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New Insight into the Biochemical Pathology of Liver in Choline Deficiency

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ABSTRACT: A diet deficient in choline can cause liver cancer in rats. The previous work since 1932 emphasized the fat-removing ability of choline from the liver. There are other dietary factors, including methionine, which, like choline, can remove fat from the liver. These factors were termed as lipotropes. Since then, choline deficiency and lipotrope deficiency are used synonoumously. Recent work since 1980 has clearly demonstrated that choline deficiency (CD) and lipotrope deficiency (LD) are not the same. Generation of free radicals, DNA alterations, liver cell death, and liver cancer that occur due to CD are not generated by LD. Generation of free radicals due to CD diet and some of the agents that counteract free radical action also prevent CD effects except for lipid accumulation in the liver. Despite the recent observations on the role of phospholipase A_2 (PLA_2) as the protector of the membranes, it has been found that by preventing the rise of PLA_2 in the liver, cell death can be prevented. These new findings give choline a distinct role in liver cell death and cancer rather than the role of lipotrope. A new hypothesis linking dietary choline deficiency and liver cancer has been discussed.

KEY WORDS: choline deficiency, lipotrope deficiency, dietary liver cancer, liver cell death, free radicals, spin traps, phospholipase A_2.

I. INTRODUCTION

The existence of choline was recognized more than a century ago.[36] The deficiency syndrome was discovered accidentally a long time thereafter.[2] While working with the depancreatized dogs whose blood glucose levels were maintained in the normal range by insulin, Best and Huntsman found that the dogs developed fatty liver, although the dogs were ingesting lean meat and carbohydrate (no fat). The fat accumulation was prevented when dogs were fed raw pancreas along with their food. The effective constituent was later discovered to be choline. This discovery set the research on choline deficiency fatty liver into motion. Later, it was found that other dietary components, including methionine, betaine, folic acid, vitamin B12, pantothenic acid, inositol, pyridoxin, and riboflavin have the ability, although to a lesser degree, to remove "fat" from the liver.[4] This property of removing

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"fat" (triglyceride [TG] or triacrylglycerol [TAG]) from the liver is described as lipotropism, and these agents are designated as lipotropes or lypotropic compounds.[3]

A. What is Choline?

Choline is a positively charged, quaternary ammonium compound. It is abundantly present in the animal and plant kingdoms as the base of phospholipid lecithin. Lecithin appears to be an integral part of most, if not all, cell biological membrane systems.

Fatty liver due to the lack of choline or its precursors has been observed in the rat, hamster, monkey, mouse, dog, rabbit, calf, pig, duckling, and guinea pig. In the guinea pig, however, it is difficult to produce fatty liver of this type, apparently because it dislikes the unnatural rations.[20] Experimentally, humans can be made choline deficient after 3 weeks on a choline-deficient diet,[33] exhibiting a decline in plasma choline concentration associated with liver dysfunction.[40]

The synthesis of phosphatidyl choline (lecithin) in the body and its turnover is very rapid.[23] For example, the half-life of lecithin in rat liver microsomes is 8 h. This rapid turnover of lecithin may explain why a choline deficiency manifests itself so quickly. Eight to ten hours of diet without choline causes accumulation of TG in the rat liver[42] (see Figure 1). It can be seen that only TG increases, while non-TG lipids remain virtually unchanged. The diet used was deficient only in choline.

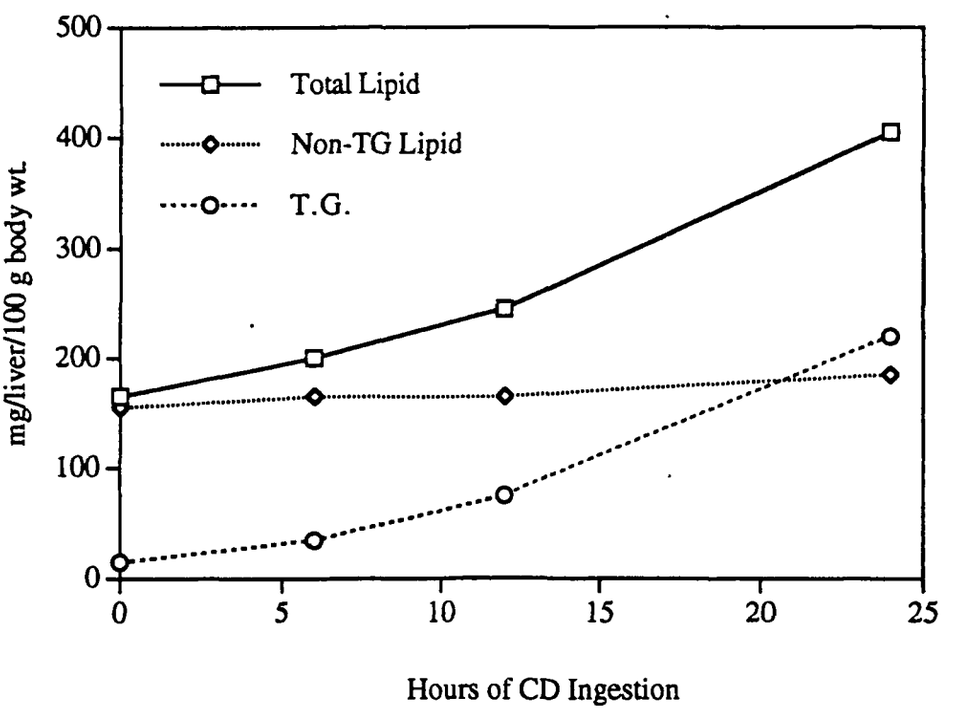

FIGURE 1. Liver lipid analysis with choline deficiency (CD diet). (From Ghoshal, A. K. and Recknagel, R. O., 1963, unpublished findings. With permission.)

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B. CD vs. Lipotrope Deficiency

Because choline has been linked from the very beginning to the removal of TG from the liver, research became focused on the mechanisms of TG removal. To generate a rapid deposition of TG, all or several of the lipotropes were removed from the diet. However, as choline deficiency was the major component, the diet continued to be designated as choline deficient rather than lipotrope deficient. This inexact terminology continues to create confusion to this day. Many reviewers of choline deficiency quote data without mentioning whether the source of the data is from "pure" choline deficiency or from lipotrope deficiency.

The importance of more exact terminology became apparent when it was discovered in the early 1980s that the removal of choline from an otherwise normal diet (without any known added carcinogen) is associated with liver cell death and eventual cancer development. Such rapid liver cell death and probably liver cell cancer were not found in lipotrope deficiency.

Although the major focus of this review article is on research done since early 1980s using "pure" CD diet, a brief discussion on the results obtained with a "pure" CD diet and those with Lipotrope-deficient diet seems appropriate.

C. Differences between CD- and Lipotrope-deficient diets

One major source for the complexity and confusion is the multiple origins for utilizable choline. Choline, as lecithin, is a common and almost ubiquitous dietary component. Choline can also be synthesized from phosphatidyl ethanolamine by methylation by *S*-adenosyl-methionine. These two major sources for choline in lecithin make it virtually impossible to devise an effective choline-deficient diet without a considerable decrease in the intake of methionine.

When methionine, vitamin B12, and folic acid are removed or greatly reduced in the diet with an absence of choline to generate a lipotrope deficiency, the diet not only affects the liver but also other organs.[26] When the diet given to rats is devoid of choline alone, the liver is affected almost exclusively.[16] An effective and acceptable diet, deficient in choline, but adequate in all the other known lipotropes, was devised by Young et al.[39] in 1956. This is the diet used by Lombardi and associates for many years and by us more recently. The methionine content, 1800 mg/kg of food, is adequate for normal growth of the young adult rat.[32]

Methionine was removed almost entirely in lipotrope-deficient diets because of the misconception that choline deficiency is nothing more than methyl deficiency.[24,26] It should be realized that choline is not very efficient as a donor for methyl groups. (1) As a methyl group donor, choline is weak and donates only one methyl group. Also, choline must first be oxidized to betaine before it can act as a methyl donor for methionine synthesis. (2) A choline-devoid (CD) diet that contains just adequate levels of methionine induces a choline deficiency even though the level of methionine in the blood and other tissues, including liver, remains in the control range.[34] (3) It requires about two to three times more methionine than choline, on a molar basis, to prevent or reverse the effects of choline deficiency.[39,41] In choline deficiency, the *S*-adenosyl-methionine (SAM) pathway increases by two- to threefold.[22] The SAM pathway synthesizes lecithin in the membrane by transferring methyl groups to ethanolamine. It appears that the lecithin synthesized under

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the usual CD regimen is not sufficient even though there is no shortage of methionine (methyl group). These considerations suggest that the pathobiology of choline deficiency is a reflection of a deficiency of choline and not a reflection of methyl group deficiency. Designating choline deficiency as methyl deficiency is not just an academic one. The pathologic consequences of choline deficiency and lipotrope-deficient (LD) diets creates misleading conclusions. Differences are shown in Table 1. As can be seen from the table, rats do not gain body weight with an LD diet. In nutritional experiments, body weight gain should not be compromised.

II. ACUTE AND LONG-TERM EFFECTS OF CD

A diet devoid of choline when given to male Fischer 344 rats induces a highly reproducible series of changes in the liver[16] (see Table 2). Within 6 to 8 h, the liver begins to accumulate triglycerides (triacylglycerols) in the hepatocytes in zone 1, spreading to zones 2 and 3 to involve the whole liver by days 4 to 5. Previously, it was considered that choline deficiency caused zone 3 (centrilobular) fat accumulation.[27] What was studied was lipotrope, not choline deficiency. Because choline has been linked to the removal of triacylglycerols (triglyceride, "fat") from the liver, research became focused on the mechanism of this fat removal. In order to generate rapid and heavy deposition of fat in the liver, not only choline but also methionine, vitamin B12, and folic acid were removed from the diet. Many authors have designated this diet as CD. A more appropriate term is LD.[3,39] This diet was not only nutritionally very inadequate but also had other complications. In other words, lipotrope deficiency and choline deficiency have radically different effects on the liver. These differences have been adequately discussed.[17]

TABLE 1
Comparison of Major Differences Between Pathologic Consequences in the Rat With Choline-Devoid and Lipotrope-Deficient Diets[17]

Consequence	Choline devoid	Lipotrope deficient
Body weight	Good weight gain	Poor or no weight gain
Fatty liver	Very rapid periportal (zone 1)	Rapid central (zone 3)
Necrosis	Early (4.5 to 5 d) focal and widespread; at least 50% of hepatocytes within 2 weeks	None; only some ill-defined hepatocyte injury associated with fatty cysts at a late period
"Fatty cysts" (lipodiastemata)	Not seen	Regularly seen after many weeks of LD diet
Cirrhosis	Very infrequent, even after 2 years	Very frequent, almost every rat
Hepatocellular carcinoma	Frequent — at least 50 to 70% of male rats by 2 years	Uncertain

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TABLE 2
Sequence of Known Liver Changes with Dietary Choline Deficiency

Time	Biochemical pathology
6 to 8 h	Fatty liver, periportal (not central) progressively involving all the liver cells by days 4 to 5
24 h	Lipid peroxidation in nuclei
48 h	Alkali-sensitive alteration in DNA
72 h	Increase in activity of phospholipase A_2 in microsomes (but not in nuclei)
$4^1/_2$ to 5 d	Onset of progressive liver cell death with at least 50% by day 14
5 d	Lipid peroxidation in mitochondria
10 weeks	Initiation of liver carcinogenesis with appearance of rare resistant hepatocytes with resistance phenotype
1 year	First appearance of hepatocellular carcinoma with metastasis

The next temporal occurrence in the liver in choline deficiency is the appearance of lipid peroxidation in the nuclear membranes as detected by diene conjugates within 24 h and by the appearance of aldehydes both histochemically and chemically.[12,30,32] This is followed by DNA alteration at 48 h.[31] Within 3 d, phospholipase A_2 increases in microsomes but not in nuclei.[21] By day 5, one can detect mitochondrial lipid peroxidation. Hepatocyte cell death appears at 4 1/2 to 5 d. This increases so that about 50% of the liver shows cell death by 14 d.[14] Initiation of hepatocellular carcinogenesis, as assessed by the appearance of rare resistant hepatocytes with a special "resistance phenotype",[10,18,29] becomes evident at about 10 weeks after beginning the exposure to the CD diet.[18] Hepatocellular carcinoma appears at about 1 year.[15,16,24,38] This temporal sequence of events is shown in Table 2. The acute changes from the triacylglycerol accumulation to the appearance of resistant hepatocytes in the liver are very reproducible and appear in 100% of the rats. More than 50% of the rats eventually develop liver cancer.[16] This CD model is very useful to study the different alterations in the liver, including cell death.

A. Evidence of Free Radical Involvement in CD

Lipid peroxidation in the nuclear membranes (both inner and outer) within 24 h of CD diet feeding is the strongest evidence of free radical activity. This was supported not only by the subsequent appearance of aldehydes[9] but also by the demonstrations that lipid peroxidation can be completely prevented by (1) dietary supplementation with a radicophil, AD_5,[19] calcium and strontium,[18] and (2) spin traps such as α-phenyl-*tert*-butylnitrone (PBN) and *tert*-nitrosobutane (tNB).[14] All these agents not only prevent peroxidation of the membrane but also prevent the further sequence of DNA damage, cell death, and cell proliferation. The spin traps also prevent the PLA_2 increase.[12] However, none of these can prevent fat accumulation in the liver; only choline supplementation can prevent the triacylglycerol accumulation.

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TABLE 3
Evidence for Free Radical Activity in Liver with Choline Deficiency

1. Lipid peroxidation in nuclei by 24 h
2. Radicophile AD_5 (*N-p*-methoxyphenylacetyldehydroso-alanine) prevents lipid peroxidation
3. Free radical trapping agents, such as α-phenyl-*tert*-butylnitrone (PBN) and *tert*-nitrosobutane (tNB) prevent lipid peroxidation
4. Calcium and strontium[18] prevent lipid peroxidation
5. Lipid peroxidation is followed in time by DNA alteration and hepatocyte cell death, and all of these effects are prevented by the above agents
6. Inhibition of phospholipase A_2 (PLA_2) increases lipid peroxidation, which now appears also in microsomes
7. Aldehydes appear in liver cells following lipid peroxidation as with other instances of lipid peroxidation in the liver (see, e.g., Esterbauer[9]), and these are prevented by PLA_2 as well as by inhibiting lipid peroxidation
8. Radicophile AD_5, PBN, and tNB prevent not only lipid peroxidation but also DNA alterations and cell death even when administered after lipid peroxidation appears in the nuclei

III. POSSIBLE ROLE OF THE AGENTS MENTIONED ABOVE IN THE PREVENTION OF LIPID PEROXIDATION

AD_5 (*N-p*-metoxyphenylacetyldehydrosoalanine) is a radicophile.[37] This compound has been shown to be a free radical scavenger for superoxide anion and hydroxy radical.[5,6] It has been shown in our laboratory[19] that AD_5 can inhibit *in vivo*-induced microsomal lipid peroxidation.

IV. CALCIUM AND STRONTIUM

How does excess Ca^{2+} protect the membrane from peroxidation? Normally, free radicals are believed to be generated in small amounts during oxidative reactions. Membranes have many defense mechanisms by which this small amount of free radicals can be counteracted. However, when excessive generation of free radicals or a reduction of defense mechanism occur, this condition can become unbalanced. What is the mechanism by which choline deficiency can unbalance the system? In this respect, choline could have a dual role. It has been shown by *in vitro* experiments[25] that lecithin (choline) can break down preformed lipid hydroperoxides. Another property of choline is its positive charge. In choline deficiency, the reduction of choline in the membrane[21] may make the membrane relatively more negatively charged and possibly susceptible to free radical attack. Calcium has two positive charges. Calcium in moderate excess may attach itself to the membrane and presumably restore the positive charge. The addition of extra calcium to a CD diet prevents all the early CD-induced changes except fat accumulation. This property of extra calcium is shared by strontium, which also has two positive charges.

V. SPIN TRAPS

The role of spin traps in the prevention of free radical attack may be more straightforward. Spin traps are used for capturing and identifying free radicals. When rats were pretreated with spin traps such as tNB and

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PBN before the CD diet was started, free radical formation, as measured by lipid peroxidation, was prevented. Pretreatment with spin trap agents prevented lipid peroxidation, DNA damage, phospholipase A_2 (PLA_2) activation, and cell death but not the accumulation of triacylglycerols.

Although there appears to be considerable evidence for the presence of free radicals in the livers of rats after CD exposure, the exact identity of the free radicals has yet to be determined.

VI. A HYPOTHESIS RELATING CD TO CARCINOGENESIS

The ability of free radicals to attack and alter DNA has been shown by *in vitro* experiments. [1,7,28,35] It has been proposed that a diet devoid of choline without any added carcinogen initiates rat hepatocytes by free radicals generated in the nuclear membrane. As depicted in Figure 2, the temporal sequence of events could be free radical generation → DNA damage → PLA_2 activation → cell death → cell proliferation → initiation → cancer. This suggested sequence for carcinogenesis with the CD diet is modeled after a current hypothesis of liver cancer induction by chemical carcinogens (Figure 3).[10] Although the involvement of free radicals in the initiation process of carcinogenesis is suggested by a reasonable database, the data to implicate free radicals in the promotion and progression phases of carcinogenesis are at best only possibilities.

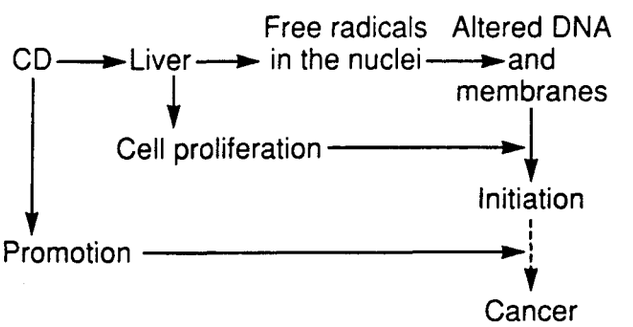

FIGURE 2. Liver carcinogenesis with CD diet: possible mechanisms.

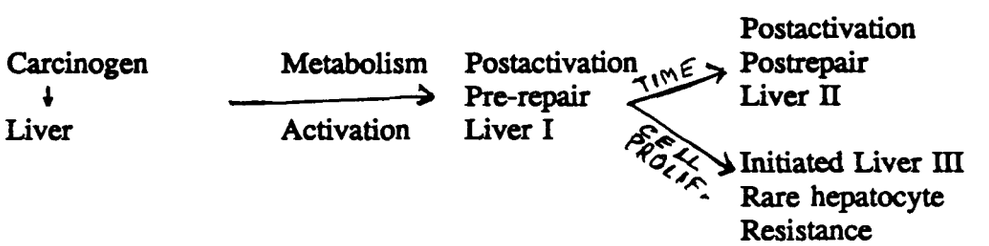

FIGURE 3. Liver cancer induction (shown up to initiation) by chemical carcinogens.

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VII. CELL DEATH IN CD AND ROLE OF PHOSPHOLIPASE A_2

A distinct role for PLA_2 in liver cell death due to CD has been shown.[13] It has been demonstrated (Table 1) that the induction of lipid peroxidation, DNA damage, excess of PLA_2 enzyme activity, and cell death in the rat liver with a CD diet can all be inhibited by pretreatment with spin trap *tert*-nitrosobutane (tNB).[12] When free radical generation and DNA damage is allowed to occur with the CD diet and then the increase in PLA_2 activity is inhibited by PBx, an oligomer (n = 6) of prostaglandin B_1,[11] cell death is also prevented.[12]

This suggests that even in the presence of excess free radicals and damaged DNA, PLA_2 induction in the liver appears to play a role in cell death due to CD. In other words, CD diet induces a free radical increase that generates liver cell death through a chain reaction in which PLA_2 activity is in the downstream.

The observation that PLA_2 preferentially hydrolyzes peroxidized fatty acid esters in phospholipid membranes is of interest in this context (Van Kujik et al., 1987). Based on this finding, it was suggested that PLA_2 may play a protector role of membrane lipids from the injury due to lipid peroxidation. Even though this suggestion is interesting, it is in contrast to our finding that inhibiting PLA_2 activity protects the cell from CD diet-induced cell death. One possible explanation that could accommodate these two considerations is that the hydrolyzed peroxidized fatty acid is converted to an aldelyde in the cytosol and exerts its cytotoxic effect. This argument explains the regain of the membrane integrity after the loss of the peroxidized fatty acid and at the same time the cytotoxicity induced by the aldelyde that is generated.

VIII. HOW DOES EXPOSURE TO A CD DIET RESULT IN EXCESS FREE RADICALS?

There are at least two general possibilities:

1. A CD diet may increase the rate of generation of free radicals such that their rate of production becomes more rapid than their rate of degradation or neutralization by the different antioxidant mechanisms in the living cell.
2. A CD diet may decrease the ability of the hepatocyte, especially the membranes of the cell, to handle and destroy lipid peroxides and other products of free radical action. Under these conditions, the rate of degradation or neutralization of free radicals and/or their consequences becomes much less than the normal rate of generation.

There is no readily evident mechanism for pursuing the first hypothesis. No data are known by this author that could support such an hypothesis.

There are, however, data that offer some support, consistent with the second hypothesis. The dietary exposure to a CD diet rapidly leads to major changes in the phospholipid composition of the hepatocyte membranes. These changes are of considerable magnitude and could theoretically interfere with the ability of the cell to exert its antioxidant-antilipid peroxide repair systems. Although the evidence is by no means conclusive, it does offer an orientation that could be exploited for subjecting the hypothesis to increasingly rigorous critical experiments. The biological consequences of choline deficiency are sufficiently important and impressive to warrant such an approach.

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IX. GENERAL COMMENTS

1. Choline as a base of phospholipid lecithin is available abundantly throughout the vegetable and animal world, with the possible exception of some parts of the world where malnutrition exists or where people eat spoiled grains. Thus, it is most likely that choline deficiency in humans is quite uncommon.
2. It should be realized that choline deficiency is not methyl deficiency. Although choline has three methyl groups, only one is donatable, after oxidation to betaine. The CD syndrome cannot be rectified fully and efficiently by methionine.
3. One cannot consider choline deficiency and lipotrope (choline, methionine, B_{12}, and folic acid) deficiencies as the same. The syndromes are quite different.
4. The occurrence of cancer by eliminating one dietary component without the addition of any carcinogen is a unique animal model in which to study, step by step, not only liver carcinogenesis but also *in vivo* free radical generation and liver cell death without the interference of a xenobiotic.

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