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Growth and liver morphology after long-term ethanol consumption of rats

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Summary

Ethanol was administered to female and male Wistar rats by mixing it with their drinking water. Ethanol concentrations were gradually increased up to either 8% or 15%.

Female rats receiving 8% ethanol in their drinking water consumed 5–13 g, males 4–10 g daily. The ethanol/total food caloric intake percentages were 13 to 20% and 9 to 15% for female and male rats, respectively. There was no difference in body weight and relative liver weight between treated rats and their controls.

Female and male rats receiving 15% of ethanol in their drinking water consumed 8–14 g ethanol per kg body weight per day. The percentages of ethanol/total food caloric intake were stabilized at about 25% for both sexes. Growth of the rats differed only slightly from controls; a tendency for a higher increase of body weight of the control rats was found. No difference in relative liver weight between ethanol-treated and control rats was observed. Microscopic examinations revealed that the ethanol treatment resulted in fat accumulation in the liver cells. A proliferation of the Smooth Endoplasmic Reticulum (SER) was more marked in the 15% dosed rats than in the 8% dosed rats and more distinct in female rats than in male rats in both dosage groups.

Keywords: Ethanol consumption; Growth; Rats; Liver cytology; Liver pathology

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Several methods of administration of ethanol to rats have been reported in the literature, varying from injection of daily doses (Farbiszewski *et al.*, 1988) to synthetic liquid diets (Lieber & DeCarli, 1967, 1982; French, 1968). Ethanol is often added to the drinking water (Thorpe & Shorey, 1965; Dobbins *et al.*, 1972; Liu *et al.*,1975; Prasad *et al.*, 1985; Väänänen & Lindros, 1985; Nadkarni & D'Souza, 1988; Benedetti *et al.*, 1988).

In this paper a method of administering ethanol to rats via the drinking water is described. A dosage procedure is developed that corresponds to 'moderate' drinking in humans. Moderate drinking is defined as long-term consumption of a maximum of 20% of the daily caloric intake as ethanol (Prasad *et al.*, 1985; Zentella de Pina *et al.*, 1989). Treated animals should have similar weight gain and caloric intake as controls. No severe hepatocellular damage should be observed, although liver enzymes could be induced.

Materials and methods

Animals

Homebred Cpb : WU (Wistar) rats were used. The initial weights varied between 100–120 g (females) and 120–150 g (males). The rats were about 6 weeks old. They were of specified pathogen free quality (antibodies against *Pneumonia* virus of mice and the pin-worm *Syphacia muris* were found, no other pathogens). Rats were kept in Macrolon type 3 cages on sterilized softwood granules as bedding with three animals per cage. Rats were housed in stainless steel metabolism cages on a wire mesh for measurements on individual rats. The rats were weighed three times a week.

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The animals were provided with RMH-TM pellets (Hope Farms bv, Woerden, The Netherlands). Groups of 12 rats were used in each experiment (6 females and 6 males). Three rats of each sex were ethanol-treated, the other three received tap water. The ethanol-treated rats had free access to water with an increasing ethanol percentage reaching 8% or 15% (called the 8% and 15% ethanol treatments) after 2 and 3 weeks, respectively. Details of the ethanol treatment schemes are given in Table 1.

Room temperature was regulated (22 ± 2°C), relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 1900 h and 0700 h so that blood ethanol concentrations could be measured during the active period of the rats. Blood samples were taken from the rats via a canula in the arteria femoralis. The animals did not need to be anaesthetized using this method.

Ethanol consumption

The amount of ethanol consumed was calculated from the volumes of the ethanol/water mixture the rats drank and food pellets were weighed. The percentage of the total caloric intake due to ethanol was calculated from these values.

Microscopy

The rats were killed by cervical dislocation at the end of the period of ethanol treatment. The livers were excised and weighed and the ratio liver to body weight was determined. The samples for light microscopy were fixed in McDowell (1% glutardialdehyde and 4% paraformaldehyde in 0·8 M $NaH_2PO_4 \cdot H_2O$ [pH = 7·4]) at room temperature for at least 24 h. They were embedded in paraplast. Sections of 6 μm were cut on a Leitz microtome and stained with a haemateine-eosine mixture. Samples for electron microscopy were fixed in 2% glutardialdehyde in 0·1 M phosphate buffer (pH = 7·4) at 4°C for 24 h and were embedded in EPON 812. Semithin (1 μm) sections for light microscopy were cut on a Reichert ultra microtome OMU3. These were stained with toluidine blue. Ultrathin sections of 60 nm were contrasted with uranylacetate and lead citrate. They were studied in a Philips EM301 Electron Microscope at 60 KV.

Table 1. Scheme of ethanol treatment of rats

8% ethanol treatment	4 days 2% (w/v) ethanol in tap water
	3 days 4% (w/v) ethanol
	7 days 6% (w/v) ethanol
	28 days 8% (w/v) ethanol
15% ethanol treatment	4 days 2% (w/v) ethanol in tap water
	3 days 4% (w/v) ethanol
	4 days 6% (w/v) ethanol
	3 days 8% (w/v) ethanol
	4 days 10% (w/v) ethanol
	3 days 12% (w/v) ethanol
	21 days 15% (w/v) ethanol

Chemicals

The ethanol used was Ethanol absolute GR supplied by Merck (Darmstadt, Germany). The ethanol in blood concentrations were determined using the Boehringer testkit Cat No. 176290 (Boehringer Mannheim GmbH, Mannheim, West Germany).

EPON 812, paraplast and stains were supplied by Merck (Darmstadt, Germany). Lead nitrate was obtained from Analar BDH Chemicals Ltd (Poole, England).

Statistical analysis

Results are presented as mean values. Statistical analysis was carried out using the SAS (Statistical Analysis System) package on a VAX785 mini-computer. The procedures used were ANOVA for analysis of variance with equal cells and GLM (general linear model) for analysis of variance with unequal cells.

Results

Blood ethanol concentrations

Ethanol concentrations in the blood of three female and three male rats consuming 8% ethanol in their drinking water varied widely between the animals (Fig. 1). There was also great variability in the values measured during 1 day. Comparison of the data from several days did not show a consistent pattern in the values for individual rats. The minimum blood ethanol

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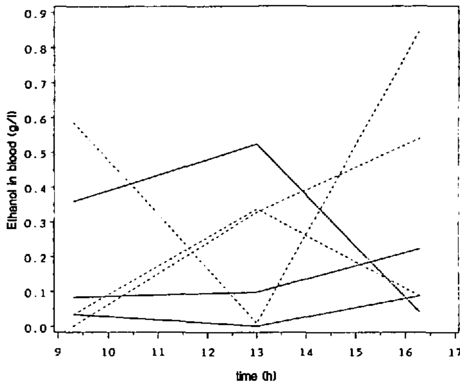


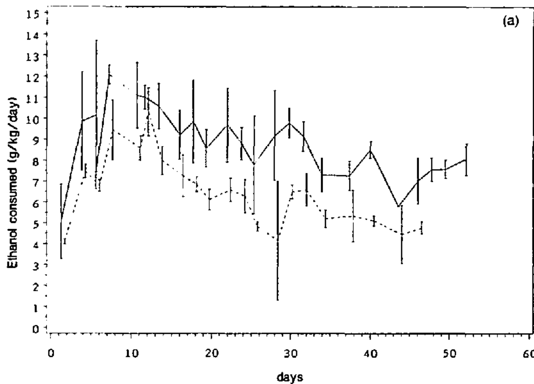
Fig. 1. Blood ethanol concentrations. Ethanol concentrations measured three times during a dark period in blood of three female and three male rats from the 8% ethanol treatment group. (——; female rats, ----; male rats).

value measured was 0 g/l, the maximum value was 0·9 g/l.

A blood sample was taken at time intervals of 4 h from the rats and the volume of water drunk was measured. The blood ethanol concentration was 0 g/l when a rat did not drink the ethanol/water mixture and greater than 0 g/l when a rat drank. However, there was no quantitative relationship between the amount of ethanol/water mixture drunk during 4 h and the blood alcohol levels at the end of that period.

Ethanol consumption and growth

The daily ethanol consumption of rats receiving the 8% treatment is shown in Fig. 2a. Female rats consumed more ethanol than male rats;



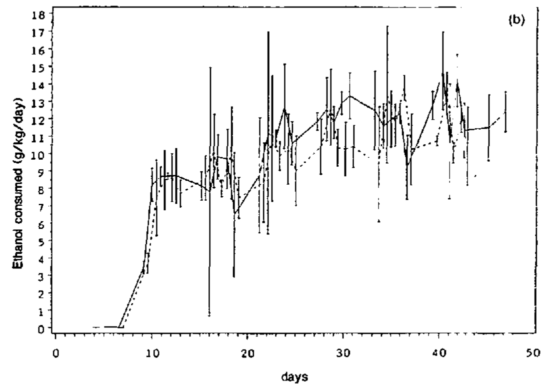
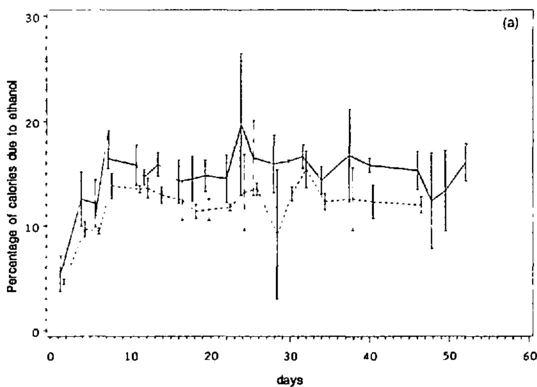


Fig. 2. Amount of ethanol consumed. The amount of ethanol (g/kg/day) consumed by female (——) and male (-----) rats with the 8% ethanol treatment (a) or 15% ethanol treatment (b). Each point represents the mean (±SEM) of three rats.



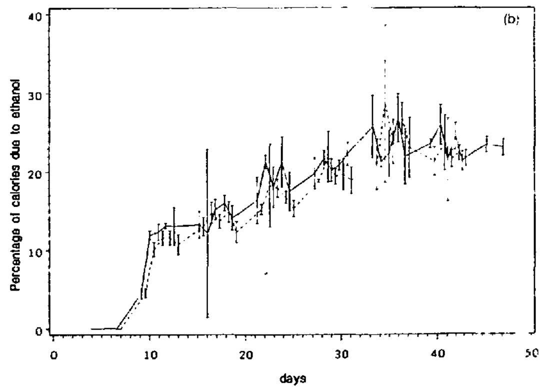


Fig. 3. Relative caloric intake. Ethanol consumption as a percentage of total caloric intake during the 8% ethanol treatment (a) or 15% ethanol treatment (b). Each point represents the mean (±SEM) of three female (——) or three male (----) rats.

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5–13 and 4–10 g/kg/day, respectively. Rats receiving the 15% ethanol treatment consumed 6–14 g/kg/day for both, female and male rats (Fig. 2b).

The ethanol consumption as a fraction of total food caloric intake during the 8% ethanol treatment is shown in Fig. 3a. Levels of 13 to 20% and 9 to 15% for female and male rats, respectively, were attained. In the 15% ethanol treatment group the level stabilized in the last 2 weeks of ethanol-treatment at about 20% of total caloric intake for both female and male rats (Fig. 3b).

The body weights of female and male rats during the 8% ethanol treatment period (Table 1) were compared with controls (Fig. 4a and b). Growth rate of ethanol-treated and control rats was equal (differences were not statistically significant). The body weights of female and male rats during the 15% ethanol treatment period (Table 1) were slightly lighter than the control rats and grew more slowly (Fig. 4c and d) but this difference was not statistically significant.

Relative liver weight

There were no statistically significant differences in liver weights expressed as a percentage of the total body weight between ethanol and non-ethanol treated rats using analysis of variance. There was also no stastistical significant difference in the relative liver weights between the two sexes (Table 2).

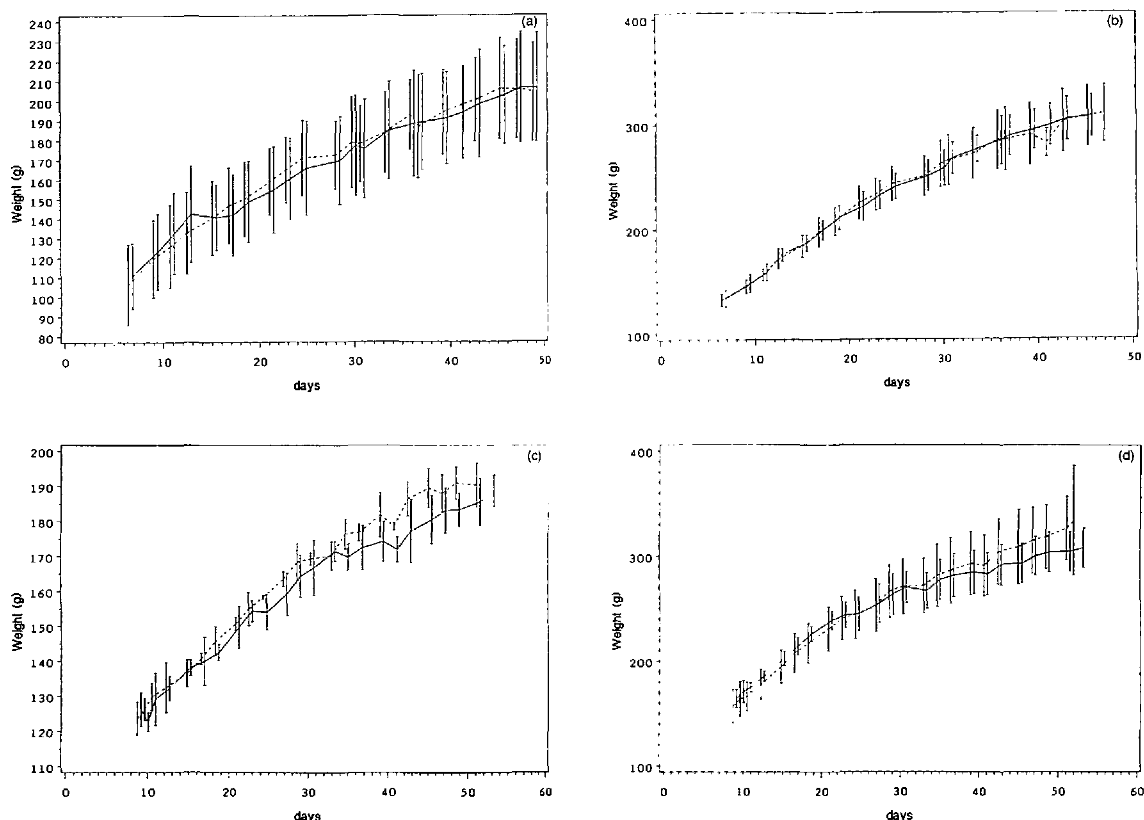

Fig. 4. Growth of rats. Weights of female rats drinking only water (----) or with the 8% ethanol treatment (a) or with the 15% ethanol treatment (c) (——). Weight of male rats drinking only water (----) or with the 8% ethanol treatment (b) or with the 15% ethanol (d) treatment (——). Each point represents the mean (±SEM) of three rats.

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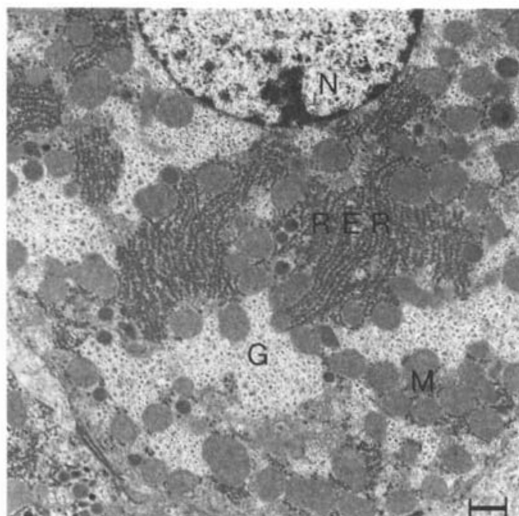
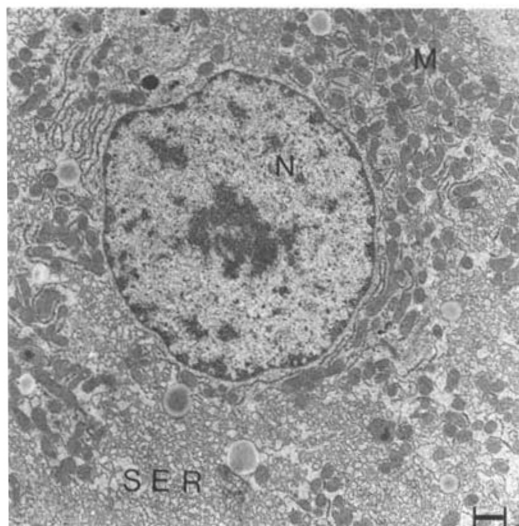
Table 2. Relative liver weight of rats after 6 weeks on different diets (liver weight/body weight)

Percentage ethanol	*Sex*	*No.*	*Relative liver weight (%)*	*SEM*
0	F	20	4·07	0·60
0	M	21	4·13	0·35
8	F	9	3·85	0·22
8	M	9	4·02	0·43
15	F	12	3·80	0·48
15	M	12	4·13	0·46



(a)



(b)

Fig. 5. Electronmicrographs of rat liver. Electronmicrograph of a control female rat liver (a) and of a liver of a female rat from 15% ethanol treatment (b). N = nucleus, RER = Rough Endoplasmic Reticulum, SER = Smooth Endoplasmic Reticulum, G = glycogen, M = Mitochondrion. (Magnification 5a = 7100, 5b = 6100 |-| = 1 μm.)

Microscopic examination

The livers of ethanol-treated and control animals appeared under light microscopy to have a rather homogeneous structure. Fat droplets were observed in the hepatocytes of ethanol-treated rats but little glycogen.

Electronmicrographs of the liver of rats on the ethanol diet showed an accumulation of fat droplets especially near the sinusoides. The proliferation of the Smooth Endoplasmic Reticulum (SER) was clearly seen; the nuclei were often lobed and sometimes possessed clumped chromatin. The Rough Endoplasmic Reticulum (RER) did not appear swollen and ribosomes were still attached to the membrane. The number of mitochondria in ethanol-treated rats was not increased and their appearance not changed; giant mitochondria and crystalline enclosed particles were not found. No bile obstruction was observed. The effects on the liver described above were more obvious in female rats than in male rats; as well as more obvious in the 15% ethanol group than in the 8% ethanol group (Fig. 5a and b).

Discussion

A common method of chronic exposure of rats to ethanol is the feeding of a liquid diet containing essential dietary components supplemented with ethanol. This diet produces a reproducible animal model for the study of several pathological events observed in 'heavy' drinking humans (Lieber & DeCarli, 1967). Another method is the addition of ethanol to the drinking water, with the rats obtaining their protein, fat, carbohydrate, vitamins and minerals from food pellets. The results obtained with the two kinds of diets differ greatly, not just because of the use of different treatment methods but also because of differences in rat strains, periods of treatment and amount of fat, protein and carbohydrate in the diet. Lieber and DeCarli (1973) observed fat deposition in the liver after feeding the liquid diet. They compared an original diet containing 35% fat with a low fat diet (12%) and found that livers were less fatty with the same ethanol ingestion in the low fat diet (Lieber & DeCarli, 1982).

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Several investigators have proposed modifications to the liquid diet. A higher content of lipotrophic factors and amino acid nitrogen (Porta *et al.*, 1968) produced less steatosis, although swelling of mitochondria and loss of cristae organization was observed by Banks *et al.* (1969). Neither chemical nor histological evidence of liver fat accumulation was found with the AIN-76A standard diet (Bieri *et al.*, 1977) containing 21% of protein, 12% of fat and 30% of carbohydrate (Schoemaker & Visek, 1988).

The importance of the carbohydrate content of the diets was emphasized by Rao *et al.*, 1987. They found that rats consumed more diet and grew faster when the concentration of ethanol in the liquid diet was decreased from 36% to 20%, and the carbohydrate content was increased. A lower fat content of the liver was found. The rats consumed an equal amount of ethanol-derived calories per day with both diets.

The food pellets used in our study contained 22·4% of the energy content as protein, 10·8% as fat and 49·3% as carbohydrates. They therefore formed a rather low fat, high carbohydrate diet.

The absolute amount of ethanol ingested in g/kg/day and the percentage of the total caloric intake due to ethanol seem good parameters to determine and compare the ethanol dose due to the different diets. The rats consumed 36% of their daily caloric intake as ethanol using the method of Lieber and DeCarli (1982). This is equivalent to an ethanol intake of 12–18 g/kg/day. Blood ethanol levels varied between 1·0 and 1·5 g/l. The ethanol intake in absolute amounts was comparable with the results of our study although the percentage of total caloric intake due to ethanol by our rats was lower; 20% compared with 36%. In our study only the blood ethanol levels in rats receiving the 8% ethanol treatment were measured and these were rather low.

Rubin *et al.* (1970) described morphological changes such as hepatic steatosis, proliferation of hepatic SER, increased microsomal protein and enlarged mitochondria using the Lieber/DeCarli diet. In our study steatosis and proliferation of the SER was also observed. These have also been observed by other investigators giving rats ethanol in their drinking water with other nutrients in food pellets. Benedetti *et al.* (1988) observed areas of proliferated SER in hepatocytes containing lipid droplets of various size. They also observed bizarre shaped mitochondria. Their laboratory animal diet contained 41% of calories as carbohydrate, 21% as protein and 6% as fat. The chronic ethanol group received ethanol solution supplemented with 6% sucrose. The ethanol concentration was gradually increased from 5% (v/v) to 25% (corresponding to a mean daily ethanol intake of 14·8 g/kg), which was reached on the 29th day. Two control groups were used; one with 6% of the calories as sucrose and one with tap water. Blood ethanol levels reached a level of 1·1 g/l in the fifth week. Dobbins *et al.* (1972) performed a morphometric analysis of the effects of ethanol upon rat liver. They compared three diets in their study; chow with 25% of calories as ethanol in the drinking water, chow with isocaloric sucrose and chow supplemented with 2% choline plus 25% ethanol. In the 25% ethanol group they observed an increased hepatic cell size, an increased total volume of mitochondria and many lipid droplets. These phenomena were partially reversed by choline supplementation. The surface area of both SER and RER was reduced to 50% in the ethanol-treated animals. The diets consisted of 19% of the calories as protein, 7·5% as fat and 43·5% as mixed starches.

Thorpe and Shorey (1965) added ethanol to the drinking water with a final concentration of 20%. Their diet was also low in fat and high in carbohydrate. The control group received diet and plain water *ad libitum*. The ethanol dose was equivalent to 5·7 g/kg/day; ethanol providing 22% of the total caloric intake. No change in liver lipid content was observed.

Most diets in which normal food pellets are used in combination with ethanol in drinking water are low in fat content and high in carbohydrate. The ethanol dose has to be at least 15% to lead to observable damage. The method

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we have described and chosen is relatively simple and led to a considerable intake of ethanol (6–14 g/kg/day) which is 20% of the daily caloric intake.

Electron microscopy of the rat livers confirmed proliferation of SER as an effect of ethanol as previously described by Rubin *et al.* (1968). The livers of female rats which received 15% ethanol in their drinking water contained more fat droplets than the livers of male rats. Small fat droplets were observed in the livers of the rats that received 8% ethanol. No effects on mitochondria nor severe pathological effects such as liver necrosis or cirrhosis were observed.

The growth and development of the rats was normal during the treatment based on the body weight curves. The ratio of liver weight to body weight did not differ significantly between ethanol-treated and control rats.

Our results confirm that addition of ethanol to the drinking water enabled the ingestion of high amounts of ethanol; 5–13 g/kg/day which is comparable to the Lieber/DeCarli diet. This resulted in a considerable caloric intake from ethanol (12–20%). Human alcoholics are defined as those who are chronically consuming at least 20% of their daily caloric intake as ethanol (Lelbach, 1974). This rat model therefore seems realistic. We think that this model is suitable for further studies on the effects of long-term ethanol exposure on the toxicokinetics of xenobiotics.

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