the expression profile in this experimental model is unknown. We found that a choline-deficient diet induced changes in renal gene expression in male weanling Wistar rats, showing 542 differential genes identified in Venn diagram, and that the source of lipids, either VO or MO, changes gene expression (Table 3; Fig 2).

Combined choline and lipid effects

Two findings were observed in the analysis to explore pathogenic mechanisms involved in the protective effect that fish oil has on this experimental model. On the one hand, a thorough analysis was carried out of the differential

expression between groups CDMO and CSMO (Group B3). There were no differential genes for $p < 0.01$. This finding would show no significant differences between the genes in both conditions. On the other hand, the analysis of the differential expression between groups CSMO and CSVO (Group B2) revealed 32 genes. Of 27342 unigenes present within the arrays, 11 were over-expressed, and 21 were under-expressed in CSMO rats.

Serum markers

As shown in Table 2, we found high serum levels of homocysteine (μmol/L) in rats fed MO (with or without choline) and high serum levels of vitamin B_{12} (pg/mL) in CDVO. Homocysteine was greatly augmented in the CSMO and CDMO groups. Homocysteine concentration in plasma may change according to different exogenous and endogenous circumstances [26]. Different factors could be involved in these groups, such as concentration of fish oil in the diet, time of feeding and lower concentration of B_{12} and folic acid. A larger synthesis of endogenous choline through a triple methylation of phosphatidyl ethanol amine by S-adenosylmethionine could also be involved. Another factor to take into consideration is an alteration in the transsulfuration pathway. Gstp1 is one of the enzymes involved in the catabolism of homocysteine, and it was also augmented in these groups. The significant increase in vitamin B_{12} in serum could be due to the release of this vitamin from the necrotic renal tubules [27, 28]. To our knowledge, there is an association between Gstp1, stress and oxidative damage, which has been proposed as biochemical mechanism of AKI [29, 30]. Choline deficiency-related AKI is associated with higher rates of lipid peroxidation and oxidative damage [30–32]. Polyunsaturated fatty acids are affected by oxidative damage in direct relation to the content of double ligatures. Fish oil is rich in polyunsaturated fatty acids; however, decreasing rather than increasing oxidative stress and lipoperoxidation occurs in the kidney. This might be related to the intrinsic content of antioxidants and to the upregulation of the Gstp1 gene. Thus, regardless of the presence or absence of choline, MO produces an upregulation of the Gstp1 gene, an enzyme involved in the glutathione regeneration pathway as xenobiotic and antioxidant. Since reduced glutathione is an antioxidant, it delays the oxidative stress and lipid peroxidation (Fig. 3). Gstp1 could be a gene target for gene therapy for AKI since the induction of an upregulation would be protecting the kidney from the renal damage induced by oxidative stress and lipid peroxidation. Figure 3 shows that in CSVO group, which is the one most similar to the standard group, Gstp1 expression has low values, a higher value in choline-deficient diet, and surprisingly higher value in the presence of MO, with or without

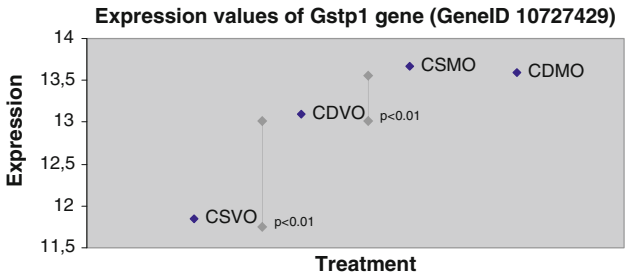

Fig. 3 Expression values of Gstp1 gene (GeneID 10727429). All the intensities are expressed in normalized log2 scale (dimensionless). CSVO (11,8524): choline-supplemented diet with vegetable oils as lipids; CDVO (13,1026): choline-deficient diet with vegetable oils as lipids (corn and hydrogenated oils); CSMO (13,6752): choline-supplemented diet with fish oil as lipid; and CDMO (13,5875): choline-deficient diet with fish oil (menhaden oil) as lipid

choline in the diet. In previous studies done and published by some members of our group, we analyzed the following methods in the renal homogenate of CDVO animals with renal necrosis [30, 32, 33]: total reactive antioxidant potential, and chemiluminescence and thiobarbituric acid-reactive substances. We reached the conclusion that in CDVO, the decrease in antioxidants and lipid peroxidation is previous to the necrosis occurring around day 7. When VO is replaced by MO in CD rats, oxidative stress and lipid peroxidation are reduced in the kidney. This is controversial since while polyunsaturated fatty acids are affected by oxidative damage in the same way as the content of double ligatures and MO is rich in polyunsaturated fatty acids, there is a decrease in oxidative stress and renal lipoperoxidation, rather than an increase. We hypothesize that this could be due to the intrinsic content of antioxidants in MO, or due to the upregulation of Gstp1 expression (enzyme involved in the metabolism of glutathione), which produces MO independently of the presence or absence of choline.

Research priorities in critical care increasingly focus on long-term outcomes and prognosis for survivors of critical illnesses. Few studies have described the long-term outcomes after AKI, which is a common disorder among hospitalized patients, accounting for 3–7 % of patients admitted to hospital and about 25–30 % of patients in the intensive care unit [34, 35]. In light of these relevant data, those patients that are more likely to develop this syndrome could receive a particular diet, such as fish oil. Since there is no curative treatment for AKI, newly discovered tissue-specific markers will have the potential to transform the disease [36]. In conclusion, this experiment was part of a large set of experiments and was used in a hypothesis-generating manner. The comprehensive analysis of genetic expression allowed confirming that menhaden oil (a type of fish oil) has a protective effect on this nutritional experimental model and identifying 32 genes that could be responsible for that protection, including Gstp1. These

results reveal that gene changes may play a role in renal injury.

Acknowledgments We thank Mariana dos Santos and Silvia Caram for technical assistance, Myrthala Duguid (Head of the Library at CEMIC University Institute), and Valeria Melia for English editing of the manuscript. This paper was partially supported by a grant from CONICET and University of Buenos Aires, Argentina.

Conflict of interest All the authors declare no conflicts of interest in this protocol.

References

1. Zeisel SH, Blusztajn JK (1994) Choline and human nutrition. Annu Rev Nutr 14:269–296
2. Institute of Medicine of the National Academies (1998) Dietary reference intakes for thiamine, riboflavin, niacin, vitamin B6, foltae, vitamin B_{12}, pantothenic acid, biotin, and choline. Choline. The National Academies Press, Washington DC, pp 390–422
3. Zeisel SH, da Costa KA (2009) Choline: an essential nutrient for public health. Nutr Rev 67:615–623
4. Montes de Oca M, Perazzo JC, Monserrat AJ, Arrizurieta de Muchnik EE (1980) Acute renal failure induced by choline deficiency: structural functional correlations. Nephron 26:41–48
5. Monserrat AJ, Musso AM, Tartas N, Nicastro MA, Konopka HF, Arienti di García I, Sanchez Avalos JC (1981) Consumption coagulopathy in acute renal failure induced by hypolipotropic diets. Nephron 28:276–284
6. Fewster ME, Hall MO (1967) The renal phospholipid composition of choline-deficient rats. Lipids 2:239–243
7. Monserrat AJ, Porta EA, Ghoshal AK, Hartman SB (1974) Sequential renal lipid changes in weanling rats fed a choline-deficient diet. J Nutr 104:1496–1502
8. Simon JB, Scheig R, Klatskin G (1968) Relationship of early lipid changes in kidney and liver to the hemorrhagic renal necrosis of choline-deficient rats. Lab Invest 19:503–509
9. O'Neal RM, Still WJS, Hartroft WS (1961) Increased lipotropic requirements with renal necrosis induced in rats by high-fat diets. J Nutr 75:309–318
10. Monserrat AJ, Romero M, Lago N, Aristi C (1995) Protective effect of coconut oil on renal necrosis occurring in rats fed a methyl-deficient diet. Ren Fail 17:525–537
11. Monserrat AJ, Cutrin JC, Coll C (2000) Protective effect of myristic acid on renal necrosis occurring in rats fed a methyl-deficient diet. Res Exp Med (Berl) 199:195–206
12. Courrèges C, Caruso C, Klein J, Monserrat AJ (2002) Protective effect of menhaden oil on renal necrosis occurring in weanling rats fed a methyl-deficient diet. Nutr Res 22:1077–1089
13. Takayama J, Takaoka M, Supino Y, Yamamoto Y, Ohkita M, Matsumura Y (2007) Sex difference in ischemic acute renal failure in rats: approach by proteomic analysis. Biol Pharm Bull 30:1905–1912
14. NIH Guide for the Care and Use of Laboratory Animals (2011) The National Academies Press, Washington
15. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ et al (2003) Exploration, normalization and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249–264
16. Smyth GK, Speed T (2003) Normalization of cDNA microarray data. Methods 31:265–273
17. Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments: Stat Appl Genet Mol Biol 3. doi:10.2202/1544-6115.1027
18. Fresno C, Llera AS, Girotti MR, Valacco MP, López JA, Podhajcer OL, Balzarini MG, Prada F, Fernández EA (2012) The multi-reference contrast method: facilitating set enrichment analysis. Comput Biol Med 42:188–194
19. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57
20. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene Ontology: tool for the unification of biology. Nat Genet 25:25–29
21. Bellomo R (2006) The epidemiology of acute renal failure: 1975 versus 2005. Curr Opin Crit Care 12:557–560
22. Ympa YP, Sakr Y, Reinhart K, Vincent JL (2005) Has mortality from acute renal failure decreased? A systematic review of the literature. Am J Med 118:827–832
23. Webb S, Dobb G (2007) ARF, ATN or AKI? It's now acute kidney injury. Anaesth Intensive Care 35:843–844
24. He JC, Chuang PY, Ma'ayan A, Iyengar R (2012) Systems biology of kidney diseases. Kidney Int 81:22–39
25. Nettleton JA (1995) Omega-3 fatty acids, and health. Chapman & Hall, New York
26. Verhoef P (2007) Homocysteine-an indicator of healthy diet? Am J Clin Nutr 85:1446–1447
27. Herbert V (1997) Vitamina B_{12}. In Conceptosd actuales sobre nutrición, 7th edn. OMS, Washington, pp 203
28. Scott JS, Treston AM, Bowman EP, Owens JA, Cooksley WG (1984) The regulatory roles of liver and kidney in cobalamin (vitamin B_{12}) metabolism in the rat the uptake and intracellular binding of cobalamin and the activity of the cobalamin-dependent enzymes in response to varying cobalamin supply. Cli Sci (Lond) 67:299–306
29. Monserrat AJ, Ghoshal A, Hartroft W, Porta E (1969) Lipoperoxidation in the pathogenesis of renal necrosis in choline-deficient rats. Am J Pathol 55:163–190
30. Repetto MG, Ossani G, Monserrat A, Boveris A (2010) Oxidative damage: the biochemical mechanism of cellular injury and necrosis in choline deficiency. Exp Mol Pathol 88:143–149
31. Newberne PM, Bresnahan MR, Kula N (1969) Effect of two synthetic antioxidants, vitamin E, and ascorbic acid on the choline-deficient rat. J Nutr 97:219–231
32. Ossani G, Dalghi M, Repetto M (2007) Oxidative damage and lipid peroxidation in the kidney of choline-deficient rats. Front Biosci 12:1174–1183
33. Ossani G (2010) Deficiencia de colina y patogenia de la insuficiencia renal aguda. PhD Thesis. University of Buenos Aires. Department of Pathology
34. Bagshaw SM (2006) The long-term outcome after acute renal failure. Curr Opin Crit Care 12:561–566
35. Brenner BM, Rector FC (2007) The kidney. Saunders, Philadelphia
36. Siew ED, Ware LB, Ikizler TA (2011) Biological markers of acute kidney injury. J Am Soc Nephrol 5:810–820