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Four-Week Inhalation Toxicity Study of 2,2-Dichloro-1,1,1-trifluoroethane (HCFC-123) in Guinea Pigs

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Abstract: Four-Week Inhalation Toxicity Study of 2,2-Dichloro-1,1,1-trifluoroethane (HCFC-123) in Guinea Pigs: Isamu KABE, *et al.* Department of Preventive Medicine and Public Health, School of Medicine, Keio University—Groups of eight male Hartley guinea pigs were exposed to 30, 100 or 300 ppm 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123) by inhalation for 6 hours a day for 4 wk. All the animals were sacrificed 48 h postexposure. Guinea pigs exposed to 300 ppm HCFC-123 had significantly lower body weight and the weight gain than controls, but there was no significant difference and no tendency in absolute and relative organ weight. ICDH, ALT and AST, which were the most sensitive indicators of halothane-induced liver injury, did not differ significantly between exposed groups and controls. In the 100 ppm group, a few vacuolar fatty changes in the portal area (zone I) were identified. In the 300 ppm group, severe fatty degeneration was observed in the portal and intermediate areas, partly centrilobule, and the incidence increased significantly compared to the controls. On the other hand, there was no histopathological change in the control or 30 ppm groups. No increase in any of the liver peroxisomal enzymes (AOX, PT, catalase) was seen in male guinea pigs exposed to HCFC-123. The activity of hepatic ALDH was significantly decreased in the 300 ppm exposed group, suggesting that HCFC-123 or its metabolite inhibited ALDH activity. In conclusion, inhalation exposure to 100 ppm or more of HCFC-123 for 4 wk produced nonfatal liver change in guinea pigs, namely hepatic fatty changes predominantly in the portal area (zone I) without any increase in AST, ALT or ICDH. The no-observed-adverse-effect level (NOAEL) of HCFC-123 for four wk in guinea pigs may be 30 ppm. Peroxisome proliferation, which may result in hepatocellular tumor induction, was not observed in guinea pigs exposed to HCFC-123.
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Key words: 2,2-Dichloro-1,1,1-trifluoroethane (HCFC-123), Liver toxicity, Chlorofluorocarbon substitute, Trifluoroacetic acid, Guinea pig, Inhalation, Fatty change, Peroxisome

2,2-Dichloro-1,1,1-trifluoroethane (HCFC-123, CAS No.306-83-2) is considered as a replacement for ozone-depleting chlorofluorocarbons (CFCs), which are very stable in the atmosphere and may cause adverse effects in humans such as skin cancer[1–3]. HCFC-123 has been used as a foam-blowing agent, a refrigerant and a solvent in cleaning jobs. It is a colorless ether-odor liquid with a boiling point of 27.6°C at 1 torr, and its ozone deletion potential is 0.02. In 2000, 300 tons of HCFC-123 was shipped to Japan according to the statistics of the Japan Fluorocarbon Manufacturers Association. The Programme for Alternative Fluorocarbon Toxicity Testing (PAFT) has performed extensive toxicological evaluations including a 2-yr bioassay in rats for CFC substitutes (http://www.afeas.org/paft/hcfc-123.html, last updated October 11, 1996). For HCFC-123, increases in the incidence of benign adenomas and in hepatic β-oxidation were observed in chronic inhalation studies in 300–5,000 ppm

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rats without life-shortening[4, 5]. There were no significant toxicological findings in a 90-d study with 300–20,000 ppm or in teratology and reproduction studies with 300–5,000 ppm[4, 6], and it was concluded that the inhalation toxicity of HCFC-123 was relatively low[5].

In 1997, however, Hoet *et al.* observed an epidemic of liver disease after accidental exposure to a mixture of HCFC-123 and 1-chloro-1,2,2,2-tetrafluoroethane (HCFC-124)[7]. In Japan we reported that repeated exposure to HCFC-123 ranging from 24 to 1,125 ppm with a geometric mean of 225 ppm for about five wk caused acute liver dysfunction[8, 9], and that, in another case where exposure to HCFC-123 exceeded 1,000 ppm during the busiest work period, HCFC-123 might be the cause of recurrent hepatitis[10]. Yoshinami N *et al.* also reported that repeated exposure to HCFC-123 caused liver dysfunction in the affected workers[11]. Marit GB *et al.* reported that histopathological changes including necrosis were observed in Hartley guinea pigs in a single exposure to HCFC-123 at 1,000 ppm for 4 h[12]. Serum biochemical parameters such as isocitrate dehydrogenase (ICDH) were also increased. The authors suggested that the mechanism of liver dysfunction due to HCFC-123 exposure might be similar to that of halothane-induced hepatitis.

In this study we conducted inhalation experiments in guinea pigs, the results of which seem to resemble HCFC-123-induced liver injury in humans, to evaluate the effect on the liver of a lower exposure level than that in earlier studies and to explore the mechanical aspect of its toxicity.

Materials and Methods

Animals

Eight-wk-old male Hartley guinea pigs were purchased from Charles River Japan Inc. (Tokyo). Two or three guinea pigs each were housed in stainless steel cages in a filtrated air-ventilated rack for animal breeding (Shinano Seisakusho). The cages were maintained at a temperature of approximately 24 ± 2°C, 60 ± 10% relative humidity, and a 12-h light/dark cycle. The animals were fed pelleted rodent chow (CRF-1, Oriental Kobo) and water *ad libitum* throughout the experiment.

Experiments

Thirty-two guinea pigs were divided into three exposure groups and one control group after one wk of acclimatization. HCFC-123 (purity: 99.9%, Sumitomo Seika), diluted with pure nitrogen to 1,000 ppm and compressed in gas cylinders, was introduced into the three exposure chambers and maintained at concentrations of 30, 100 and 300 ppm by diluting it with filtered room air for six hours a day, five days a week for four wk. The flow rate of air was kept at 2 *l* per min. Guinea pigs in the control group were exposed to filtered room air in the same manner as the exposure groups. HCFC-123 concentrations were measured with a gas chromatograph (GC-8A, Shimazu) at 30-min intervals[13].

The behavior and external appearance of the guinea pigs were observed every day. The body weight was measured before and after exposure every week. Twenty-four h after the end of the final exposure, food was removed, and 48 h later, blood was collected from the inferior vena cava under anesthesia with i.p. injection of sodium pentobarbital. Their liver, kidneys, pancreas and testes were promptly removed and weighed. Liver tissues used for immunoblot analysis were frozen on dry ice immediately, and the rest were then fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with hemotoxylin-eosin for light microscopic evaluation. They were also stained for fatty assessment with a solution of Sudan 7B and oil red O for visualization of neutral fatty acid, and examined microscopically. Histopathological findings were assessed by a pathologist.

Serum biochemical and hematological examinations

Serum biochemical indices examined were ICDH, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamytransferase (γGTP), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, indirect bilirubin, lactic dehydrogenase (LDH), LDH isozymes (LDH1, LDH2, LDH3, LDH4, LDH5), total cholesterol, triglyceride, blood urea nitrogen (BUN), creatinine and fasting blood glucose (FBG), which were measured by the standard clinical chemistry method (Hitachi 7450).

Hematological parameters included erythrocyte count (RBC), hemoglobin (Hb) and the total and differential counts of leukocytes (WBC, Stab, Seg, Lymph, Mono) measured by means of an automatic hemocytometry (Sysmex XE2100).

Analysis of β-oxidation enzymes and ALDH

The frozen liver sections were kept at − 80°C until analysis. Liver extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 3% skim milk, the membranes were incubated with the primary antibody followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson, West Grove). The primary polyclonal antibodies were prepared with purified acyl-CoA oxidase (AOX)[14], peroxisomal thiolase (PT)[15] and catalase[16], respectively. The signals obtained by immunoblot analysis were quantified by scanning densitometry and the mean of the values from the guinea pigs in the control group were assigned the value 1.0.

Total aldehyde dehydrogenase (ALDH) activity in the supernatant from liver centrifuged at 700 g was examined by measuring the rate of nicotinamide adenine dinucleotide (NADH) formation with acetaldehyde as a substrate.

Table 1. Body weight, organ weight and histopathological findings in the guinea pigs (n=8) exposed to HCFC-123

	HCFC-123 concentration			
	Control (air only)	30 ppm	100 ppm	300 ppm
Body weight (g)	689 ± 22	677 ± 24	698 ± 28	652 ± 27*
Body weight gain (g)	159 ± 22	141 ± 16	155 ± 21	113 ± 21**
Organ weight				
Liver (g)	21.3 ± 1.9	22.7 ± 2.0	20.8 ± 2.2	21.4 ± 2.3
Liver (% body weight)[#]	3.1 ± 0.1	3.4 ± 0.1	3.0 ± 0.1	3.3 ± 0.1
Kidney (g)	6.1 ± 0.4	5.8 ± 0.6	5.7 ± 0.6	6.1 ± 0.4
Spleen (g)	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.8 ± 0.1
Testis (g)	3.5 ± 0.6	3.4 ± 0.5	3.5 ± 0.3	3.4 ± 0.4
Histopathological findings[##]				
Diffuse vascular (fatty) change	0 / 8	0 / 8	1 / 8	5 / 8[+]
Multifocal random degeneration	0 / 8	0 / 8	3 / 8	8 / 8[++]

Weight data expressed are the mean ± standard deviation. [#]: Liver (% body weight): relative liver weight. [##]: Incidence data expressed as number with finding/number examined. *, **: $p<0.05$, $p<0.01$ (Student's t-test), +, ++: $p<0.05$, $p<0.01$ (Fisher's test).

Statistical analysis

Statistically significant differences between mean values for body weight, biochemical and hematological items including β-oxidation enzymes and ALDH were assessed by a Student's t-test. The analysis of histopathological findings was performed by Fisher's exact method. For serum biochemical and hematological indices, samples hemolyzed macroscopically were excluded from the analysis.

Results

Exposure concentrations, animal observation, body weights and organ weights

The arithmetic means ± S.D. of the HCFC-123 concentration in the exposure chambers for four wk were 27 ± 1, 94 ± 9 and 301 ± 10 ppm, respectively. No significant differences between the control and exposed guinea pigs in behavior or external appearance were observed. Mean body weight and body weight gain was significantly lower in the group exposed to 300 ppm HCFC-123 from the eighth day of exposure (Table 1). There was no significant difference between the experimental and the control animals and no tendency in the absolute and relative organ weights of livers, kidneys, spleens, and testes (Table 1).

Serum biochemical and hematological examinations

There was no exposure-related effect on the hematological parameters or biochemical values except for ALP and FBG (Table 2). ICDH, ALT and AST did not differ significantly among the exposed and control groups.

Histopathological examination

Air-only and 30 ppm HCFC123-exposed groups had no significant histopathological changes in the liver.

In the 100 ppm group, focal and mild fatty changes were observed mainly in the portal area (zone I) (Fig. 1). Some hepatocytes in the area stained red with the fatty stain, Sudan 7B and oil red O.

The degree of fatty changes increased in the 300 ppm group. The areas involved were zone I and zone II (intermediate area), but hepatocytes in centrilobular area (zone III) were spared (Fig. 2). The lesions observed included zonal and multifocal random hepatocellular degeneration. The fatty staining revealed various fatty droplets in the hepatocytes surrounding the portal tract (Fig. 3). On the other hand, those surrounding the central vein changed only slightly.

Neither hepatic necrosis nor the infiltration of macrophages or lymphocytes was found in any of the groups.

The prevalence of the histopathological findings is shown in Table 1. The prevalence in the 300 ppm group increased significantly compared to the control. Histopathological examination revealed exposure-related hepatic degeneration.

Induction of hepatic β-oxidation activity (peroxisome proliferation)

Expressions of the three peroxisomal enzymes were examined in order to investigate peroxisome proliferation after HCFC-123 exposure in guinea pigs. HCFC-123 exposure did not increase the level of liver peroxisome proliferation. No increase in any of the liver peroxisomal

Table 2. Hematological and serum biochemical effects in the guinea pigs (n=8) exposed to HCFC-123

	HCFC-123 concentration							
	Control (air only)		30 ppm		100 ppm		300 ppm	
	n[#]	GM (GSD)[##]	n[#]	GM (GSD)[##]	n[#]	GM (GSD)[##]	n[#]	GM (GSD)[##]
Hematological examinations								
RBC (× $10^4/\mu l$)	5	552 (1.1)	6	619 (1.0)	5	561 (1.1)	7	610 (1.1)
Hb (mg/m*l*)	5	14.4 (1.1)	6	16.3 (1.1)	5	14.2 (1.1)	7	15.6 (1.1)
Hct (%)	5	50.4 (1.2)	6	56.7 (1.1)	5	49.8 (1.1)	7	53.9 (1.1)
WBC (× 10/μl)	5	280 (1.3)	6	310 (1.3)	5	235 (1.5)	7	392 (1.4)
Seg (%)	5	38.7 (1.6)	6	39.4 (1.4)	5	45.1 (1.2)	7	43.4 (1.2)
Eosin (%)	5	2.2 (1.8)	6	1.7 (1.9)	5	4.2 (1.4)	7	3.5 (2.9)
Lymph (%)	5	54.7 (1.3)	6	55.9 (1.2)	5	48.7 (1.3)	7	53.4 (1.2)
Platelet (× $10^4/\mu l$)	5	41.7 (1.3)	6	33.8 (1.6)	5	43.9 (1.1)	7	45.5 (1.3)
Serum biochemical examinations								
Total bilirubin (mg/d*l*)	5	0.1 (1.5)	6	0.3 (6.4)	6	0.1 (2.1)	7	0.1 (3.4)
Direct bilirubin (mg/d*l*)	5	0.1 (2.8)	6	0.2 (5.8)	6	0.1 (1.8)	7	0.0 (4.5)
AST (IU/*l*)	5	78.3 (1.6)	7	81.6 (2.1)	6	56.8 (1.4)	7	77.1 (2.3)
ALT (IU/*l*)	5	47.3 (1.1)	7	44.5 (1.3)	6	40.6 (1.2)	7	50.3 (1.5)
LDH(IU/*l*)	5	289 (2.0)	7	496 (3.0)	6	218 (1.7)	7	246 (2.5)
ALP (IU/*l*)	5	418 (1.3)	7	400 (1.2)	6	341 (1.1)	7	320 (1.1)*
rGTP (IU/*l*)	5	7.3 (1.2)	7	8.9 (1.5)	6	8.1 (1.3)	7	6.7 (1.6)
Triglyceride (mg/d*l*)	5	25.4 (1.2)	7	30.8 (1.6)	6	56.0 (1.7)	7	36.5 (1.6)
Total cholesterol (mg/d*l*)	5	36.5 (1.4)	7	37.7 (1.3)	6	40.3 (1.1)	7	37.9 (1.3)
BUN (mg/d*l*)	5	23.2 (1.2)	7	24.4 (1.1)	6	24.3 (1.1)	7	24.3 (1.6)
Creatinine (mg/d*l*)	5	0.3 (1.1)	7	0.4 (1.3)	6	0.4 (1.3)	7	0.5 (2.8)
FBG (mg/d*l*)	5	143 (1.1)	7	118 (1.2)	6	114 (1.1)	7	103 (1.1)**
ICDH (IU/*l*)	4	63.8 (1.2)	7	70.3 (1.7)	6	53.9 (1.2)	7	70.1 (2.0)

For serum biochemical and hematological indices, samples hemolyzed macroscopically were excluded from the analysis. [#]: Due to the small sample size, some samples could not be examined. [##]: GM: geometric mean, GSD: geometric standard deviation. *: $p<0.05$, **: $p<0.01$ compared to control guinea pigs by Student's t-test.

enzymes (AOX, PT and catalase) was seen in male guinea pigs exposed to HCFC-123. On the other hand, there was a significant decrease in the activity of ALDH, the metabolic enzyme which produces trifluoroacetic acid, in the 300 ppm group compared with the controls (57.4, 60.7, 58.0, 35.3 nmol NADH/mg protein/min (geometric mean) for 0, 30, 100, and 300 ppm, respectively).

Discussion

The results of this study indicate that repeated exposure to low-concentration HCFC-123 for four wk causes hepatic fatty changes predominantly in the portal area (zone 1) of the guinea pig liver at 100 ppm. The severity and prevalence of the changes, mainly in the portal and intermediate areas, were increased and body weight gain was suppressed at 300 ppm though neither hepatic necrosis nor any increase in serum liver-derived enzymes was observed. Fatty degeneration can be produced by various chemicals such as carbon tetrachloride, tetracycline, and dimethylformamide[17]. For HCFC-123, a single exposure in guinea pigs resulted in centrilobular vacuolar (fatty) change, multifocal random degeneration and necrosis of the liver with increase in serum AST, ALT and ICDH at 1,000 ppm or more[12, 18]. It was therefore considered that HCFC-123 is hepatotoxic, and the no-observed-adverse-effect level (NOAEL) for four-wk exposure to HCFC-123 in guinea pigs would be 30 ppm.

In a subchronic inhalation study with rats, Rusch *et al.* reported that histopathological findings were minimal and only induction of peroxisomal activity was seen at 300 ppm HCFC-123 for 90 d. There was no death or even marked signs of toxicity with 4-wk inhalation at 20,000 ppm[4]. They therefore concluded that HCFC-123 had low inhalation toxicity. Moreover, the 2-yr inhalation toxicity of HCFC-123 was also low in rats at concentrations of 300–5,000 ppm[5]. PAFT therefore concluded that the inhalation toxicity of HCFC-123 is relatively low though an increased incidence of benign, but not life-threatening, tumors was observed in rats[4–6].

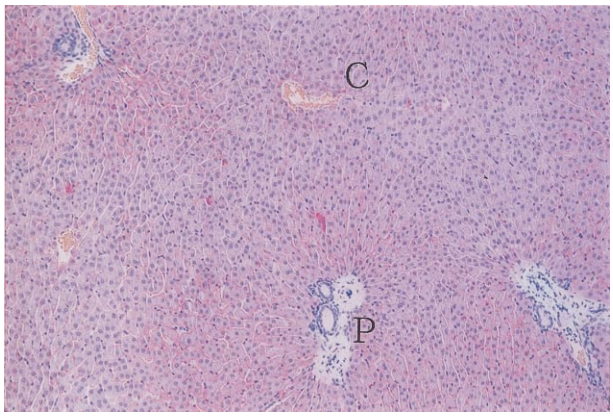

Fig. 1. A few fatty changes in the hepatic portal area (zone I) in a guinea pig exposed to 100 ppm HCFC-123. P: portal tract. C: central vein. Sudan 7B and oil red O. × 25.

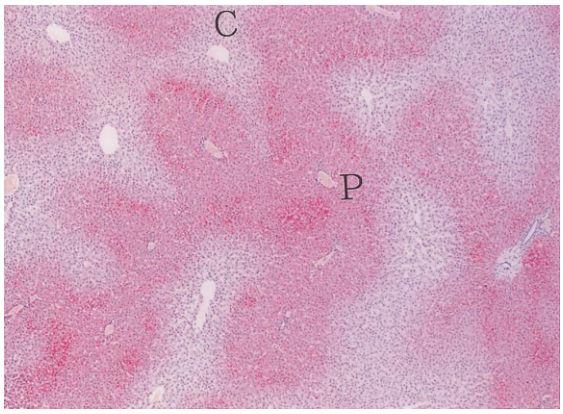

Fig. 2. Hepatic multifocal and zonal fatty degeneration in a guinea pig exposed to 300 ppm HCFC-123. P: portal vein. C: central vein. Sudan 7B and oil red O. × 10.

These observations in rats are different from those in guinea pigs and in human subjects.

Reasons for such discrepancy may be explained by the species difference in the metabolic pathway of HCFC-123. The chemical structure of HCFC-123 is very similar to that of halothane (1-bromo-1-chloro-2,2,2-trifluoroethane), a well-known hepatotoxic chemical. In guinea pigs, halothane was mainly metabolized through the oxidative pathway by CYP 2E1, and formed reactive trifluoroacetylchloride that reacted with water to form trifluoroacetic acid (TFA) and trifluoroacetyl-protein adducts[19, 20]. In rats, on the other hand, pretreatment with the P450 inducer, e.g. phenobarbital or hypoxia, was necessary to reproduce halothane toxicity in the liver, which means that reductive dehalogenation is the main pathway under normal conditions in rats[21, 22]. The main metabolic pathway of halothane in humans is also the oxidative one with CYP 2E1, as in guinea pigs; a guinea pig model could therefore be more sensitive and more appropriate than a rat model to evaluate halothane hepatotoxicity in humans[19, 20, 23, 24]. It has been observed that HCFC-123 was also dehalogenated mainly in the oxidative pathway similar to halothane[18, 25, 26]. Higher TFA concentrations in urine have been detected among workers repeatedly exposed to HCFC-123[9, 11, 13], and trifluoroacetyl-adducted proteins in the liver and serum CYP 2E1 autoantibodies have also been identified among workers repeatedly exposed to HCFC-123 and HCFC-124[9]. Trifluoroacetyl-adducted proteins may cause immune hepatitis through neo-antigen formation. As both the structure and biotransformation of HCFC-123 is similar to those of halothane, a guinea pig should be a better model for evaluating HCFC-123-induced hepatotoxicity in humans. On the other hand, the toxicological evaluation of HCFC-123 in PAFT has been mainly performed in rats.

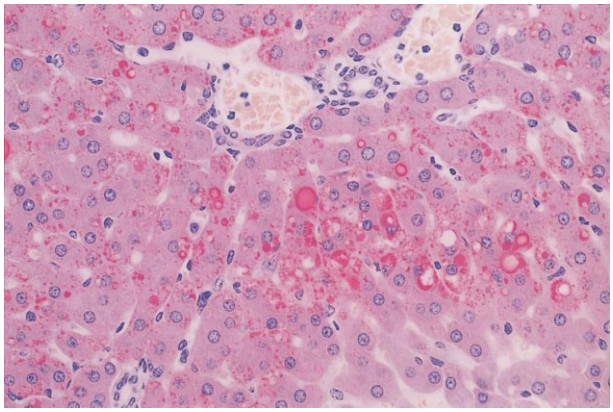

Fig. 3. Various fatty droplets observed in the hepatocytes surrounding the portal tract in a guinea pig exposed to 300 ppm HCFC-123. Sudan 7B and oil red O. × 80.

Malley *et al.* reported hepatic beta-oxidation activity was higher in rats exposed to HCFC-123 at 300, 1,000 and 5,000 ppm (male) and 1,000 and 5,000 ppm (female) for 1 yr, but compound-related differences in the rate of hepatic cell proliferation were not observed at any concentrations[5]. In this study in guinea pigs, no induction of liver peroxisomal enzymes was found in any exposed group. Non-genotoxic hepatocarcinogenesis due to peroxisome proliferators has been well established in rodents, but this response largely depends on the species, i.e. humans and guinea pigs are relatively insensitive[27, 28]. Various experiments indicated that induction of β-oxidation with hepatic peroxisome proliferators was weak in guinea pigs and in humans compared to that in rats and mice[29, 30]. In this study, significant reduction of hepatic ALDH activity in the 300 ppm group may partly explain such low response to the peroxisome proliferator because a similar phenomenon was observed with

trichloroethylene[31]. The mechanism of peroxisome proliferation has been investigated, and might be mediated by the peroxisome proliferator activated receptor α ($PPAR_\alpha$)[32]. Inhibition of hepatic ALDH activity causes the metabolic production of TFA, which may be an obstacle to the induction of $PPAR_\alpha$. Hepatocellular tumor induction caused by HCFC-123 therefore may not be relevant in guinea pigs or in humans.

It has been suggested that $PPAR_\alpha$ expression in humans is lower than that in rodents, but it is sufficient to mediate such beneficial effects as hypolipidemic and hypoglycemic effects[27, 33]. We observed that the blood glucose level decreased in the 300 ppm group even though triglyceride and cholesterol levels were not affected in this experiment. As for the lack of induction of $PPAR_\alpha$ in the 300 ppm group, hepatic fatty changes may not result from the presence of $PPAR_\alpha$ but from the toxic effects of HCFC-123.

Male guinea pigs exposed to 300 ppm had significantly lower body weight and lower body weight gains than controls, and significantly decreased glucose. Previous studies reported that rats exposed to more than 300 ppm not only had lower body weight and lower gains but also decreased serum cholesterol and glucose[4, 5]. The effect on body weight may be related to the compound-related disturbance in lipid and carbohydrate metabolism leading to hepatic fatty changes.

In conclusion, inhalation exposure to 100 ppm or more of HCFC-123 for 4 wk produced nonfatal liver damage in guinea pigs, namely hepatic fatty changes predominantly in the portal area (zone I) without increases in AST, ALT and ICDH. The NOAEL of HCFC-123 for four wk in guinea pigs may be 30 ppm. Peroxisome proliferation, which may result in hepatocellular tumor induction, was not observed in guinea pigs exposed to HCFC-123.

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