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Protective effect of myristic acid on renal necrosis occurring in rats fed a methyl-deficient diet

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Abstract. Weanling rats fed a methyl-deficient diet develop acute renal failure, the morphological features of which vary from focal tubular necrosis to widespread cortical necrosis. We and others have shown that coconut oil, rich in saturated fatty acids, has a renal protective effect in this experimental model. In the experiment we are reporting now, we studied which fatty acid is involved in the protection afforded by coconut oil by feeding five groups of methyl-deficient rats a mixture of corn oil and hydrogenated vegetable oil, C6-C8-C10 fatty acids, C12 fatty acid, C14 fatty acid and C16-C18 fatty acids. Five groups of rats receiving the same diets supplemented with choline chloride were used as controls. The group of methyl-deficient rats fed C14 fatty acid (myristic acid) showed a greater percentage of surviving animals and lower renal damage than the other groups of methyl-deficient rats, indicating that the protective effect of coconut oil found in previous experiments is due to its high content of myristic acid.

Key words: Acute renal failure – Choline deficiency – Methyl group deficiency – Myristic acid – Renal necrosis

Introduction

The importance of methyl metabolism is widely recognised both in humans and in experimental animals. Nutrients involved in this metabolism are also known as lipotropic factors and are supplied in the diet mainly as methionine and choline, though folate and vitamin B_{12} are necessary for a normal metabolism [3, 11, 37]. Choline, in addition to being a source of labile methyl groups, is a precursor for the synthesis of choline containing phospholipids and acetylcholine [37, 39].

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

Diet component	Group				
	1, 6	2, 7	3, 8	4, 9	5, 10
Soy bean protein[a]	20	20	20	20	20
Sucrose	48.5	48.5	48.5	48.5	48.5
Cellulose[b]	4	4	4	4	4
Vitamin mixture (without B_{12} and choline)[c]	4	4	4	4	4
Salts (W)[d]	2	2	2	2	2
L-Cystine[e]	0.5	0.5	0.5	0.5	0.5
Safflower oil[e]	1	1	1	1	1
Hydrogenated vegetable oil[g]	14.3	–	–	–	–
Corn oil[h]	5.7	–	–	–	–
Caproic acid[e] (C6)	–	6.6	–	–	–
Caprylic acid[e] (C8)	–	6.8	–	–	–
Capric acid[e] (C10)	–	6.6	–	–	–
Lauric acid[e] (C12)	–	–	20	–	–
Myristic acid[e] (C14)	–	–	–	20	–
Palmitic acid[e] (C16)	–	–	–	–	10
Stearic acid[e] (C18)	–	–	–	–	10

Diets in groups 6–10 were supplemented with 0.35% choline chloride
[a] Soybean protein grade II, U.S. Biochemical Corp., USA
[b] Celufil. Non-Nutritive Bulk, U.S. Biochemical Corp., USA
[c] Vitamin Diet Fortification Mixture, U.S. Biochemical Corp., USA
[d] Salt Mixture Wesson Modification, U.S. Biochemical Corp., USA
[e] U.S. Biochemical Corp., USA
[f] Flora Dánica, Buenos Aires, Argentina
[g] Mazola, Refinerías de Maíz, Buenos Aires, Argentina

In the past few years, Ghoshal and Farber [8] have proposed that choline deficiency should be differentiated from methyl group deficiency because the pathology induced by the two deficiencies, as well as the pathogenetic mechanisms involved, could be different. The diets we implemented in the present experiment should be considered methyl-deficient diets rather than choline-deficient diets.

Rats fed a diet deficient in lipotropic factors may develop changes in different organs, mainly the liver (steatosis, cellular death, cirrhosis and cancer) [3, 8, 11, 26], the heart (steatosis and necrosis) [2, 34] and the kidneys. Weanling rats fed a methyl-deficient diet show acute renal failure, the morphological features of which vary from focal tubular necrosis to widespread cortical necrosis or eventual morphological evidence of reparative processes [11, 20].

Along with others, we have found that coconut oil (rich in saturated fatty acids) in the diet has a protective effect in relation to the renal lesions observed in methyl-deficient rats [19, 34, 36]. The purpose of the experiment we are reporting was to further explore which fatty acid is involved in the protection afforded by coconut oil. This would be important to improve our knowledge of pathogenic mechanisms involved in renal tubular necrosis and cortical necrosis.

Materials and methods

Eighty conventional weanling Wistar male rats from the Facultad de Farmacia y Bioquímica of the University of Buenos Aires were divided into ten different groups. The rats in groups 1–5 (ten rats in each group) were fed ad libitum the methyl-deficient diets shown in Table 1, while those in groups 6–10 (six rats in each group) were used as controls for the methyl-deficient animals and were fed the same diets supplemented with 0.35% choline chloride. All diets had 1% safflower oil in order to provide essential fatty acids. The different lipids were given at a 20% concentration, because this is the concentration of coconut oil that afforded protection in previous experiments [19, 36]. Groups 1 and 6 received a mixture of corn oil and hydrogenated vegetable oil to supply different fatty acids; these are the type of lipids we have used in previous experiments [2, 17, 19, 20] and groups 1 and 6 were thus used as controls for those rats receiving the individual fatty acids. All animals had free access to a drinking bottle with tap water, were individually housed in suspended wire-bottomed cages in an air-conditioned room and were exposed to light from 7.00 a.m. to 7.00 p.m. Body weight and food intake were measured daily.

The rats were kept on the diets until they died or, if death did not occur, they were killed on the 21st day. Under light ether anesthesia, blood was drawn from the abdominal aorta without an anticoagulant, and urea and creatinine were determined in serum by standard procedures. The heart, kidneys and liver were removed and weighed. Tissue samples were fixed in buffered formalin and embedded in paraffin, and sections were stained with hematoxylin-eosin or special stains such as periodic acid-Schiff (PAS), phosphotungstic acid-hematoxylin (PTAH), Masson's trichrome or von Kossa [15]. Frozen sections of the liver were stained with oil red O [35].

Fatty changes in the liver were evaluated in the frozen sections stained with oil red O and the changes classified as mild, moderate or severe according to the extent (zonal to diffuse) of the fat deposition in the liver lobule.

For topographical localisation of different segments of the kidney, well-known terminology was used [30]. A previously proposed classification was applied to evaluate the renal pathology [17]:

A Kidney without necrosis

B Acute tubular necrosis
– Grade 1: isolated foci of cellular necrosis in some tubules
– Grade 2: small group of tubules with necrosis
– Grade 3: zones of tubular necrosis
– Grade 4: confluent zones of tubular necrosis

C Cortical necrosis
– 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
– Grade 8: massive cortical necrosis

D Repair (characterized by different degrees of interstitial fibrosis, tubular atrophy, tubular regeneration, glomerular fibrosis, etc.; according to its extension it is divided into four grades)

The Fisher's exact test was employed to statistically evaluate mortality, while renal and body weights and length of survival as well as creatinine and urea in serum were expressed as mean ± standard error; comparisons were made by one-way analysis of variance (ANOVA). In the case of significant differences, ANOVA was followed by the Tukey-Kramer test. Differences which resulted in P values lower than 0.05 were considered significant. A personal computer version of the InStat program was used (GraphPad Software, San Diego, version 2, 1990.

Table 2. Initial, maximum and final body weights (mean ± SE)

Group	Initial body weight (g)	Final body weight (g)		Maximum body weight (Dead rats)	
		Died	Killed	g	Day
1	46.0±1.1 (10)	53.1±1.9 (10)[a]	–	64.0±2.0 (10)[d]	5.5±0.2
2	45.3±1.0 (10)	41.6±3.4 (8)	75.5±13.5 (2)	51.1±2.0 (8)	6.3±1.5
3	46.3±1.1 (10)	31.8±1.0 (10)[b]	–	46.3±1.1 (10)[b]	0.0±0.0[e]
4	44.9±1.0 (10)	58.5±5.5 (2)[c]	86.5±3.8 (8)	68.0±5.0 (2)	8.5±0.6
5	45.6±0.7 (10)	47.7±1.3 (10)	–	55.7±1.4 (10)	6.1±0.7
6	46.3±1.6 (6)	–	122.8±7.3 (6)	–	–
7	45.2±1.5 (6)	31.0 (1)	85.2±4.4 (5)	45.0 (1)	0.0
8	45.7±1.3 (6)	31.2±1.1 (6)	–	45.7±1.3 (6)	0.0
9	45.5±1.4 (6)	28.0 (1)	108.2±6.5 (5)	40.0 (1)	0.0
10	45.2±1.6 (6)	–	94.8±7.3 (6)	–	–

Figures in parentheses indicate the number of rats
[a] Signficiantly different from groups 2 and 3
[b] Signficiantly different from groups 4 and 5
[c] Signficiantly different from group 2
[d] Signficiantly different from groups 2, 3 and 5
[e] Signficiantly different from groups 1, 2, 4 and 5

Table 3. Mortality, survival time, urea and creatinine (mean ± SE)

Group	Rats (*n*)	Died (*n*)	Length of survival (days)		Urea (mg/dl)[a]	Creatinine (mg/dl)[a]
			Mean	Median		
1	10	10[b]	9.2±0.4	9.0	–	–
2	10	8[b]	10.8±1.5[c]	11.0	151±118[b]	2.1±1.2
3	10	10	5.7±1.3	4.0	–	–
4	10	2	9.0±0.0	9.0	53±12	1.5±0.6
5	10	10	8.5±0.8	9.0	–	–
6	6	0	21.0±0.0	21.0	18±2	0.8±0.1
7	6	1	2.0±0.0	2.0	25±4	0.7±0.1
8	6	6	4.7±1.1	3.5	–	–
9	6	1	2.0±0.0	2.0	23±4	0.7±0.1
10	6	0	21.0±0.0	21.0	26±1	0.8±0.1

[a] Only rats that were killed
[b] Significantly different from group 4
[a] Significantly different from group 3

Results

The initial, final and maximum body weights are indicated in Table 2. As indicated by maximum body weights, methyl-deficient rats in groups 1, 2, 4 and 5 grew adequately well during the first 5–8 days. Later on, as a consequence of the severe renal damage, they lost weight (difference between maximum and final body weights in dead rats). The rats in group 3 steadi-

Table 4. Renal weights (mean ± SE)

Group	Dead		Killed	
	g	g/100 g BW	g	g/100 g BW
1	1.813±0.115 (10)[a]	3.394±0.132	–	–
2	1.076±0.178 (8)	2.455±0.264	1.354±0.384 (2)	1.945±0.567
3	0.542±0.026 (10)[b]	1.706±0.071[b]	–	–
4	1.843±0.227 (2)	3.140±0.100	1.164±0.058 (8)	1.356±0.074
5	1.396±0.111 (10)	2.913±0.209	–	–
6	–	–	1.542±0.073 (6)	1.262±0.038
7	0.580 (1)	1.870	1.033±0.038 (5)	1.220±0.059
8	0.531±0.027 (6)	1.708±0.087	–	–
9	0.433 (1)	1.546	1.354±0.131 (5)	1.244±0.057
10	–	–	1.133±0.082 (6)	1.200±0.041

Figures in parentheses indicate the number of rats
[a] Significantly different from groups 2 and 3
[b] Significantly different from groups 1, 2 and 4

Table 5. Renal histopathology (*NN* no necrosis, *TN* tubular necrosis, *CN* cortical necrosis, *R* repair)

Group	Dead rats				Killed Rats			
	NN	TN	CN	R	NN	TN	CN	R
1								
(*n*)	–	2	7	1	–	–	–	–
Grade[a]	–	4.0±0.0	5.7±0.3	3.0	–	–	–	–
2								
(*n*)	3	4	1	–	–	1	–	1
Grade[a]	–	3.0±0.0	5.0	–	–	3.0	–	1.0
3								
(*n*)	10	–	–	–	–	–	–	–
Grade	–	–	–	–	–	–	–	–
4								
(*n*)	–	2	–	–	4	–	–	4
Grade[a]	–	3.0±1.0	–	–	–	–	–	2.3±0.5
5								
(*n*)	1	5	4	–	–	–	–	–
Grade[a]	–	3.8±0.2	5.0±1.4	–	–	–	–	–

[a] Mean ± SE

ly lost body weight. Choline-supplemented rats also grew well except those fed lauric acid (group 8) and those that died in groups 7 (n=1) and 9 (n=1).

Mortality, length of survival and urea and creatinine in serum are shown in Table 3. As expected, mortality was high (100%) in the methyl-deficient rats in group 1; the lowest mortality rate (20%) was found in group 4 (myristic acid). Rats fed lauric acid showed mortality rate of 100% in both the methyl-deficient and the choline-supplemented rats. In both these groups,

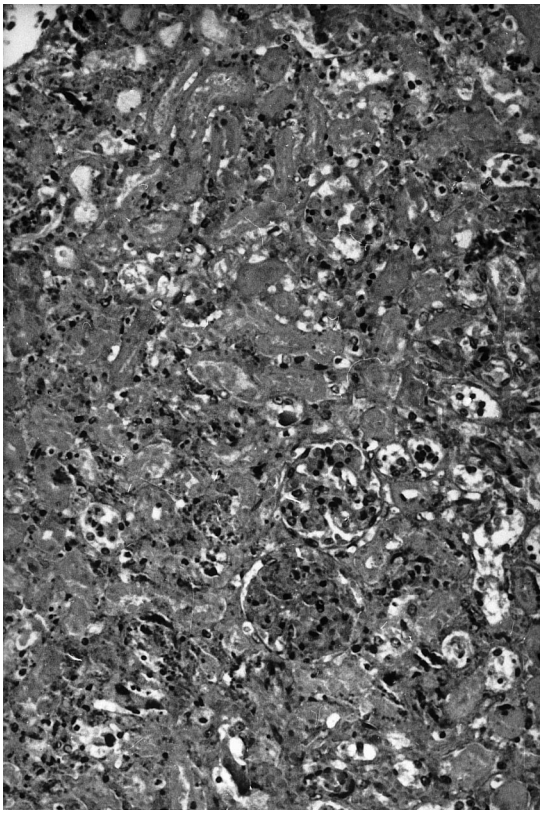

Fig. 1. Cortical necrosis in a choline-deficient rat in group 1 found dead on day 8. H&E, ×225

the length of survival (mean and median) was shorter than that observed in the other methyl-deficient groups.

Urea and creatinine in serum were within the normal range in surviving choline-supplemented rats, while they were markedly elevated in those in group 2 and slightly elevated in those in group 4.

Renal weights (Table 4) were higher in those rats whose death was attributable to methyl deficiency. Renal weight was not increased in rats fed lauric acid (groups 3 and 8) or in the choline-supplemented animals.

The acute renal changes were grossly characterized by an increase in size and weight and by a purplish-red discoloration. Necrosis mainly involved proximal convoluted tubules and was characterized by increased eosinophilia, changes in tinctorial affinities with Masson's thrichrome and PTAH, pyknosis and mainly kariolysis; in more advanced stages, glomerular and vascular necrosis were also observed. Concomitant with tubular necrosis, hyalin casts were found. Repair was characterized by the disappearance of necrotic tubules and casts and by different degrees of regeneration, tubular atrophy and fibrosis The result of the histological study is shown in Table 5. Most rats in groups 1, 2 and 5 showed the typical renal lesions of tubular or

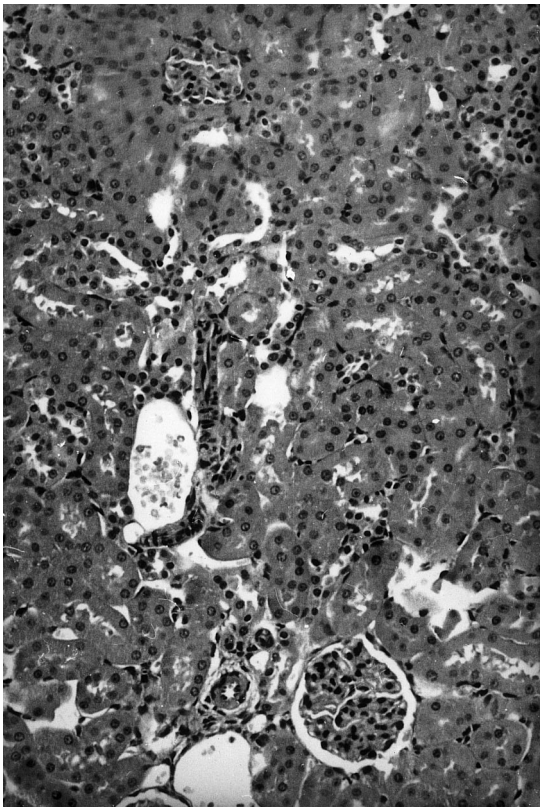

Fig. 2. No renal damage in a choline-deficient rat in group 4 killed on day 21. H&E, ×225

cortical necrosis found in methyl deficiency (Fig. 1). Three dead rats in group 2 did not show renal necrosis, and the deaths cannot be attribute to methyl deficiency. Myristic acid showed a clearly protective effect. Two dead rats in group 4 (myristic acid) displayed tubular necrosis, while, of the eight rats killed on the 21st day, four did not show renal damage (Fig. 2) and four displayed reparative changes (Fig. 3). Methyl-deficient rats fed lauric acid did not show renal necrosis, and choline-supplemented rats did not show renal damage (Fig. 4).

Rats in group 1 displayed a moderately or severely fatty liver, rats in group 4 a severely fatty liver, and those in group 5 a moderately fatty liver. In most cases, fatty liver was of the macrovesicular type. Rats in group 2 showed mild steatosis or no fatty changes. Rats in group 3 did not show a fatty liver. Animals fed the choline-supplemented diets did not show steatosis. The weight of the liver increased according to the extent of fat deposition

Histological changes in the heart were as follows: in group 1, two rats showed necrosis and one focal interstitial myocarditis; in group 2, two rats displayed necrosis; in group 3, four rats showed mild interstitial myocardi-

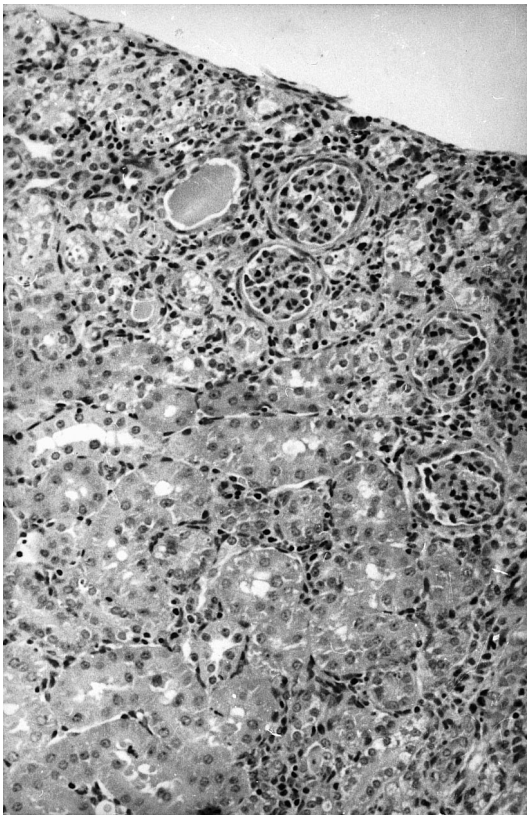

Fig. 3. Repair in a choline-deficient rat in group 4 killed on day 21. H&E, ×225

tis; in group 4, one rat showed necrosis; and in group 5, six rats showed focal myocarditis, one necrosis and nine multiple foci of calcifications clearly shown by von Kossa's method. In the choline-supplemented rats, foci of interstitial myocarditis were found in one rat in group 8.

Discussion

Though Best and Huntsman [4] showed that choline prevented the deposition of fat in the liver in 1932, it was not until 1939 that Griffith and Wade [10] discovered that weanling rats fed a choline-deficient diet develop renal necrosis.

Primary tubular changes or ischaemia have been postulated as pathogenic mechanisms [16, 21, 32]. Local intravascular coagulation has been proposed as the intermediate mechanism between tubular and cortical necrosis [17].

The possible pathogenic role of changes in the renal lipids has, in this experimental model, been repeatedly studied without obtaining clear evidence of a particular lipid change in relation to the renal necrosis [7, 18, 27]. On

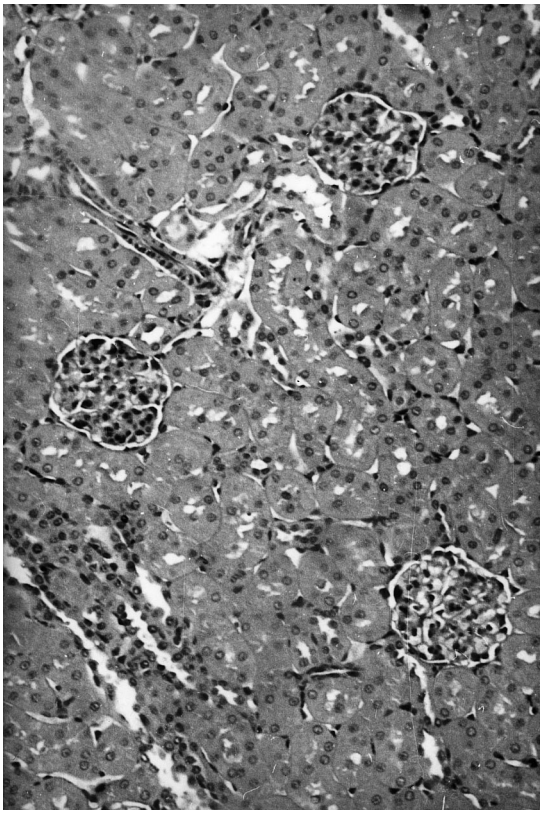

Fig. 4. Normal features in a choline-supplemented rat in group 6 killed on day 21. H&E, ×225

the other hand, it is known that, in rats fed a choline-deficient diet, both the quantity and the quality of dietary lipids can modulate the renal lesions [9, 23].

In 1954, Wilgram et al. [34] stated that renal lesions did not develop when the rats were fed a coconut oil-enriched, choline-deficient diet; furthermore, Zaki et al. [36] showed that coconut oil prevented the renal lesions induced by choline-deficiency. Recently, we [19] have shown that coconut oil at a 20% concentration has a kidney-protective effect in this experimental model, particularly if 1% safflower oil is added to the diet. Coconut oil is rich in saturated fatty acids, whereas safflower oil is rich in linoleic acid.

The protective effect of coconut oil in relation to the renal necrosis observed in weanling rats fed a methyl-deficient diet raises the question of which fatty acids afford the protective effect.

Zaki et al. [36] studied the effects of short- (C6–C10), medium- (C12–C14) and long chain (C16–C18) fatty acids in relation to the liver and kidney of choline-deficient rats. They found a protective effect of the C12–C14 fraction in relation to mortality and renal lesions.

In the experiment we are now reporting, we have studied this point further by feeding five groups of methyl-deficient rats a mixture of corn oil and hydrogenated vegetable oil (group 1), C6–C8–C10 fatty acids (group 2), C12 fatty acids (group 3), C14 fatty acids (group 4), or C16–C18 fatty acids (group 5); rats receiving the same diets supplemented with choline chloride were used as controls. All groups received 1% safflower oil in order to provide essential fatty acids. Our results indicate that the protective effect of coconut oil is due to C14 (myristic acid).

The type of lipid present in the diet in experiments in vivo or in the culture medium in experiments in vitro can alter the lipid composition of cellular structures, influence many cellular activities and induce a different behaviour vis-à-vis cellular injury [1, 13, 25, 28, 31, 33]. Thus the severity of cellular injury induced by H_2O_2 in OK cells was changed according to the polyunsaturated fatty acid content of the cell membrane [25]. An increase in lipid peroxidation is observed after the intake of polyunsaturated fatty acids [6]. Cultured endothelial cells supplemented with polyunsaturated fatty acids have increased toxicity and lipid peroxidation following exposure to H_2O_2 [12].

Sequential in vivo and in vitro experiments on changes in renal lipid peroxidation in weanling rats fed a methyl-deficient diet [16] and on the in vivo protective effect of antioxidants [16, 22] strongly support the hypothesis that free radicals play an important pathogenic role in the tubular necrosis found in this model.

It is tempting to speculate that the protective effect of saturated fatty acids is due to the fact that they are less susceptible to peroxidation than unsaturated fatty acids. However, all diets had 1% safflower oil and, furthermore, this fact does not explain why such a protective action was not observed in animals in groups 2 (C6–C8–C10) and 5 (C16–C18). It seems that the protection is due to the specific effect of myristic acid rather than to a general action due to saturated fatty acids.

In animals fed a choline-deficient diet, the fatty liver is due to an impairment in the assembly of lipoproteins [37]. The exogenous supply of fatty acids affects the secretion of very low density lipoproteins (VLDL) [31]. As expected, in this experiment, no fatty liver was found in the choline-supplemented animals. Rats fed the choline-deficient diet showed a moderate to severe fatty liver in groups 1, 4 and 5, while mild fatty liver was observed in four rats in group 2 fed C6–C8–C10 fatty acids. These results are similar to those obtained by Stetten and Salcedo [29]. No fatty liver was found in rats in group 3 fed lauric acid.

It is known that choline deficiency induces cardiac lesions, including cardiac necrosis [2, 24]. In this experiment, we found cardiac necrosis attributable to choline deficiency in two rats in group 1, two in group 2, one in group 4 and one in group 5. It is interesting to note the high frequency (nine out of ten) of calcifications in rats in group 5.

Kesten et al. [14] found that 15 out of 16 rats fed a choline-deficient diet with 35% lauric acid died. The average survival time was 4.4 days. The addition of 0.5% choline chloride prevented death in four out of four animals. The authors described “widespread interstitial myocarditis associated usually with necrosis of isolated muscle cells”; the addition of choline chloride “completely prevented the myocardial changes”.

By ingestion lauric acid has an LD_{50} of 12 g/kg [5], so the ingestion of lauric acid in rats in groups 3 and 8 was above LD_{50}. Mortality in group 8 obviously cannot be attributed to methyl deficiency. On the other hand, in group 3, in which rats were fed a methyl-deficient diet and lauric acid, the average survival time (4.0 days) was too short to attribute death to choline deficiency and, furthermore, the typical renal changes of choline deficiency ("haemorrhagic kidneys") were not found in animals in group 3. Of the rats fed lauric acid, interstitial myocarditis was found in four out of ten methyl-deficient and in one out of six choline-supplemented rats.

In relation to the renal lesions, experiments are now in progress to study the influence of other types of fatty acids and to analyse the mechanism of action of the protective effect of myristic acid. It is possible to speculate that its effect is due to changes in cell signalling [38].

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