

# Influence of dietary nucleotides on liver structural recovery and hepatocyte binuclearity in cirrhosis induced by thioacetamide

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## Abstract

**Intake of thioacetamide in drinking water causes liver cirrhosis in rats, which exhibit many changes similar to human disease. Nucleotides play an important part in major cellular functions, and recent studies suggest that dietary nucleotides may be considered 'semi-essential' nutrients in situations when an inadequate dietary supply may affect the growth of tissues with a rapid turnover rate. The aim of this study was to assess the effect of dietary nucleotides on lesions in thioacetamide-cirrhotic rats, and to calculate the proportion of mono and binucleated hepatocytes in different experimental groups. Rats were given cirrhosis by oral intake of thioacetamide in the drinking water (300 mg/l) for four months. One group was treated with a standard nucleotide free diet, and another group was treated with the same diet supplemented with 250 mg of nucleotides per 100 g of diet for one and two weeks. A striking reduction (mean (SEM)) in the proportion of binucleated cells was seen in thioacetamide-cirrhotic rats (4.8 (1.3) v 21.4 (1.0)), showing a change in the mitotic mechanism in focal lesions. Cirrhotic rats that consumed a semipurified diet supplemented with nucleotides during two weeks showed considerable histological regeneration of the injured liver. These animals had significantly higher proportion of binucleated cells than did animals at the beginning of the recovery period (8.2 (1.2) v 4.8 (1.3)). In the second week of recovery, both types of diet ( $F=5.54$ ,  $p<0.05$ ) and the previous administration of thioacetamide ( $F=142.82$ ,  $p<0.001$ ) had significant effects on the percentage of binucleated hepatocytes.**

(Gut 1996; 38: 260-264)

Keywords: cirrhosis, thioacetamide, dietary nucleotides, binuclearity, liver recovery.

Thioacetamide (TAA) induced cirrhosis in rats is characterised by single cell necrosis, fibrosis, nodular parenchyma, and disruption of the vascular pattern.<sup>1-6</sup> In a number of animal models, TAA cirrhosis seems to resemble the important features of human disease.<sup>7-10</sup>

Severe protein energy malnutrition, common in patients with advanced liver disease, can seriously undermine the capacity for liver regeneration and functional restoration.<sup>11 12</sup>

Disturbance in carbohydrate, protein, and lipid metabolism are most relevant for, and responsive to, nutritional supplementation. None the less, an adequate supply of energy, protein, and other nutrients is important for liver regeneration. Nucleotides are the basic unit of nucleic acids and play an important part in all major aspects of metabolism.<sup>13</sup> Recent studies suggest that dietary nucleotides may be considered 'semi-essential' nutrients in special situations when an inadequate dietary supply may affect the growth of tissues with a rapid turnover rate.<sup>13-16</sup>

Binucleated cells are present in a variety of organs, including hepatocytes. The proportion of binucleated liver cells depends on variables such as species, age, growth, regeneration, response to genotoxic or non-genotoxic carcinogens, and neoplasia.<sup>17</sup> Jack *et al*<sup>18</sup> noted the importance of hepatocyte hypertrophy in contributing to the development of pre-neoplastic lesions. Changes in the per cent of binucleated and mononucleated hepatocytes may be important in explaining the significance of hypertrophy in changed hepatocytes. A reduction in binuclearity apparently influences total nuclear and cellular ploidy. These authors also reported a change in the regulation of the mitotic cycle and, consequently, of cell growth.<sup>18</sup>

In this study we assess the effects of dietary nucleotide administration on cirrhosis in rats, to find out if an exogenous supply of these nutrients benefits the optimal functioning of hepatocytes and structural recovery in the damaged liver. We also estimated the percentage of mono and binucleated hepatocytes in liver tissues from different experimental groups, in an attempt to determine the effect of TAA, and the extent to which dietary supplementation with nucleotides restored normal numbers of these cells after TAA induced cirrhosis.

## Methods

Female Wistar rats were maintained under standardised conditions in our laboratory. The animals were divided into two groups: one group was treated with 300 mg/l TAA dissolved in drinking water (provided ad libitum); the other group, which served as the control, was given water without TAA. For three months all animals were fed a standard chow diet, then a semipurified control diet was substituted and given for one month (Table I). Composition of semipurified diet complied with ILAR rules.<sup>19</sup>

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Accepted for publication  
12 July 1995

TABLE I Semipurified control diet composition (Research Department of PULEVA, Spain)

Composition	g	Chemical composition	%
Calcium caseinate	225.5	Proteins	20
Cellulose	50	Carbohydrates	67.18
Sugar	150	Lipids	10
Corn starch	446.27	Minerals	2.4
VKO	100	Vitamins	0.0129
Minerals*	24	D1-Methionine	0.3
Vitamins*	0.129	Choline chloride	0.11

Expressed as kg of diet. VKO: mixture of olive oil (66%), soya oil (23%), and medium chain triacylglycerides from refined coconut oil (11%). \*Mineral and vitamin values as specified by ILAR.<sup>16</sup>

All animals were housed and treated in accordance with the recommendations of the American Physiological Society.<sup>20</sup>

After four months, six control and seven TAA treated animals were killed to evaluate the changes produced by the administration of TAA. At this time, treatment with TAA was stopped and the rest of the animals in the TAA and control groups were divided into the following groups:

(a) *Control group* – the control group was divided into two subgroups. In one (control-control), the animals received the semipurified diet for one (CC1, n=5) or two weeks (CC2, n=7). In the other (control-nucleotide), rats were given the semipurified diet supplemented with 50 mg each of AMP, IMP, CMP, GMP, and UMP per 100 g feed during one (CN1, n=5) or two weeks (CN2, n=7).

(b) *TAA group* – the TAA group was also divided into subgroups. In the TAA-control subgroup, the animals received the semipurified diet for one (TC1, n=8) or two weeks (TC2, n=9). In the TAA-nucleotide subgroup, the semipurified diet was supplemented with 50 mg each of AMP, IMP, CMP, GMP, UMP per 100 g feed during one (TN1, n=9) or two weeks (TN2, n=10).

#### Histological studies

After the animals were killed, the liver was immediately removed and samples were taken from the left lobe. Tissue was fixed in 3% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.3), and postfixed in 1.5% osmium tetroxide. The samples were then dehydrated in acetone and embedded in Epon 812 resin. Semithin sections (1 µm) stained with toluidine blue were used for light microscopic examination.

Underlying cirrhosis and fibrosis were confirmed histologically in all cases.

TABLE II Body weight and liver weight/body weight ratio in rats with cirrhosis induced by oral intake of TAA for four months, and after one and two weeks of recovery and feeding with either a semipurified control diet or a diet supplemented with nucleotides

Group	Body weight (g)		Liver weight/body weight × 10 <sup>3</sup>	
	1 Week	2 Weeks	1 Week	2 Weeks
TAA	212.4 (14.9)*§		40.5 (0.0)*§	
Control	304.3 (12.0)		22.2 (0.0)	
<i>Experimental groups</i>				
TAA-nucleotides	260.7 (9.5)†‡	281.5 (8.4)†§‡\$	33.9 (0.0)†‡	30.2 (0.0)†
TAA-control	237.9 (10.3)‡\$	255.4 (7.0)†	32.7 (0.0)†‡	33.9 (0.0)†§‡\$
Control-nucleotides	392.6 (23.0)*	381.7 (24.1)*	25.0 (0.0)	27.1 (0.0)
Control-control	335.0 (12.8)	357.3 (15.5)*¶	23.9 (0.0)	26.5 (0.0)

Results are expressed as mean (SEM).

\*Significance *v* control; †significance *v* thioacetamide; ‡significance between experimental TAA group after recovery and its control group. §p<0.001; ||p<0.01; ¶p<0.05.

#### Number of mononucleated and binucleated hepatocytes

Sections measuring 1 µm thick were stained with toluidine blue and used to count mono and binucleated hepatocytes in each animal. All counts were made with a grid fitted in the eyepiece of the microscope at 63× magnification. Three random fields in each of 10 sections were counted, and the mean values were calculated for each animal.

In sections from cirrhotic rats the fibrous septae were not taken into account. To estimate the number of mono and binucleated hepatocytes in each group, we calculated the relative proportions of each cell type in each animal with the formulas number of mononucleated cells/number of binucleated cells×100, and number of binucleated cells/total number of hepatocytes×100.

#### Statistical analysis

All results are expressed as the mean (SEM). A one way analysis of variance (ANOVA) was used to compare TAA treatment *v* the control factor. A two way ANOVA was used to evaluate the combined effects of two variables (TAA treatment and diet).<sup>21</sup> Comparison of the means was done using 'a posteriori' Bonferroni test; p<0.05 was considered statistically significant. All data were evaluated for statistical significance with BMDP software.<sup>22</sup>

#### Results

Water intake by TAA treated rats was significantly lower than in control animals (12.92 (0.33) *v* 29.52 (0.82) ml/day). In addition, food intake was slightly lower in the experimental TAA group (18.60 (0.40) *v* 20.21 (0.53) g/day). During the second week of recovery, intake of the semipurified diet increased significantly in the experimental group (21.27 (1.48) g/day in group TC2 *v* 16.77 (0.44) g/day in group CC2), although the difference in food intake between the subgroups given the nucleotide supplemented diets did not reach significance (20.03 (0.09) g/day in group TN2 *v* 16.69 (3.51) g/day in group CN2).

The degree of liver hypertrophy induced by TAA was evaluated on the basis of body weight and the liver to body weight ratio (Table II). After four months of TAA administration, body weight was lower, and the liver to body weight ratio higher, in TAA fed rats. An increase in body weight was seen in all TAA treated animals after one and two weeks of recovery, but they failed to reach their corresponding control values. The liver to body weight ratio in previously TAA treated animals that were treated with different diets remained high, except in the TN2 group.

#### Histological findings

Cirrhosis with extensive organic injury was detectable four months after the beginning of the experiment. The liver of TAA treated animals showed a generalised nodular

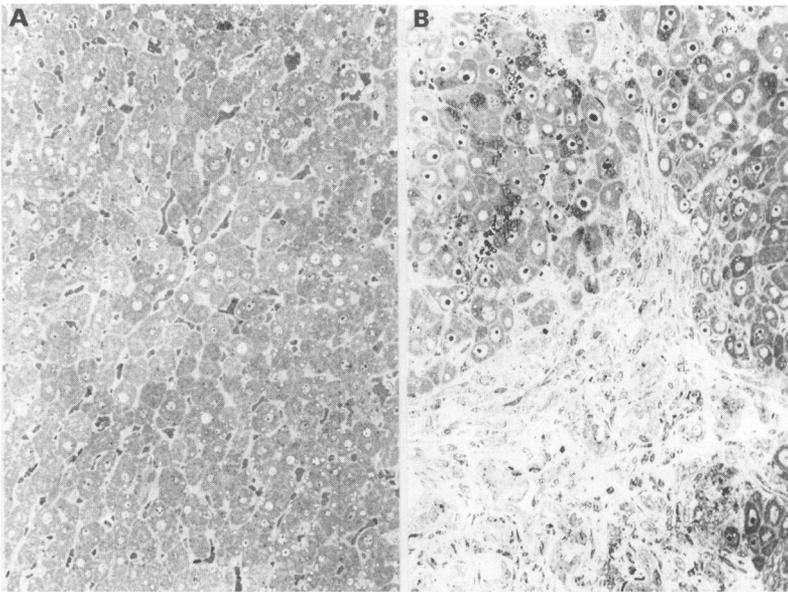


Figure 1: Light micrographs of rat livers (A): control group, (B): TAA group. In the group treated with TAA, fibrous septa, ductular proliferation, and inflammatory cell infiltration of the portal tract are seen. (Original magnification  $\times 80$ .)

appearance. Regenerative parenchymatic nodules were seen surrounded by septa of fibrous tissue. There was a considerable increase in bile canaliculi, fat storing cells, and Kupffer cells (Fig 1).

The layers of connective tissue were thickened, and the parenchymal areas were considerably reduced. The hepatocytes seemed to be larger and structurally abnormal,

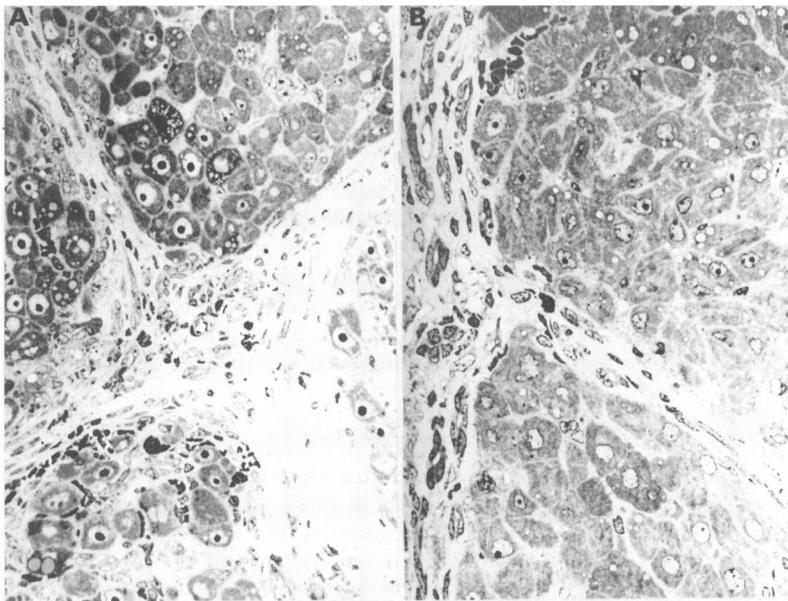


Figure 2: Light micrographs of liver from TAA group (A), TAA control group (TC2) (B), and TAA nucleotides group (TN2) (C). TN2 group showed an appreciable histological recovery of liver injured, with a reduction in the fibrous septa. (Original magnification  $\times 160$ .)

with an irregular, voluminous nucleus and prominent nucleolus. The cytoplasm contained large numbers of fat droplets (Fig 1).

Rats that consumed the diet supplemented with nucleotides during two weeks after treatment with TAA showed appreciable histological regeneration of the injured liver. In this group we saw a considerable reduction in the extent of fibrous septae, and an increase in the surface occupied by normal hepatic parenchyma.

In rats given the control diet for two weeks, recovery was evident, but structural improvement was less complete than in animals that received nucleotide supplements (Fig 2). One week was found to be too short a period for there to be significant changes suggestive of hepatic recovery.

#### Binucleated and mononucleated cells

A significant decrease in the percentage of binucleated cells, with a corresponding increase in the percentage of mononucleated cells, was seen in the TAA group (Table III).

In animals treated with TAA and subsequently fed either a semipurified diet or a diet supplemented with nucleotides, we did not see significant changes in the relative proportions of mono and binucleated hepatocytes after one week with respect to the start of the recovery period. In group TN2 animals, however, the percentage of mononucleated cells after two weeks was significantly lower than at the beginning of the recovery period, so that the proportion of binucleated cells had almost doubled. At the second week of recovery both the type of diet and the previous administration of TAA had significant effects on the percentage of binucleated hepatocytes ( $F_{\text{diet}}=5.54$ ,  $p<0.05$ ,  $F_{\text{TAA}}=142.82$ ,  $p<0.001$ ).

#### Discussion

Treatment of rats with TAA at doses and for periods similar to those used in this study cause cirrhosis-like hepatic lesions with histological and metabolic characteristics similar to those of human cirrhosis of different origins.<sup>7-10</sup> In this study liver injury induced by TAA was characterised by the presence of cellular necrosis and parenchymal nodularity; the increase in liver weight was mainly caused by non-parenchymal tissue, most of which consisted of connective tissue and vascular lumina. Our findings suggest that dietary supplementation with nucleotides influences the recovery from changes in body weight and liver weight caused by nodular cirrhosis.

The liver, especially in rats, has a considerable capacity for regeneration, which occurs as surviving hepatocytes undergo mitosis to offset the loss of cells caused by hepatotoxic agents. Research interest has centred on the mechanisms of regeneration, and it has been suggested that they may be specific, to some degree, for cellular proliferation aimed at restoring the initial number of cells and re-establishing hepatic function.<sup>23</sup>

The selective destruction of liver cells after TAA is given was followed by proliferative

TABLE III Relative proportions of mono and binucleated hepatocytes in rats with cirrhosis induced by oral intake of TAA for four months, and one and two weeks after withdrawal of the hepatotoxic agent and feeding with either a semipurified diet or a diet supplemented with nucleotides

Group	% Mononucleated		% Binucleated	
TAA	95.2 (3.0)*		4.8 (1.3)*	
Control	78.6 (2.3)		21.4 (1.0)	
Experimental groups	1 Week	2 Weeks	1 Week	2 Weeks
TAA-nucleotides	95.0 (1.6)‡	91.8 (2.6)†‡‡	5.0 (0.7)‡	8.2 (1.2)†‡‡
TAA-control	93.4 (2.6)‡	93.6 (3.0)‡	6.6 (1.1)‡	6.4 (1.3)‡
Control-nucleotides	79.6 (1.8)	77.8 (1.9)§ <sup>a</sup>	20.4 (0.8)	22.2 (0.9)§ <sup>a</sup>
Control-control	76.8 (3.1)	81.2 (2.3)	23.2 (1.4)	18.8 (1.0)

Results are expressed as mean (SEM).

\*Significance  $\nu$  control; †significance  $\nu$  TAA; ‡significance between experimental TAA group after recovery and its control group; §significance  $\nu$  control-control 2 weeks.

||p<0.001; †p<0.01; ‡p<0.05.

processes, as shown by the finding of dedifferentiated populations of hepatocytes.

Michalopoulos *et al* have suggested that obstruction of hepatocyte proliferation may be programmed phenotypically, or may result from internal signals.<sup>24</sup>

Changes in the population of binucleated cells suggest that mitosis is changed in the cirrhotic liver. A reduction in the number of binucleated cells may also affect total and cellular ploidy. Díez-Fernández *et al* have recently described relations between genomic DNA ploidy and parameters of liver damage during necrosis and regeneration induced by intraperitoneal administration of TAA.<sup>23</sup> Although the role of mitotic mechanisms remains speculative, the results of our study confirm that a reduction in the number of binucleated cells is consistently seen in TAA induced nodular cirrhosis. This cell population may decline because of a decrease in the rate of formation of binucleated hepatocytes, and because of the disappearance of existing binucleated cells.<sup>13</sup>

Dietary supplementation with nucleotides influenced the percentage of binucleated hepatocytes not only in TAA induced nodular cirrhosis, but also in the non-pathological state, especially after two weeks of recovery. In rats fed for two weeks on a diet supplemented with nucleotides, the proportion of binucleated cells increased. Although the relative proportions of cell types did not recover the normal values found in control animals, they were nearly double the proportions obtained in TAA treated rats after four months. These results suggest that nucleotides affect the regulation of the mitotic cycle, and thus influence cell growth.

The mechanism by which dietary nucleotides influence tissue repair remains unclear. One hypothesis is that dietary nucleotides may affect the intracellular nucleotides pool and the rate of nucleic acids synthesis. Ohyanagi *et al* have shown that nucleotides increase the growth of cultured hepatocytes,<sup>16</sup> and Ogoshi *et al* have seen that intravenous mixtures of nucleosides and nucleotides promote hepatic regeneration of 70% of hepatectomised rats or liver injury induced by D-galactosamine.<sup>15</sup> In addition, dietary nucleotides increase glycogen biosynthesis in the liver.<sup>14</sup> Our working group recently found that dietary nucleotides enhance the aggregation of hepatocyte

ribosomes to form polysomes, and increase the intracellular pool of acid soluble nucleotides in adult rats. Moreover, Palombo *et al* have reported that dietary nucleotides contribute to maintain the hepatocyte concentrations of ATP in cold ischaemic rats.<sup>25</sup>

In conclusion, our findings show the beneficial effects of dietary nucleotides on hepatocyte repair in rats with liver cirrhosis. In animals given a nucleotide supplemented diet, the liver showed widespread evidence of structural repair of cellular damage, for example, the disappearance of nodules and fibrous septae, and tended to recover normal numbers of binucleated cells. Changes suggestive of recovery became apparent after as little as two weeks on the nucleotide supplemented diet. The degree of structural recovery, however, depends on several factors, with diet being only one of the most important.

Our results suggest that studies in humans with liver cirrhosis will provide further information on the potentially beneficial effect of dietary nucleotides on hepatic structure and function.

This study was supported by the Project SAL90-0615 CICYT, and Abbott Laboratories, Granada, Spain. We thank Ms Karen Shashok for translating the original manuscript into English.

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