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# Short-term Menhaden Oil Rich Diet Changes Renal Lipid Profile in Acute Kidney Injury

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**Abstract: Weanling male Wistar rats fed a choline-deficient diet develop acute kidney injury. Menhaden oil, which is a very important source of omega-3 fatty acids, has a notorious protective effect. The mechanism of this protection is unknown; one possibility could be that menhaden oil changes renal lipid profile, with an impact on the functions of biological membranes. The aim of this work was to study the renal lipid profile in rats fed a choline-deficient diet with menhaden oil or vegetable oil as lipids. Rats were divided into 4 groups and fed four different diets for 7 days: choline-deficient or choline-supplemented diets with corn and hydrogenated oils or menhaden oil. Serum homocysteine, vitamin $B_{12}$, and folic acid were analyzed. Renal lipid profile, as well as the fatty acid composition of the three oils, was measured. Choline-deficient rats fed vegetable oils showed renal cortical necrosis. Renal omega-6 fatty acids were higher in rats fed a choline-deficient diet and a choline-supplemented diet with vegetable oils, while renal omega-3 fatty acids were higher in rats fed a choline-deficient diet and a choline-supplemented diet with menhaden oil. Rats fed menhaden oil diets had higher levels of renal eicosapentaenoic and docosahexaenoic acids. Renal myristic acid was increased in rats fed menhaden oil. The lipid renal profile varied quickly according to the type of oil present in the diet.**

**Key words:** menhaden oil, choline deficiency, fatty acids, acute kidney injury, lipid profile

## 1 INTRODUCTION

Fatty acids have multiple functions, they produce energy by oxidation; they are structural components of membranes and precursors of prostaglandins, among others. Phospholipids are the main component of biological membranes, and can be classified into two types: glycerophospholipids, the most important, and sphingolipids[1)]. Glycerophospholipids are made of a hydrophilic head and hydrophobic tails consisting of fatty acids of 16 to 24 carbons with 0 to 6 double bonds[2)]. Differences in fatty acids saturation have an impact on the fluidity of membranes: the higher the unsaturation, the higher the fluidity[3)]. Biological membranes have multiple biological functions, such as exchange of materials, location of enzymes, energy transduction, cell-cell interaction, among others. Changes in membrane structure may alter any of its functions[3)].

Weanling male rats fed a choline-deficient (CD) diet develop acute kidney injury (AKI) with morphological alterations that vary from focal tubular necrosis to massive cortical necrosis[4)], steatosis, cirrosis and hepatocarcinoma; heart necrosis and ocular hemorrhage[5, 6)]. Choline is a quaternary amine involved in multiple metabolic pathways. It plays a role in the synthesis of phospholipids, in the synthesis of both acetyl choline and very low density lipoprotein. This is also a source of labile methyl groups through betaine formation[7)]. Dietary intakes for human beings have been established[8)]. Choline deficiency syndrome is rare in healthy humans, since this amine is widely distributed in food. However, the requirement of choline varies according to the rate of growth of each individual, period of life, such as childhood and pregnancy, and to complex interactions between choline and methionine, folic acid, and vitamin $B_{12}$. The metabolism of choline is closely related to the me-

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tabolism of methionine, homocysteine, vitamin $B_{12}$ and folic acid, which are all involved in the transmethylation pathway in the activated methyl cycle[9]. Methionine can be regenerated by the transference of a methyl group from the N-5-methyltetrahydrofolate to homocysteine. This reaction can only be done with the presence of vitamin $B_{12}$ (cobalamin methyl) or betaine (product of the oxidation of choline).

The pathogenesis of renal necrosis due to choline deficiency is uncertain. Lipoperoxidation has been proposed as the pathogenic mechanism of tubular necrosis, and local intravascular coagulation has been proposed as the link between tubular and cortical necrosis[10, 11]. Both the quantity and quality of lipids have an impact on the development of renal necrosis in this experimental model[12, 13]. The potential pathogenic role of changes in renal lipids has been repeatedly studied, however a correlation between a particular lipid change and renal histology has not been clearly evidenced[14–16]. Renal damage increases with a high-fat diet[12, 17]. Coconut oil has a protective effect due to the presence of myristic acid[13, 18, 19]. Menhaden oil is a kind of fish oil with a high amount of myristic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. This is a very important source of omega-3 (ω3) fatty acids, and has a notorious protective effect, whose mechanism is unknown[20]. One possibility is that menhaden oil could change renal lipid profile, with an impact on biological membranes function. Given the protective effect of menhaden oil, the aim of this work was to study the renal lipid profile in rats fed a CD diet with menhaden oil or vegetable oil as dietary lipids.

## 2 EXPERIMENTAL

Thirty-two Wistar male weanling rats (21 days of age) from the Animal Facility at the Centre for Experimental and Applied Pathology were divided into 4 groups (n = 8) and fed the following diets: CD diet with corn and hydrogenated oils as lipids; choline-supplemented (CS) diet with corn and hydrogenated oils as lipids; CD diet with menhaden oil as lipid; CS diet with menhaden oil as lipid (Table 1). Diets were kept at 4℃ in the dark and were replaced every day. Authors have adhered to appropriate NIH Guide for the Care and Use of Laboratory Animals[21]. This protocol was approved by the Animal Care and Use Committee (protocol number 1276/2012) at the School of Medicine, University of Buenos Aires. Animals were anesthetized with thiopental sodium (40 mg/kg body weight) and sacrificed after receiving the experimental diets for 7 days. Biochemical markers in serum were analyzed. The right kidney was cryopreserved for lipid profile analysis. The left kidney was fixed in buffered-formalin and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin to analyze histopathological alterations and determine the existence of tubular or cortical necrosis.

Homocysteine (ABBOTT), vitamin $B_{12}$ (Siemens) and folic acid (Siemens) were measured according to the manufacturer's instructions. Homocysteine assay is a chemiluminiscent microparticle immunoassay for the quantitative determination of total L-homocysteine. Vitamin $B_{12}$ and folic acid were simultaneously measured in solid phase no boil radioassay by proteic competition.

For renal lipids determinations, the cryopreserved

**Table 1** Diets.

| Diet components (g/100 g) | CDVO | CSVO | CDMO | CSMO |
|---|---|---|---|---|
| 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 | 49.50 | 49.15 | 49.50 | 49.15 |
| 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 | 0.00 | 0.35 | 0.00 | 0.35 |

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 menhaden oil as lipid; and CSMO: Choline-supplemented diet with menhaden oil as lipid; g: grams. (1) MP Biomedicals (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.

kidney was wrapped with aluminum foil and broken with a hammer previously wrapped with tape paper on a counter covered in aluminum. The pieces of the kidney were placed in a mortar with liquid nitrogen to keep the cryopreservation and were pulverized with a pestle. Nitrogen was added as it evaporated. The tissue was broken up until complete pulverization. Powder was placed with a spatula in a tube with hexane: iscpropylic alcohol 3:2 (15 mL). Fatty acid composition of the three oils used in the diets was also measured. Renal lipids were extracted, and fatty acids containing 12 to 24 carbons were measured by gas-liquid chromatography (Agilent 7890, column SP 2560, carrier gas hydrogen).

Fatty acids from kidney lipids extracts (and also oils used in the experiment) were transesterificated with acetylchloride in a methanol:benzene (4:1) solution at 100℃ for one hour. Chromatographic analysis was done by a 7890A Agilent gas chromatograph with flame ionization detector (FID). The carrier gas was hydrogen (99.999%), which was kept at a constant flow of 1 mL $min^{-1}$. SP 2560 capillary column was used for analyte separations (100 m, 250 μm ID, 0.2 μm film). For analysis, 1.0 μL of the benzene phase was injected in the split mode at 225℃. The temperature program used for the chromatographic separation was as follows: 100℃ for 4 min, temperature was increased at 3℃ min-1 to 240℃, held for 10 min, and FID temperature was maintained at 285℃[22].

The normality of the variables was studied by graphic (Q-Q Plot, Box-Plot) and analytic methods (Kolmogorov-Smirnov). The groups of variables with normal distribution (16:0; 22:5 ω3; 22:6 ω3; ω6; PUFA, saturated fatty acids; vitamin $B_{12}$ and folic acid) were compared using ANOVA, followed by Tukey test when $p<0.05$. On the contrary, variables without normal distribution (14:0; 18:2 ω6; 18:3 ω3; 20:3 ω6; 20:4 ω6; 20:5 ω3; ω; homocysteine) were compared with the Kruskal-Wallis, followed by Mann-Whitney test. In order to decrease the risk of type I error due to several comparisons a Bonferroni correction was applied in

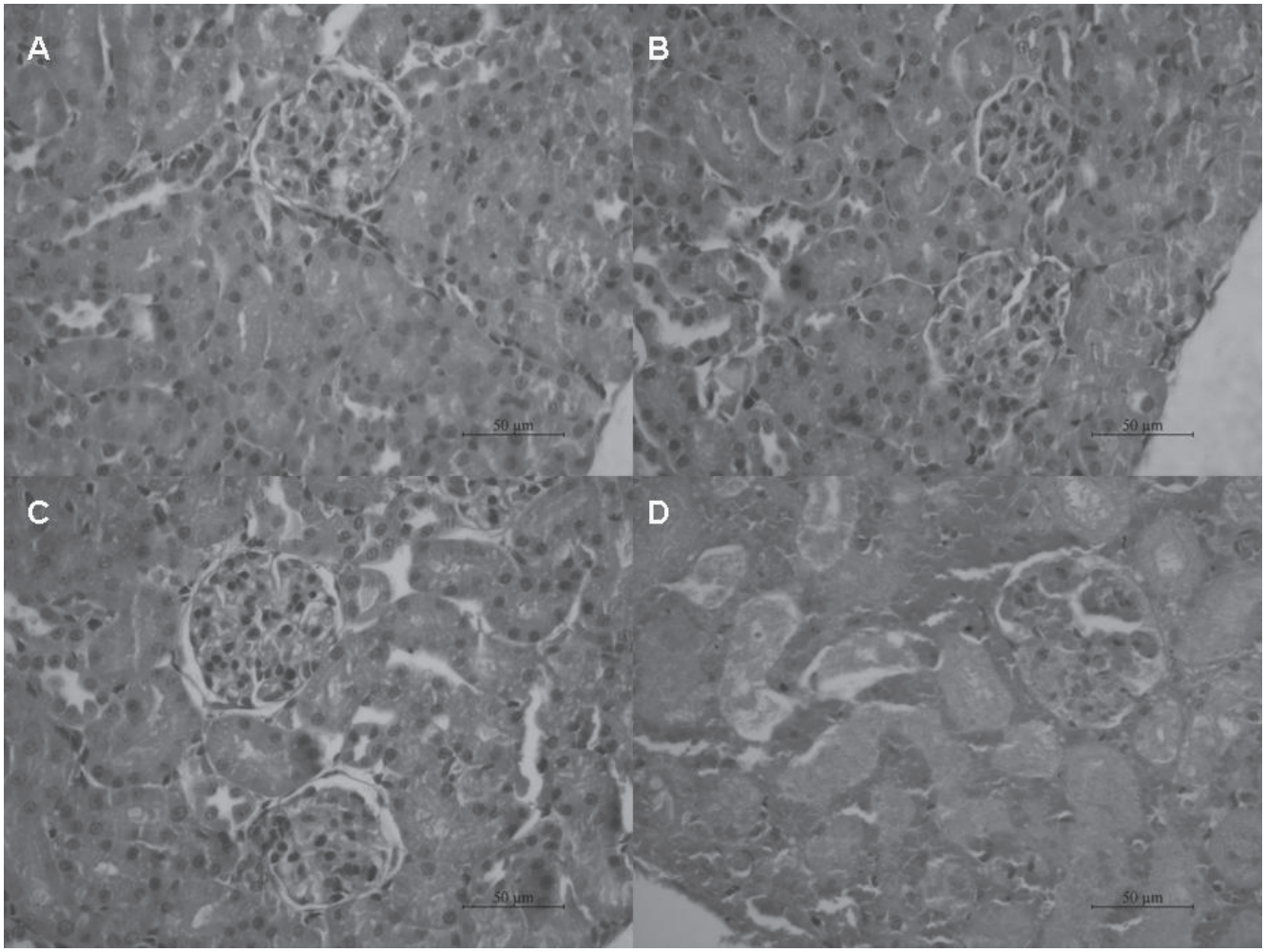


**Fig. 1** A: Kidney from CSVO showing no renal alterations. B: Kidney from CDMO showing no renal alterations. C: Kidney from CSMO showing no renal alterations. D: Kidney from CDVO showing cortical necrosis.

Kruskal-Wallis as well as in Mann-Whitney tests.

## 3 RESULTS

### 3.1 Histopathology

The histopathological classification of renal necrosis was taken from Monserrat *et al*., who divide renal alterations in kidney without necrosis (grade 0), kidney with tubular necrosis (grades 1 to 4), kidney with cortical necrosis (grades 5 to 8) and repair[11]. All rats fed a choline supplemented (CS) diet and a CD diet with menhaden oil as lipid showed no renal alterations (Fig. 1 ABC), while CD with vegetable oil rats evidenced renal cortical necrosis (grade 5) (Fig. 1 D). Renal damage was characterized by increased size and weight and by purplish red discoloration. Necrosis involved tubules and glomeruli, and was characterized by pyknosis, karyolysis and increased eosinophilia.

### 3.2 Fatty acid composition

Fatty acid composition of the three oils used in diets is shown in Table 2. Renal lipids fatty acid in all groups is shown in Table 3. Values are expressed as percentages of total fatty acids. Menhaden oil diet resulted in an important increase in ω3 fatty acids.

### 3.3 Serum markers

Table 4 shows the biochemical analysis of the serum from the four groups of animals and the results expressed as the mean ± standard deviation of the determinations of

**Table 2** Fatty acid composition of the three oils (wt%).

| Fatty acids | Menhaden oil | Hydrogenated oil | Corn oil |
|---|---|---|---|
| 12:0 | 0.11 | 0 | 0 |
| 14:0 | 8.43 | 0.36 | 0 |
| 14:1 | 0.41 | 0 | 0 |
| 15:0 | 0.8 | 0 | 0 |
| 15:1 | 0 | 0 | 0 |
| 16:0 | 18.22 | 17.63 | 12.4 |
| 16:1 trans | 0.2 | 0 | 0 |
| 16:1 | 10.82 | 0.12 | 0.13 |
| 17:0 | 0.71 | 0.17 | 0 |
| 17:1 trans | 0 | 0 | 0 |
| 17:1 | 0 | 0 | 0 |
| 18:0 | 3.49 | 24.82 | 2.25 |
| 18:1 trans | 1.22 | 0.63 | 0 |
| 18:1 ω9 | 6.57 | 14.89 | 33.51 |
| 18:1 ω7 | 3.1 | 0.84 | 0.59 |
| 18:2 trans | 0.69 | 0.35 | 0.2 |
| 18:2 ω6 | 3.7 | 34.8 | 48.43 |
| 20:0 | 0.26 | 0.4 | 0.52 |
| 18:3 ω6 | 0 | 0 | 0 |
| 20:1 | 1.14 | 0.44 | 0.28 |
| 18:3 ω3 | 1.71 | 3.57 | 0.89 |
| 20:3 ω9 | 0 | 0 | 0 |
| 22:0 | 0.18 | 0 | 0.15 |
| 20:3 ω6 | 0 | 0 | 0 |
| 20:4 ω6 | 1 | 0 | 0 |
| 20:5 ω3 EPA | 12.58 | 0 | 0 |
| 22:5 ω3 DPA | 2.42 | 0 | 0 |
| 22:6 ω3 DHA | 12.7 | 0 | 0 |

**Table 3** Renal fatty acid composition (wt%).

| Lipids | CDVO (1) (n=8) | CSVO (2) (n=8) | CDMO (3) (n=8) | CSMO (4) (n=8) | Test (df=3); significance | Post Test (*p*=) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1 vs 2 | 1vs 3 | 1 vs 4 | 2 vs 3 | 2 vs 4 | 3 vs 4 |
| 14:0 | 1.72 (0.23) | 0.89 (0.25) | 3.02 (1.06) | 3.07 (0.83) | X2=22.95; $p<0.001$ | 0.001 | 0.027 | 0.001 | 0.001 | 0.001 | 0.81 |
| 16:0 | 19.67 (1.67) | 20.33 (2.04) | 22.82 (2.87) | 21.36 (2.43) | F=3.17; $p=0.039$ | – | – | – | – | – | – |
| 18:2 ω6 | 23.02 (3.19) | 23.95 (2.89) | 5.22 (1.88) | 5.45 (2.23) | X2=24.09; $p<0.001$ | 0.6 | 0.001 | 0.001 | 0.001 | 0.001 | 0.773 |
| 18:3 ω3 | 0.96 (0.23) | 0.76 (0.19) | 0.57 (0.37) | 0.51 (0.17) | X2=11.96; $p=0.008$ | – | – | – | – | – | – |
| 20:3 ω6 | 0.81 (0.41) | 0.68 (0.22) | 0.41 (0.08) | 0.51 (0.08) | X2=9.797; $p=0.020$ | – | – | – | – | – | – |
| 20:4 ω6 | 8.99 (3.95) | 10.23 (2.13) | 7.28 (1.41) | 8.2 (1.77) | X2=5.925; $p=0.115$ | – | – | – | – | – | – |
| 20:5 ω3 | 0.21 (0.07) | 0.11 (0.03) | 5.23 (1.21) | 5.21 (1.07) | X2=26.016; $p<0.001$ | 0.004 | 0.001 | 0.001 | 0.001 | 0.001 | 0.7 |
| 22:5 ω3 | 0.54 (0.3 ) | 0.27 (0.47) | 1.55 (0.43) | 2.34 (0.64) | F=41.87; $p<0.001$ | 0.57 | 0.001 | 0.001 | 0.001 | 0.001 | 0.003 |
| 22:6 ω3 | 1.66 (0.88) | 1.26 (0.27) | 6.25 (1.64) | 7.83 (1.37) | F=62.92; $p<0.001$ | 0.91 | 0.001 | 0.001 | 0.001 | 0.001 | 0.046 |
| ω6 | 32.82 (1.55) | 34.86 (3.85) | 12.91 (1.95) | 14.16 (3.02) | F=24.777; $p<0.001$ | 0.141 | 0.001 | 0.001 | 0.001 | 0.001 | 0.336 |
| ω3 | 3.36 (0.97) | 2.4 (0.34) | 13.6 (3.2 ) | 15.89 (2.58) | X2=25.881; $p<0.001$ | 0.046 | 0.001 | 0.001 | 0.001 | 0.001 | 0.068 |
| PUFA | 36.39 (2.35) | 37.44 (4.17) | 26.59 (4.25) | 30.17 (2.78) | F=18.14; $p<0.001$ | 0.93 | 0.001 | 0.007 | 0.001 | 0.001 | 0.18 |

**Table 4** Vitamin $B_{12}$, folic acid and homocysteine.

| | CDVO (1) (n=8) | CSVO (2) (n=8) | CDMO (3) (n=8) | CSMO (4) (n=8) | Test (df=3); Significance | Post Test (*p*=) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1 vs 2 | 1 vs 3 | 1 vs 4 | 2 vs 3 | 2 vs 4 | 3 vs 4 |
| Vitamin $B_{12}$ (pg/mL) | 2628 (1087) | 1215 (223) | 1394 (382) | 1164 (207) | F=13.56; p<0.001 | 0.001 | 0.001 | 0.001 | 0.916 | 0.998 | 0.842 |
| Folic Acid (ng/mL) | 14.54 (2.48) | 23.53 (8.58) | 27.63 (7.67) | 20.22 (6.04) | F=5.41; p=0.004 | 0.052 | 0.003 | 0.428 | 0.65 | 0.524 | 0.086 |
| Homocysteine (μmol/L) | 5.8 (2.02) | 3.06 (1.29) | 7.88 (4.21) | 3.31 (2.54) | X2=16.66; p=0.001 | 0.005 | 0.224 | 0.009 | 0.002 | 0.627 | 0.007 |

homocysteine, vitamin $B_{12}$ and folic acid.

### 3.4 Final Body Weight, Kidney Weights

Table 5 shows that renal weight is higher in rats fed CD with vegetable oil diet because of the development of necrosis.

## 4 DISCUSSION

Acute kidney injury may occur due to multiple causes. Some of the patients who develop this syndrome will never regain full renal function. This fact results in end-stage renal failure, which requires either lifelong dialysis or kidney transplant[23]. Since the mechanisms underlying the origins and progression of kidney diseases are not fully understood, the development of new AKI models is mandatory. The rat is a useful experimental model for human AKI[24]. The pathogenesis of the CD diet-induced AKI is uncertain.

**Table 5** Body and Kidney weights (g).

| Diet | Final Body Weight (g) | Right Kidney Weight (g) | Left Kidney Weight (g) |
|---|---|---|---|
| CDVO | 76.20 ± 11.29 | 0.83 ± 0.14 | 0.77 ± 0.11 |
| CSVO | 76.04 ± 13.44 | 0.44 ± 0.05 | 0.44 ± 0.07 |
| CDMO | 51.72 ± 2.77 | 0.32 ± 0.02 | 0.31 ± 0.02 |
| CSMO | 54.23 ± 1.58 | 0.33 ± 0.02 | 0.31 ± 0.02 |

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 menhaden oil as lipid; and CSMO: Choline-supplemented diet with menhaden oil as lipid; g: grams.

As it has already been mentioned, the quantity and quality of lipids influence the development of renal necrosis in this experimental model. Menhaden oil, which is a very important source of ω3 fatty acids, has a notorious protective effect[20, 25].

The purpose of this study was to analyze this protective effect of menhaden oil through changes in renal lipid profile in rats fed menhaden or vegetable oils as lipids. Animals were fed diets containing different oils for 7 days since they develop renal necrosis after 6-7 days of receiving a choline-deficient diet with vegetable oils as lipids after weanling[10]. This short period of time was enough to drastically modify the lipid profile of their kidneys. As it could be expected, ω6 fatty acids were higher in rats fed CD and CS diets with vegetable oils as lipids, while ω3 fatty acids were higher in rats fed CD and CS diets with menhaden oil as lipid. The levels of EPA and DHA were higher in rats fed diets with menhaden oil as lipid, while the levels of 18:3 ω3 were higher in rats fed diets with vegetable oil. In rats fed menhaden oil, an increase in myristic acid was observed.

The notorious increase in vitamin $B_{12}$ in the plasma of rats fed a CD with vegetable oil diet could be due to the

necrosis of the renal tubules that are rich in this vitamin.

Homocysteine levels were higher in rats fed CD diets. As above mentioned, choline through betaine allows the regeneration of methionine from homocysteine. In case of choline deficiency, this transformation does not occur, or if it does, it occurs in lower percentages. The lower levels of folic acid in rats fed a CD with vegetable oil diet could be due to the increase in vitamin $B_{12}$, which consumes folic acid. This could be the reason why levels of homocysteine were not as high as in rats fed a CD with menhaden oil diet.

Results showed changes in the renal lipid profile of rats fed menhaden oil or vegetable oils. Menhaden oil could develop its protective effect through changes in 1) membrane structure, or 2) the generation of second messengers and cell signal transduction pathways, or 3) the synthesis of different prostanoids. It is worth highlighting that although animals received the experimental diet for a very short period of time, ω3 fatty acids were incorporated into their renal membranes. In previous studies we observed that oxidative stress and damage precedes histological changes in kidneys of rats fed a choline-deficient diet. We measured thiobarbituric acid reactive substances (TBARS), an indicator of phospholipids peroxidation and oxidative damage and chemiluminescence, indicator of the content of lipophylic antioxidants, in kidney homogenates. TBARS and chemiluminescence were higher in choline-deficient rats. TBARS were higher in the animals receiving vegetable oil and lower in the rats receiving menhaden oil. Chemiluminescence increased earlier in CD rats fed vegetable oil than in choline-deficient rats fed menhaden oil. Total reactive antioxidant potential (TRAP) is a measure of hydrosoluble molecules. In kidney of CD rats, its value decrease from day 0 to 7[25, 26]. Higher values of antioxidant enzymes were found in Wistar rats fed fish oil diets[27]. The oral administration of ω3 supplement may reduce oxidative stress, histological damage and kidney dysfunction after renal reperfusion injury in Sprague Dawley rats[28].

We have recently investigated the potential protective effects of menhaden oil on the basis of kidney transcriptomic data on this experimental model. Differentially expressed genes were analyzed. 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. Thus, regardless of the presence or absence of choline, menhaden oil produces an upregulation of the Gstp1 gene, involved in the glutathione regeneration pathway as xenobiotic and antioxidant[29].

## 5 CONCLUSION

In conclusion, changes in the lipid renal profile in this nutritional AKI model varied according to type of oil contented in the diet.

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