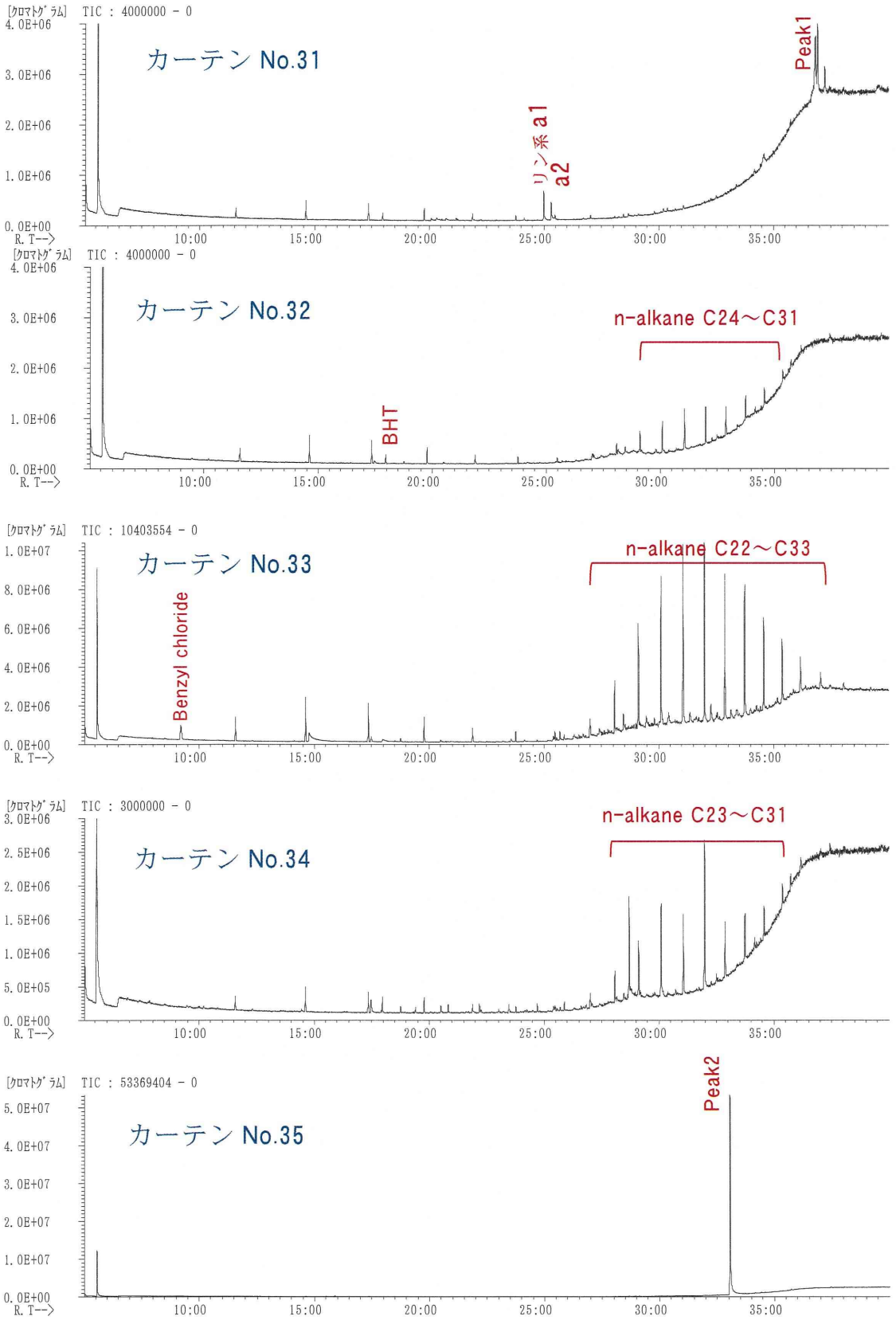


図 6 トータルイオンクロマトグラム

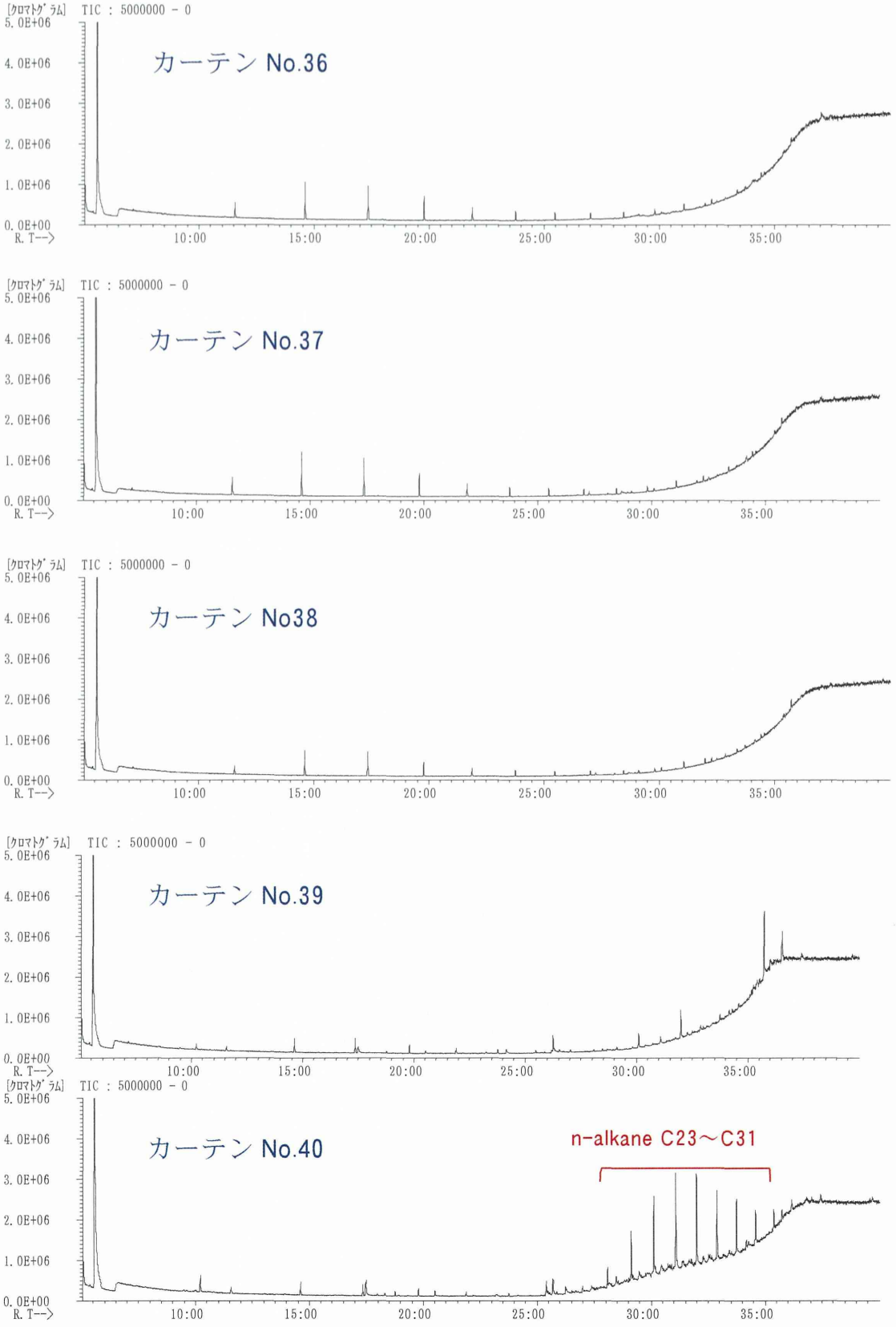
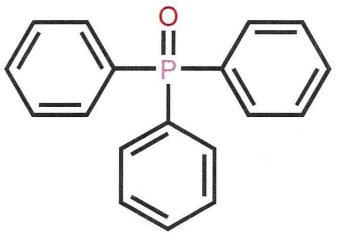
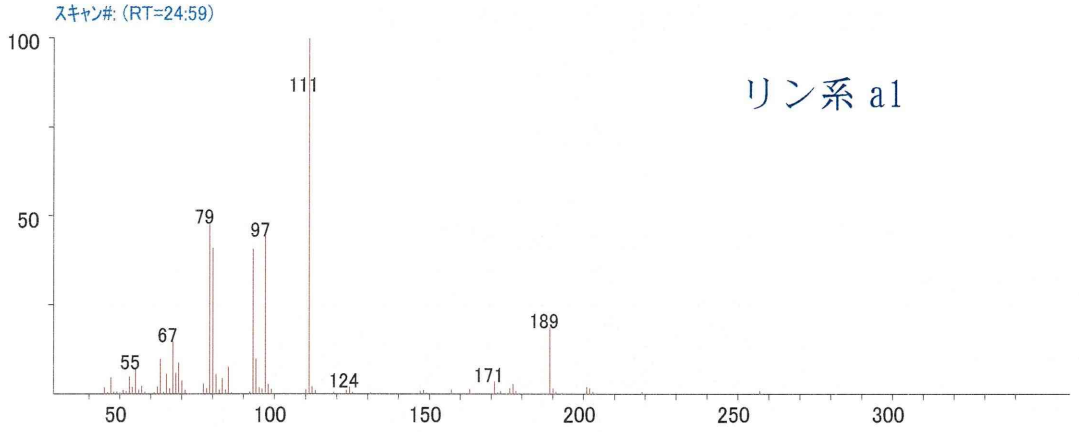


図 6 トータルイオンクロマトグラム



Triphenylphosphine Oxide
Chemical Formula: C18H15OP
Exact Mass: 278.09
Molecular Weight: 278.29

図 7 TPPO の構造式



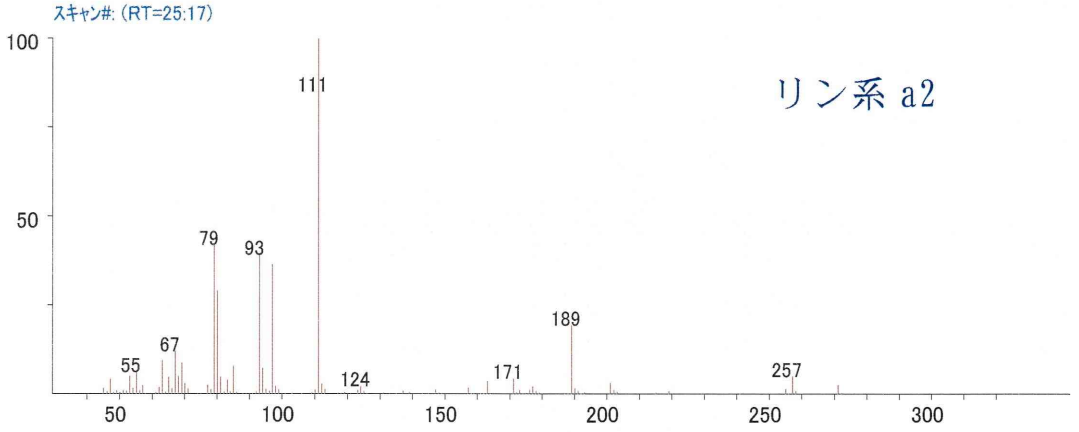


図 8　No. 19 (RT24. 5 及び RT25. 17) のスペクトル

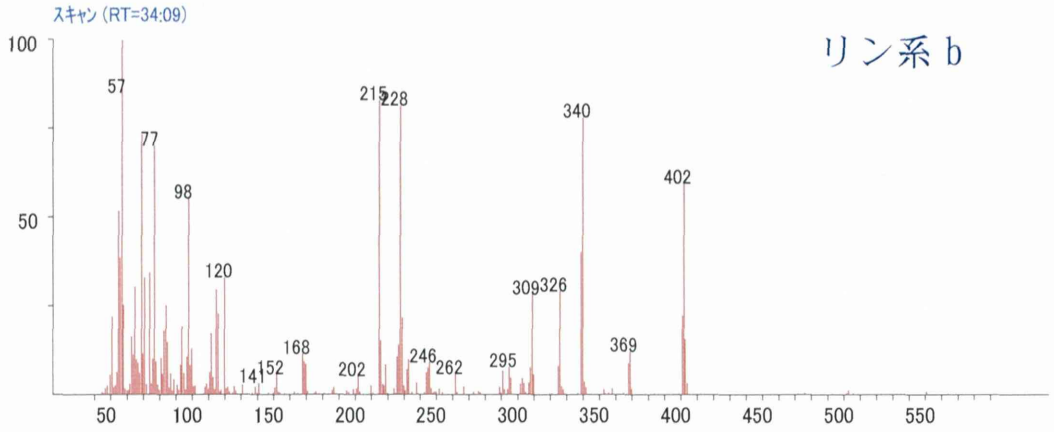


図 9　No. 14 (RT34. 09) のスペクトル

表 4 サンプリングバック法による放散試験結果

①NO.13カーテン

Compounds	60℃×6h		28℃×3d	
	Air(μg/m3)	壁面(μg)	Air(μg/m3)	壁面(μg)
*TDCPP	<0.33	2.22	<0.33	0.47
*TPhP	<0.33	<0.04	<0.33	<0.04
TCsP	<0.33	<0.04	<0.33	<0.04
*TDBPIC	<1.7	<0.20	<1.7	<0.20

②NO.19カーテン

	60℃×6h		28℃×3d	
	Air(μg/m3)	壁面(μg)	Air(μg/m3)	壁面(μg)
TDCPP	<0.33	<0.04	<0.33	<0.04
*TPhP	<0.33	<0.04	<0.33	<0.04
*TCsP	<0.33	2.38	<0.33	0.44
*TDBPIC	<1.7	<0.20	<1.7	<0.20

＊含有難燃剤実態調査で検出された化学物質

別添５

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
雨谷敬史，三宅祐一	室内環境中の未規制物質の問題	環境科学会誌	27巻	339-401	2014年
Takasu, S., Ishii, Y., Matsushita, K., Kuroda, K., Kijima, A., Kodama, Y., Ogawa, K., Umemura, T.	No effect of high fat diet-induced obesity on spontaneous reporter gene mutations in gpt delta mice.	Asian Pac J Cancer Prev.	15巻17号	7149-52	2014年
Onami, S., Cho, Y.-M., Toyoda, T., Mizuta, Y., Yoshida, M., Nishikawa, A. and Ogawa, K.	A 13-week repeated dose study of three 3-monochloropropane-1,2-diol fatty acid esters in F344 rats.	Arch. Toxicol.	88巻	871-880	2014年
Toyoda, T., Cho, Y.M., Mizuta, Y., Akagi, J., Ogawa, K.	A 13-week subchronic toxicity study of ferric citrate in F344 rats.	Food Chem Toxicol.	74巻	68-75	2014年

環境科学シンポジウム 2014

9. 室内環境中の未規制物質の問題

1. シンポジウムの趣旨

我々は1日の大半を室内環境で過ごすことなどから，室内環境は，我々の健康に直接大きな影響を与えると考えられる。本シンポジウムは本年度から開始されている厚生労働科学研究費・化学物質リスク事業「室内環境中の未規制物質の問題」で今後解決すべき問題について，議論することを目的とした。

1990年代に社会問題化したシックハウス・シックスクール問題は，建築基準法によるホルムアルデヒドなどの使用制限，13種の化学物質の室内環境指針値の策定により規制物質については改善されたが，未規制物質の問題が残っている。この問題に対しては，室内で使用され，健康影響についての報告がある個々の化学物質に対する規制と共に，化学物質と我々の生活との関わりを戦略的に考える必要がある。そこで，現在問題となっている未規制物質にどのような種類があり，それらがどのような有害性を示し，どのように曝露されているのかといったことが，どこまで判っているか議論した。

2. 各講演の概要

1) 室内空気中の揮発性有機化合物：室内濃度指針値見直しのスキーム

話題提供者：神野透人（国立医薬品食品衛生研究所）

1997～2002年に揮発性/準揮発性有機化合物13物質及び総揮発性有機化合物（TVOC）にそれぞれ室内濃度指針値，暫定目標値が設定されてから10年余りが経過した。この間，室内濃度指針値策定物質については行政施策等による低減化が図られてきた。しかし，その一方で代替溶剤等による新たな室内環境汚染の可能性が指摘されているものの，その実態は十分に把握されているとは言い難い状況である。また，WHO Guidelines for Indoor Air Quality: Selected Pollutants（2010）に収載された室内濃度指針値未策定物質による汚染実態の把握も急務となっている。このような背景から，国立医薬品食品衛生研究所生活衛生化学部では厚生労働省化学物質安全対策室の委託事業として地方衛生研究所との協働による「室内空気環境汚染化学物質調査：全国実態調査」を3年間にわたって実施してきた。本シンポジウムでは，これらの一連の調査結果を基に日本の室内空気質の現状を概説するとともに，シックハウス（室内空気汚染）問題に関する検討会において事務局から提案された「室内濃度指針値見直しのスキーム」を紹介した。

2) リン酸エステル系難燃剤・ベンゾトリアゾール系UV吸収剤のハウスダスト中残留レベル

話題提供者：磯部友彦（国立環境研究所）

近年，ハウスダストに含まれる有機リン系難燃剤（PFRs）・ベンゾトリアゾール系UV吸収剤（BUVSs）による室内環境汚染とヒト曝露が懸念されている。PFRsは，防燃や延焼防止を目的に樹脂製品や繊維製品などに添加される化学物質であり，世界で年間約30万tが生産されている。BUVSsは，紫外線による皮膚への悪影響や樹脂の劣化を防ぐために日焼け止め剤，プラスチックなどに添加され，需要量の増大が報告されている。これらの日用品に含まれる化学物質は，製品から揮発・溶出してハウスダストに蓄積する。そのため，ヒトの曝露や影響を評価する上で，ハウスダストの蓄積レベルの把握が重要となるが，包括的な分析法は確立されておらず，汚染実態に関する情報は限定的である。一方で，近年大量の電気電子機器廃棄物（e-waste）が資源リサイクル目的で先進国から途上国へ輸入されており，それらの不適切な処理によるヒトの化学物質曝露に対して懸念が高まっているものの，途上国における調査事例は皆無である。そこで本シンポジウムでは，ハウスダスト中のPFRsおよびBUVSsを分析することで，室内環境汚染の実態を解明するとともに，ハウスダストを介したヒトの曝露量推定について紹介した。

3) エコチル調査パイロット調査におけるハウスダスト中樹脂添加剤等の測定

話題提供者：鈴木　剛（国立環境研究所）

ヒトは，大気や水，食品，各種製品等，様々な媒体を通して化学物質を摂取している。中でも，樹脂添加剤（難燃剤，可塑剤等）として使用されている一部の有機ハロゲン化合物は，身の回りの製品を介して摂取され，人の健康に悪影響を及ぼしている可能性が指摘されている。室内で使用されている製品

中化学物質の摂取経路としては，製品から放散した化学物質の呼吸による吸入だけでなく，手等に付着したハウスダスト中化学物質の摂取がある。ハウスダスト中有機ハロゲン化合物等の摂取は，“hand-to-mouth”行動をする幼児で特に重要視されており，子どもの健康と環境に関する全国調査（エコチル調査）においても注目されている。本研究では，エコチル調査本体における家庭環境測定時に採取されるハウスダストの分析にあたって，パイロット調査で採取された試料を用いて測定項目の優先順位付けや試料処理・分析法の検討を行った。

本研究は，(1) 測定対象物質の選定とダスト試料のふるい処理の評価を目的とする「包括分析」，(2) 選定物質をモニタリングして検出一般性と濃度レベルを評価する「ターゲット分析」，(3) 選定物質について居住者の体内負荷量との関係性を評価する「生体試料分析」の三段階の調査を計画している。(1) の包括分析では，(2) で実施するモニタリング調査のための予備調査と位置付け，測定対象物質の選定とふるい作業の評価を行った。具体的には，測定対象物質の選定評価では，ハウスダスト中化学物質の濃度レベルを評価し，(2) の測定対象物質を選定した。

包括分析では，ハウスダストを採取した60家庭のうち，難燃剤等の樹脂添加剤の濃度が比較的高いと想定されるハウスダストを数試料選定して，難燃剤や可塑剤等の樹脂添加剤，POPs（残留性有機汚染物質）や金属類等の分析評価を実施した。本シンポジウムでは，包括分析の結果と，ターゲット分析の進捗状況について紹介した。

4) 臭素系難燃剤とその代替物質のリスクトレードオフ評価

話題提供者：徳村雅弘（横浜国立大学）

有害な化学物質から人間の健康と環境を守るため，市場で流通している化学物質に有害性が懸念された場合，しばしば予防的に代替物質への移行が検討される。被代替物質と比べ，すべての点において代替物質の方が優っているケースは稀であり，多くの場合は被代替物質と代替物質との間でリスクトレードオフが発生する。効果的な代替を行うためにはリスクトレードオフの評価を行うことが望ましいが，代替物質の候補としてあげられる物質は膨大であり，毒性などの有害性に関する情報が不足している場合もあり，十分な評価が行えない場合が多い。代替物質の候補となる膨大な数の化学物質に対し一つ一つ毒性試験を行うことは，コストや時間的にみても現実的ではない。

ヘキサブロモシクロドデカン（HBCD）は平成25年5月にストックホルム（POPs）条約の付属書A（廃絶）に追加されることが決定された臭素系難燃剤であり，国内でも平成16年に第一種監視化学物質に，平成22年に第三種監視化学物質に指定されたことから，自主的に代替が推し進められている。一方，HBCDの国内のリスク評価結果によれば，健康リスクも生態リスクも懸念されるレベルにはない。

本シンポジウムでは，定量的構造活性相関（Quantitative Structure Activity Relationship：QSAR）と呼ばれる毒性予測手法を用いた，毒性が未知の代替物質を対象としたリスクトレードオフ評価の手法について，HBCDを被代替物質とした研究例について紹介した。

5) 室内環境中の未規制物質へのアプローチ

話題提供者：雨谷敬史（静岡県立大学）

建築物の高気密化により発生する化学物質の問題は，室内空気質ガイドラインの作成によりその一部が解決されたが，室内の化学物質は多種多様であり，ヒト健康影響や環境影響が懸念される例も報告されている。特に，電気電子製品やカーテン等の繊維などに使用されている難燃剤や可塑剤には，環境残留性，生物蓄積性及び有害性が高い物質が含まれており，早急な代替化が求められている。ただし，これらの代替物質についても，ハザード評価が十分でない場合が多く，新たなリスクとなることが懸念されている。

本研究プロジェクトでは，(1) 室内に存在する製品や化学物質の情報（物性，含有形態，含有率など）を収集することで室内化学物質ライブラリを作成し，(2) ライブラリを用いたスクリーニングから抽出された高懸念物質のハザード評価を行う。さらには (3) これらの化学物質のヒトへの正確な曝露状況の検討を行うこととしている。本シンポジウムでは，プロジェクトで提案している室内環境中の未規制物質に対する網羅的な調査方法の概要を紹介し，その進捗状況について報告した。

3. ま と め

室内環境中に存在すると考えられている未規制化学物質のうち，主としてハウスダストに付着していると考えられる樹脂添加剤，繊維添加剤等について議論した。健康リスクの観点からは，対策に急を要する物質は既に規制されているものがほとんどであるが，多種多様な化学物質，工業的に作られている化学物質が検出されてきており，ハザード評価，曝露評価を含めたリスク評価の重要性が益々高まってきていると感じた。また，シックハウス症候

群（化学物質過敏症）のような，低濃度でも問題になるような疾病への対策も今後の課題として残っており，嗅覚受容体のメカニズムや臭い刺激のメカニズムの解明とともに考えていくべき問題と考えられた。

オーガナイザー　雨谷敬史・三宅祐一
（静岡県立大学）

RESEARCH ARTICLE

No Effect of High Fat Diet-Induced Obesity on Spontaneous Reporter Gene Mutations in *gpt* Delta Mice

Shinji Takasu[1], Yuji Ishii[1], Kohei Matsushita[1], Ken Kuroda[1], Aki Kijima[1], Yukio Kodama[2], Kumiko Ogawa[1], Takashi Umemura[1]*

Abstract

A large number of epidemiological studies have demonstrated that obesity is a risk factor for several human cancers. Several animal studies using rodents with diet-induced or genetic obesity have also demonstrated that obesity can promote tumor development. However, the effects of obesity on the early stages of carcinogenesis, and especially on the spontaneous occurrence of somatic gene mutations, remain unclear. To investigate the effects of obesity on the rate of spontaneous gene mutations, we performed reporter gene mutation assays in liver, kidney, and colon, organs in which obesity appears to be associated with cancer development on the basis of epidemiological or animal studies, in mice with high fat diet (HFD)-induced obesity. Six-week-old male and female C57BL/6 *gpt* delta mice were fed HFD or standard diet (STD) for 13 or 26 weeks. At the end of the experiments, reporter gene mutation assays of liver, kidney, and colon were performed. Final body weights and serum leptin levels of male and female mice fed HFD for 13 or 26 weeks were significantly increased compared with corresponding STD-fed groups. Reporter gene mutation assays of liver, kidney, and colon revealed that there were no significant differences in *gpt* or Spi$^-$ mutant frequencies between STD- and HFD-fed mice in either the 13-week or 26-week groups. These results indicate that HFD treatment and consequent obesity does not appear to influence the spontaneous occurrence of somatic gene mutations.

Keywords: Obesity - *in vivo* mutagenicity

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Introduction

A large number of epidemiological studies have demonstrated that obesity is a risk factor for several human cancers. A previous systematic review and meta-analysis showed that a 5 kg/m^2 increase in body mass index (BMI), commonly used as a marker of body fatness, is strongly associated with increased risk of several human cancers including colon [risk ratio (RR) 1.24] and renal cancers (RR 1.24) in men, and renal cancer (RR 1.34) in women (Renehan et al., 2008). A greater BMI is also a modifiable risk factor for colon cancer for Asia population (Morrison et al., 2013). On the basis of epidemiological studies, the World Cancer Research Fund and the American Institute for Cancer Research suggested that greater body fat is associated with increased risk for several cancers, including colorectal, kidney, pancreas, breast (postmenopausal) and endometrial cancer, and has been suspected as a causative factor for gallbladder cancer. In addition, there is limited evidence suggesting that greater body fatness increases the risk of liver cancer (World Cancer Research Fund, 2007).

Several animal studies using rodents with diet-induced or genetic obesity have also indicated that obesity has the potential to promote tumor development. Excessive feeding of dietary fat such as corn oil has been reported to promote azoxymethane (AOM)-induced colon carcinogenesis in F344 rats (Reddy and Maeura, 1984). The *db/db* mouse, a genetically altered animal model with obesity due to a functional defect in the long-form leptin receptor (Lee et al., 1996), is sensitive to chemical carcinogenesis such as AOM-induced colon carcinogenesis (Hirose et al., 2004) and diethylnitrosamine (DEN)-induced liver carcinogenesis (Iwasa et al., 2010). In previous studies, various pathophysiological mechanisms linking obesity to cancer have been suggested. Insulin resistance and subsequent abnormal activation of insulin-like growth factor (IGF) and the IGF-1 axis has been considered to play a key role in carcinogenesis (van Kruijsdijk et al., 2009; Shimizu et al., 2011). Other possible mechanisms include dysfunction of adipose tissue due to dysregulation of adipocytokines such as adiponectin, leptin, and plasminogen activator inhibitor-1 resulting from the enlargement of adipocytes (Shimizu et al., 2011). Thus, the effects of obesity on the promotion of tumor development and the associated cancer cell biology

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have been widely investigated. Nevertheless, the effects of obesity on the early stages of carcinogenesis, and especially the spontaneous occurrence of somatic gene mutations, remain unclear.

Animals carrying reporter genes, such as *gpt* delta transgenic animals, have been utilized to estimate the *in vivo* mutagenicity of environmental chemicals (Nohmi et al., 2000). In addition, these animals have also been used to investigate spontaneous gene mutations affected by specific genetic defects such as p53 or Nrf2 knockout (Aoki et al., 2007; Masumura et al., 2011). In the present study, to determine the effects of obesity on spontaneous gene mutations, we performed reporter gene mutation assays of liver, kidney, and colon, which are organs in which obesity has been associated with cancer development on the basis of epidemiological or animal studies, in high fat diet (HFD)-fed obese mice.

Materials and Methods

Animals

Five-week-old male and female C57BL/6 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 per haploid genome were randomized by body weight into 2 groups. They were housed in polycarbonate cages with hardwood chips for bedding in a conventional animal facility, air-conditioned to 23±2°C and 55±5% humidity, on a 12 hour light-dark cycle. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan).

Animal treatments

Following 1 week of acclimatization, 6-week-old male and female C57BL/6 *gpt* delta mice were fed HFD (Quick fat diet, crude fat; 13.6% [CLEA Japan, Tokyo, Japan]) or standard diet (STD) (CE-2 diet, crude fat; 4.3% [CLEA Japan]) *ad libitum* for 13 or 26 weeks. Body weights were measured once a week. At the end of the experiments, all animals were euthanized under deep anesthesia. Livers, kidneys, and colon mucosa were collected and frozen immediately in liquid nitrogen and stored at -80°C for *in vivo* mutation assays. A portion of each of the harvested livers was fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. At necropsy, blood samples were collected from the abdominal aorta for analysis of serum leptin levels using the mouse leptin Quantikine ELISA Kit (R&D systems, MN).

In vivo mutation assays

6-Thioguanine (6-TG) and Spi$^-$ selection were performed as previously reported by Nohmi T. et al. (Nohmi et al, 2000). Briefly, genomic DNA was extracted from liver, kidney, and colon mucosa and lambda EG10 DNA was rescued as phages by *in vitro* packaging. For 6-TG selection, packaged phages were incubated with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, infected cells were also poured onto plates containing chloramphenicol without 6-TG. The plates were then incubated at 37°C. Positively selected colonies were counted on day 3 and collected on day 4. The *gpt* mutant frequencies (MFs) were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi$^-$ selection, packaged phages were incubated with E. coli XL1-Blue MRA for survival titration and E. coli XL1-Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. On the next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes and the agar plugs suspended in SM buffer. The Spi$^-$ phenotype was confirmed by spotting the suspensions on three types of plates on which XL1-Blue MRA, XL1-Blue MRA P2, or WL95 P2 strains were spread with soft agar. Spi$^-$ mutants, which produced clear plaques on every plate, were counted.

Statistical analysis

All results were expressed as mean±SD. The data for body weights, *gpt* and Spi$^-$ MFs, and serum leptin levels were analyzed by Student's test.

Results

Obesity development

Body weight curves are shown in Figure 1. Final body weights of male mice fed STD or HFD for 13 weeks were 30.6±1.9 g and 36.2±3.2 g, and those of mice fed the diets for 26 weeks were 35.5±3.7 g and 45.2±3.4 g, respectively. Final body weights of female mice fed STD or HFD for 13 weeks were 23.2±1.1 g and 25.7±1.9 g, and those of mice fed the diets for 26 weeks were 26.0±2.0 g and 35.6±5.3 g, respectively. Final body weights of male and female mice fed HFD for 13 or 26 weeks were significantly

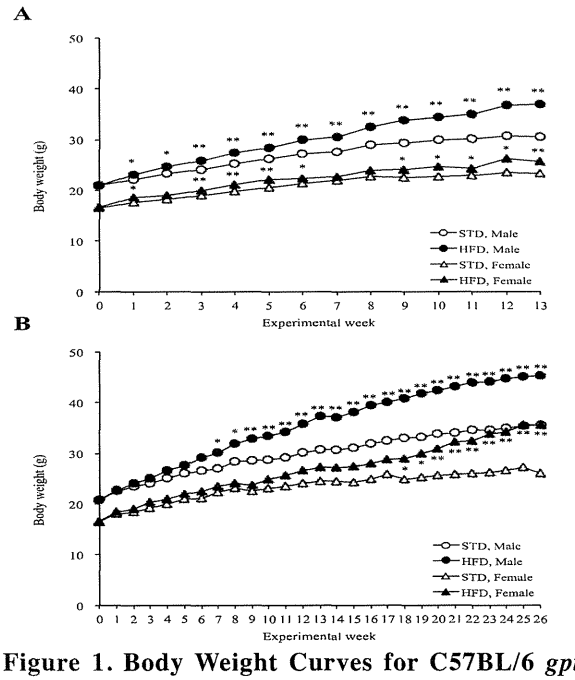

Figure 1. Body Weight Curves for C57BL/6 *gpt* Delta Mice Fed HFD for 13 A) or 26 B) Weeks. *,**, Significantly different from the STD Group at $p<0.05$ and $p<0.01$, respectively

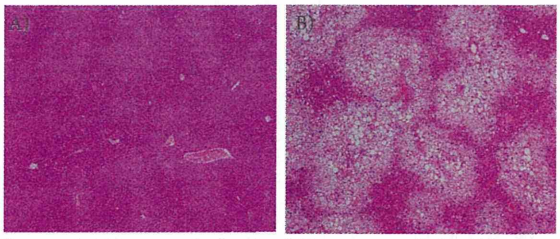

Figure 2. Histopathological Features of the Livers of C57BL/6 *gpt* Delta Mice Fed STD A) or HFD B) for 26 Weeks. HE Stain

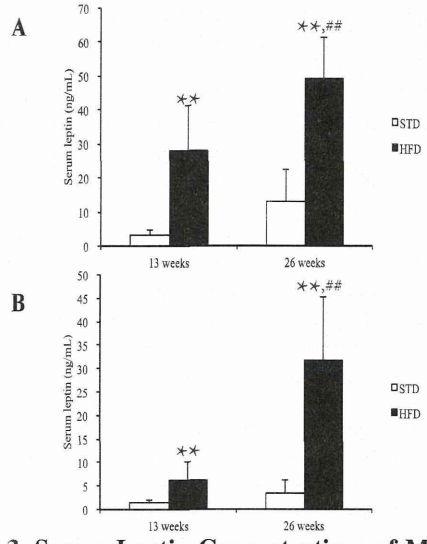

Figure 3. Serum Leptin Concentrations of Male (A) and Female (B) C57BL/6 *gpt* Delta Mice Fed HFD for 13 or 26 weeks. **Significantly different from the STD group at $p<0.01$. ##, significantly different from the group fed HFD for 13 weeks at $p<0.01$. Values are means±SD

Table 1. Gpt Mutant Frequencies in the Liver, Kidney and Colon of Mice Treated with STD or HFD

Organ	sex	Mutant frequency ($x10^{-5}$)[a]			
		13 weeks		26 weeks	
		STD	HFD	STD	HFD
Liver	Male	0.24±0.13[b]	0.22±0.15	0.73±0.11	0.61±0.17
	Female	0.72±0.24	0.45±0.19	0.57±0.19	0.52±0.21
Kidney	Male	0.27±0.17	0.36±0.28	0.67±0.59	0.49±0.26
	Female	0.27±0.12	0.22±0.11	0.48±0.32	0.36±0.21
Colon	Male	0.38±0.12	0.51±0.19	0.95±0.64	0.92±0.31
	Female	0.50±0.19	0.63±0.31	1.10±0.52	0.80±0.42

[a]Mutant frequency were calculated by dividing the number of chloramphenicol and 6-TG resistant clonies by the number of chloramphenicol resistant clonies. [b]Mean±SD

increased compared with corresponding STD-fed groups. Histopathological examination showed that the livers of mice fed HFD showed substantial steatosis (Figure 2). In addition, the final body weights of male and female mice fed STD or HFD for 26 weeks were also significantly increased compared with those of mice fed STD or HFD for 13 weeks. Serum leptin levels of male and female mice fed HFD for 13 or 26 weeks were significantly increased compared with corresponding controls (Figure 3). In addition, animals that underwent HFD feeding for 26 weeks showed significantly increased serum leptin levels compared with those of animals fed HFD for 13 weeks.

Table 2. Spi$^-$ Mutant Frequencies in rhe Liver, Kidney and Colon of Mice Treated with STD or HFD

Organ	sex	Mutant frequency ($x10^{-5}$)[a]			
		13 weeks		26 weeks	
		STD	HFD	STD	HFD
Liver	Male	0.15±0.09[b]	0.19±0.08	0.37±0.29	0.14±0.04
	Female	0.16±0.07	0.12±0.04	0.50±0.35	0.23±0.08
Kidney	Male	0.17±0.07	0.31±0.23	0.16±0.05	0.30±0.13
	Female	0.16±0.05	0.19±0.09	0.29±0.15	0.19±0.01
Colon	Male	0.20±0.16	0.15±0.04	0.28±0.11	0.23±0.09
	Female	0.35±0.17	0.21±0.11	0.31±0.30	0.34±0.11

[a]Mutant frequency were calculated by dividing the number of plaques within WL95 (P2) by the number of plaues within XL-1 Blue MRA; [b]Mean±SD

gpt and Spi$^-$ MFs in liver, kidney, and colon

Data for *gpt* and Spi$^-$ MFs in liver, kidney, and colon from mice fed HFD for 13 or 26 weeks are summarized in Table 1 and 2. There were no significant differences in *gpt* and Spi$^-$ MFs between the STD- and HFD-treated mice for either duration of feeding.

Discussion

In the present study, body weights of mice fed HFD were significantly higher than those of mice fed STD. The degree of body weight gain was increased with HFD feeding duration. These results imply that HFD feeding is responsible for the observed increase in body weight. In addition to the increase in body weights, histopathological analysis revealed that the livers of mice fed HFD exhibited steatosis, indicating that the mice fed HFD under the present experimental conditions became obese due to a metabolic disorder.

In recent years, a strong association between obesity and tumor development has come to be widely accepted. Some studies using rodents have suggested that obesity has the potential to promote tumor development (Hirose et al., 2004; Iwasa et al., 2010; Padidar et al., 2012). Genetically altered obese animal models such as *db/db* mice, which show hepatic steatosis accompanied by obesity (Iwasa et al., 2010; Trak-Smayra et al., 2011), are reported to have a high susceptibility to chemical carcinogenesis of the liver and colon (Hirose et al., 2004; Iwasa et al., 2010).

Meanwhile, since some adipocytokines such as leptin and adiponectin have been reported to affect metabolism, inflammation, angiogenesis, and cell-cycle regulation (Vansaun, 2013), breakdown of their homeostasis due to obesity has been proposed to play an important role in the promotion of tumor development (van Kruijsdijk et al., 2009). Indeed, one of the above-mentioned studies documenting the effects of obesity on the development of colon preneoplastic lesions reported that serum leptin concentrations of mice fed a high fat diet (60% fat by energy) were 4.2 times higher than those of mice fed a low-fat diet (10% fat by energy) (Padidar et al., 2012). Leptin, which is a 16kD bioactive protein mainly secreted from adipocytes, acts as a regulator of energy balance to regulate satiety and modulate glucose and insulin homeostasis (Bjorbaek and Kahn, 2004). Leptin elicits activation of Janus-activated kinase 2 and subsequent phosphorylation of insulin receptor substrates, and subsequently initiates activation of phosphoinositide

3-kinase (PI3K)/Akt pathways through the short-form leptin receptor (Ceddia, 2005). In addition, leptin binding to the long-form leptin receptor activates signal transducer and activator of transcription (STAT) 3 and STAT5 (Ceddia, 2005). Activation of these pathways leads to proliferation of various cells, including neoplastic cells, and promotes growth (van Kruijsdijk et al., 2009). Thus, it has been proposed that hyperproduction of leptin may contribute to tumor development. In the present study, serum leptin concentrations of male mice fed HFD for 13 and 26 weeks were 9.3 and 3.8 times higher, respectively, than those of mice fed STD for the same time periods. Among female mice, serum leptin concentrations in HFD-fed mice were 4.3 and 9.7 times higher than those of mice fed STD for the same time periods. These findings indicate that the degree of obesity under the present experimental conditions was sufficient for investigation of the effects of obesity on spontaneous gene mutations. The tissues examined in the present study, liver, kidney, and colon, were selected because they have been proposed to be associated with obesity-related cancer development on the basis of epidemiological studies (Renehan et al., 2008; World Cancer Research Fund, 2007). Thus, the organs examined in present study were considered to be reasonable target tissues in which to investigate the effects of obesity on the spontaneous occurrence of somatic gene mutations.

To investigate the effects of obesity on spontaneous gene mutations, we performed reporter gene mutation assays of liver, kidney, and colon from mice, and demonstrated that there were no significant differences between the STD- and HFD-fed groups. Our previous data demonstrated that the spontaneous *gpt* MFs in the colon of male C57BL/6 *gpt* delta mice at 11 weeks of age was 0.82±0.43 (Means±SD, n=5) (Okamura et al., 2010). The values at 19 and 32 weeks of age in the present study were 0.38±0.12 and 0.95±0.64, respectively. Therefore, the values of *gpt* MFs in the present study seem to be within the same range of data from the previous study. Thus, the present data showing no significant increase in spontaneous gene mutations in the organs associated with obesity-related cancer even under conditions of severe obesity suggest that HFD treatment and consequent obesity do not affect the frequency of spontaneous somatic gene mutations in spite of the condition being effective on the tumor promotion.

In conclusion, we demonstrated that obesity induced by HFD did not influence the frequency of reporter gene mutations in liver, kidney, or colon from *gpt* delta mice. Therefore, the tumor-promoting effects of obesity may not involve spontaneous gene mutations, suggesting that obesity may contribute only to the promotion of carcinogenesis.

Acknowledgements

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REGULATORY TOXICOLOGY

A 13-week repeated dose study of three 3-monochloropropane-1,2-diol fatty acid esters in F344 rats

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Abstract 3-monochloropropane-1,2-diol (3-MCPD), a rat renal and testicular carcinogen, has been reported to occur in various foods and food ingredients as free or esterified forms. Since reports about toxicity of 3-MCPD esters are limited, we conducted a 13-week rat subchronic toxicity study of 3-MCPD esters (palmitate diester: CDP, palmitate monoester: CMP, oleate diester: CDO). We administered a carcinogenic dose (3.6×10^{-4} mol/kg B.W./day) of 3-MCPD or these esters at equimolar concentrations and two 1/4 lower doses by gavage with olive oil as a vehicle five times a week for 13 weeks to F344 male and female rats. As a result, five out of ten 3-MCPD-treated females died from acute renal tubular necrosis, but none of the ester-treated rats. Decreased HGB was observed in all high-dose 3-MCPD fatty acid ester-treated rats, except CDO-treated males. The absolute and relative kidney weights were significantly increased in the ester-treated rats at medium and high doses. Relative liver weights were significantly increased in the esters-treated rat at high dose, except for CMP females. Significant increase in apoptotic epithelial cells in the initial segment of the epididymis of high-dose ester-treated males was also observed. The results suggested that although acute renal toxicity was lower than 3-MCPD, these three 3-MCPD fatty acid esters have the potential to exert subchronic toxicity to the rat kidneys and epididymis, to a similar degree as 3-MCPD under the present conditions. NOAELs (no-observed-adverse-effect levels) of CDP, CMP and CDO were suggested to be 14, 8 and 15 mg/kg B.W./day, respectively.

Keywords 3-MCPD fatty acid esters · Rat · Toxicity · Palmitate · Oleate · Epididymis

Abbreviations

3-MCPD	3-monochloropropane-1,2-diol
CDP	3-MCPD palmitate diester
CMP	3-MCPD palmitate monoester
CDO	3-MCPD oleate diester
HVP	Hydrolyzed vegetable proteins
JECFA	Joint FAO/WHO Expert Committee on Food Additives
NOAEL	No-observed-adverse-effect level
LOEL	Lowest observed effect level
PMTDI	Provisional maximum tolerable daily intake
BMDL	Lower confidence limit of benchmark dose
BMD	Benchmark dose

Introduction

3-Monochloropropane-1,2-diol (3-MCPD), belonging to a group of compounds called chloropropanols, is known to be one of the most common food-processing contaminants. It was first detected in acid-hydrolyzed vegetable proteins

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(acid-HVP) (Velíšek et al. 1980). But several subsequent studies have also demonstrated its presence in various foodstuffs such as cereal-derived products (bread, biscuits), malt-derived products, coffee, cheese, smoked food, meat and salted fish (Baer et al. 2010; Crews et al. 2001).

Concerning the toxicological profile, 3-MCPD is known to be genotoxic in most in vitro assays (WHO 2002) but not in vivo assays (El Ramy et al. 2007; Robjohns et al. 2003; SCF 2001). In an animal bioassay, no evidence of carcinogenic potential was revealed in B6C3F1 mice receiving drinking water containing 3-MCPD at 30, 100 and 300 (changed to 200 at day 100) ppm (equivalent to 4.2, 14.3, and 33.0 mg/kg B.W./day for males and 3.7, 12.2 and 31.0 mg/kg B.W./day for females, respectively) (Jeong et al. 2010). However, in the rat, it is considered to be a carcinogen targeting the kidneys (renal tubule carcinomas) and testes (Leydig cell tumor) from studies of drinking water administration at 25, 100 and 400 ppm (equivalent to 1.97, 8.27 and 29.50 mg/kg B.W./day for males and 2.68, 10.34 and 37.03 mg/kg B.W./day for females, respectively) in the SD strain (Cho et al. 2008b) and at 20, 100 and 500 ppm (equivalent to 1.1, 5.2 and 28 mg/kg B.W./day for males and 1.4, 7.0 and 35 mg/kg B.W./day for females, respectively) in F344 animals (Sunahara et al. 1993). 3-MCPD is highly suspected to be a non-genotoxic carcinogen, but there have been suggestions that the kidney tumors might be secondary to chronic nephropathy and the Leydig cell tumors might be strain-specific and/or associated with hormonal imbalance (Lynch et al. 1998; WHO 2002). In 2001, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the provisional maximum tolerable daily intake (PMTDI) of 3-MCPD as 2 μg/kg body weight based on the lowest observed effect level (LOEL) of 1.1 mg/kg B.W./day found in a long-term study (Sunahara et al. 1993) and a safety factor of 500, which included a factor of five for extrapolation from an LOEL to an no-observed-effect level (NOEL) (WHO 2002). In this study, the LOEL was derived from consideration of renal tubular hyperplasia observed in the low-dose 3-MCPD group (1.1 mg/kg B.W./day) (Sunahara et al. 1993).

Importantly, further analyses have revealed that 3-MCPD occurs in various foods either as a free form or more commonly esterified with long-chain fatty acids. High concentrations of 3-MCPD fatty acid esters have been reported in hydrogenated fats, palm oil and palm oil fractions, and solid flying fats (ILSI 2009; Zelinková et al. 2006). Moreover, the occurrence of 3-MCPD fatty acid esters in human breast milk has been documented (Zelinková et al. 2008). However, data for toxicity of 3-MCPD fatty acid esters is quite limited, in spite of the considerable human exposure to these chemicals.

As evidence of acute oral toxicity of 3-MCPD 1-monopalmitic ester (CMP) and 3-MCPD dipalmitic ester (CDP), Liu et al. (2012) reported induction of renal tubular necrosis and decrease of spermatids in the seminiferous tubules of Swiss mice, CMP exerting greater effects than CDP (Liu et al. 2012). A ninety-day toxicological study of CDP conducted by Barocelli et al. (2011) at doses of 9.78, 39.19 and 156.75 mg/kg B.W./day by daily oral gavage with corn oil (2 mL/kg B.W.) in Wistar rats demonstrated renal and testicular changes induced by high-dose CDP are similar to that in phenotype but milder than that with equimolar doses of 3-MCPD. For CDP, the authors therefore proposed the benchmark doses (BMD_{10}) as 41.1 and 64.1 mg/kg B.W./day and corresponding lower confidence limits of the benchmark doses ($BMDL_{10}$) as 17.4 and 44.3 mg/kg B.W./day from severe renal and testicular damage, respectively (Barocelli et al. 2011).

3-MCPD fatty acid esters include various forms with different fatty acids thought to be metabolized to 3-MCPD in the body (Abraham et al. 2013; Buhrke et al. 2011; Seefelder et al. 2008). However, it is unclear whether their toxicological profiles are all identical to that of 3-MCPD. As a further concern, 3-MCPD might be metabolized to glycidols of a genotoxic carcinogen, though this reaction is characteristically observed in bacteria (Wijngaard et al. 1989). Therefore, prompt attention should be paid to potential toxicity of 3-MCPD fatty acid esters (Bakhiya et al. 2011).

To evaluate the toxicological profiles of three 3-MCPD fatty acid esters, CDP, CMP and 3-MCPD oleate diester (CDO) for comparison with 3-MCPD, we conducted the present rat subchronic toxicity study with oral gavage administration using olive oil, which is reported to be minimally contaminated by 3-MCPD fatty acid esters (Destaillats et al. 2012; Zelinková et al. 2006).

Materials and methods

Test chemicals

Olive oil, 3-MCPD (98 % pure), CDP (98 % pure), (*sn1*)-CMP (98 % pure) and CDO (98 % pure) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Initially, (*sn2*)-CMP was also synthesized by Wako for analysis, but was found to be unstable and naturally converting into (*sn1*)-CMP. For this reason, we analyzed only (*sn1*)-CMP as a monoester.

Animals, diet and housing conditions

Six-week-old male and female F344 rats were obtained from Charles River Japan (Kanagawa, Japan). The animals were housed in polycarbonate cages (three or four rats per cage) with soft chips for bedding in a room with a barrier system, maintained under conditions of controlled temperature (24 ± 1 °C), humidity (55 ± 5 %), air change (12 times per hour) and lighting (12 h light/dark cycle), and were given free access to a CRF-1 basal diet (Oriental Yeast, Tokyo, Japan) and tap water.

Experimental design

After a one-week acclimatization period, 120 adult male F344 rats weighing 97.5–128.7 g and 120 adult female F344 rats weighing 81.5–103.8 g were used in this experiment. Rats were allocated with body-weight-basis randomization to twelve groups per sex, each consisting of 10 males and 10 females. The groups of both sexes were control (non-treatment group), olive oil (vehicle control group: 5 mL/kg B.W.), 3-MCPD (40 mg/kg B.W.) and various doses of 3-MCPD fatty acid ester groups. Doses of 3-MCPD fatty acid esters were determined to be 14, 55 and 220 mg/kg B.W. for CDP, 8, 32 and 130 mg/kg B.W. for CMP and 15, 60 and 240 mg/kg B.W. for CDO, the highest doses in each case being equimolar concentrations to the 40 mg/kg B.W. for 3-MCPD, selected on the basis of previous carcinogenicity testing (Cho et al. 2008b). 3-MCPD and 3-MCPD fatty acid esters were dissolved in olive oil at the time of dosing and administered five times a week by intragastric intubation for 13 weeks. During the period of administration, the animals were observed daily for any clinical signs and mortality. Body weights were measured weekly. At the end of the study, after an overnight fast, all the animals were anesthetized with isoflurane, weighed and blood samples were collected from the abdominal aorta for serum biochemistry and hematology. The animals were then killed by exsanguination from the abdominal aorta. The present experimental protocol was basically in accordance with Guidelines for Designation for Food Additives and for Revision of Standards for Use of Food Additives of Japan (1996), with minor changes, and approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (MHLW 1996).

Serum biochemistry

Parameters for serum biochemistry shown in Supplementary Table S3 and S4 were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation of whole blood.

Hematological examination

Parameters for hematology shown in Supplementary Table S5 and S6 were analyzed using an automated hematology analyzer, K-4500 (Sysmex Corp., Hyogo, Japan). Differential leukocyte and reticulocyte counts were performed with a MICROX HEG-50S (Sysmex Corp.).

Histopathology

All surviving animals were necropsied at 13 weeks. The brain, heart, lungs, liver, kidneys, spleen, thymus, adrenal glands and testes were weighed. Relative organ weights were calculated as the values relative to body weights. In addition to these organs, the nasal cavity, trachea, aorta, pituitary, thyroids, parathyroids, adrenal gland, salivary gland, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, epididymides, prostate, seminal vesicles, ovaries, uterus, vagina, mammary gland, skin, lymph node, sternum, femur including bone marrow, sciatic nerve, trigeminal nerve, spinal cord (cervical, thoracic and lumber cords), eyes and thigh muscle were fixed in 10 % neutral buffered formalin. Testes were fixed in Bouin's solution overnight and then transferred into 10 % neutral buffered formalin. Eyeballs, nervi opticus and Harderian glands were fixed in Davidson's solution overnight and then transferred into 10 % neutral buffered formalin. Tissues that needed decalcification, such as the nasal cavity, spinal cord with bones, sternum and femur, were treated with a mixture of 10 % formic acid and 10 % neutral phosphate-buffered formalin. Adequately trimmed tissues were embedded in paraffin, sectioned at 3 μm thick for hematoxylin and eosin staining, and examined under a light microscope. Histopathological examinations were carried out for the highest dose and control groups. If a chemical treatment-related change appeared at the highest dose, the relevant tissue(s) from the lower-dose groups were then also examined. Animals found dead or moribund were also analyzed as far as possible.

Immunohistochemical analysis

For the evaluation of cell proliferation in the kidney, Ki-67 labeling indices were immunohistochemically analyzed with anti-Ki67 rabbit monoclonal antibodies (SP6, 1:500: abcam, Cambridge, UK) in the paracortical area of proximal tubular epithelium per at least 2,000 proximal tubular epithelium cells per animal.

Statistical analysis

Variance in the data for body weights, organ weights, serum biochemistry and hematology was checked for homogeneity by Bartlett's procedure. After analysis of variance, the significance of differences in the multiplicity of lesions was evaluated using Tukey's test. For incidences of histopathological findings, the Fisher's exact probability test was applied. p values <0.05 between the olive oil group and treated groups were considered statistically significant.

Results

General condition, body weight

Survival curves are shown in Fig. 1. One male rat treated with 3-MCPD died in the third week, one receiving

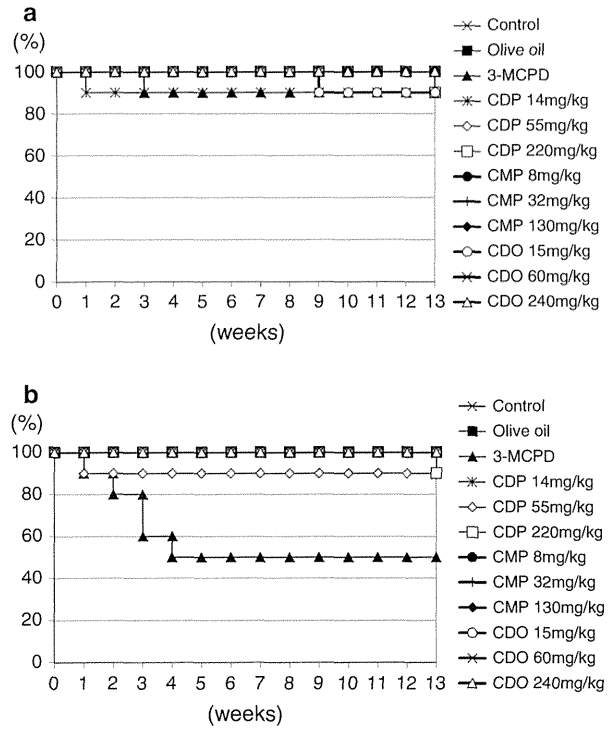

Fig. 1 Survival (%) for F344 rats treated with 3-MCPD fatty acid esters for 13 weeks: **a** males; **b** females

14 mg/kg CDP in the first week, one given 220 mg/kg CDP in the 13th week and one administered 15 mg/kg CDO in the ninth week. Four female rats treated with 3-MCPD died, one each in the first, second, and fourth weeks. One female given 55 mg/kg CDP died in the first week and one receiving 220 mg/kg CDP on the 13th week. One female rat treated with 3-MCPD was observed to be moribund and therefore was immediately killed in the third week.

Except for these 12 rats, there was no deterioration in the general conditions observed in any of the groups.

Body weight data are shown in Supplementary Fig. S1. The body weights of all groups increased gradually and progressively with age. Significantly elevated body weights were detected in the male non-treated control group compared to the vehicle control (olive oil) group in the 12th and 13th weeks. No significant differences in female body weight gain were detected among the treatment groups.

Organ weights

Final body weights and the absolute and relative organ weights are summarized in Supplementary Tables S1 and S2. Significant increase in absolute and relative kidney weights was observed in male and female rats treated with 3-MCPD and with all 3-MCPD fatty acid esters at medium or high doses (Fig. 2). Significant increase in relative liver weights was also noted in 3-MCPD and all high-dose 3-MCPD fatty acid ester-treated male and female groups, except for CMP females. These changes observed in 3-MCPD fatty acid ester groups showed dose dependence (Fig. 2).

The absolute liver and relative heart and spleen weights were significantly increased in high-dose CMP males. The relative heart weight was significantly increased in high-dose CDP and CMP females. The relative brain weights were significantly increased in high-dose CMP and CDO females.

Serum biochemistry

The results of serum biochemical analysis are summarized in Supplementary Tables S3 and S4. Significant increase in chlorine bilirubin (Bil) was observed in medium-dose CDP females. Significant decrease of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was observed in medium-dose CMP females, high-dose CMP or CDO females and medium- or high-dose 3-MCPD fatty acid ester males, respectively. Significant decrease of blood urea nitrogen (BUN) was observed in CMP females. Significant decrease of creatinine (Cre) was observed in the 3-MCPD and 3-MCPD fatty acid ester-treated groups, except for low-dose CMP males, and low-dose CDP and CDO females.

Significant decrease of sodium (Na) and chlorine (Cl) was observed in low and medium CMP females, while significant increase was observed in high-dose CMP males.

Hematological examination

The results of hematological examination are summarized in Supplementary Tables S5 and S6. Significant decrease of HGB was observed in the groups treated at high dose with all 3-MCPD fatty acid esters, except in COD males (Fig. 3). Additionally, it was observed in low-dose CDP females and medium-dose CMP females. Significant decrease of HCT was observed in high-dose CMP males and high-dose CDP, CMP and CDO females. Significant decrease of RBC was also observed in females of low-dose and high-dose CDP, high-dose CMP and high-dose COD groups. Significant decrease of MCH was observed in females given 3-MCPD and high doses of any of the 3-MCPD fatty acid esters. Significant decrease of MCHC was observed in females treated with 3-MCPD and high-dose CDP and CDO. These changes reflecting anemia were seen with tendencies for dose dependence, especially in 3-MCPD fatty acid ester-treated females.

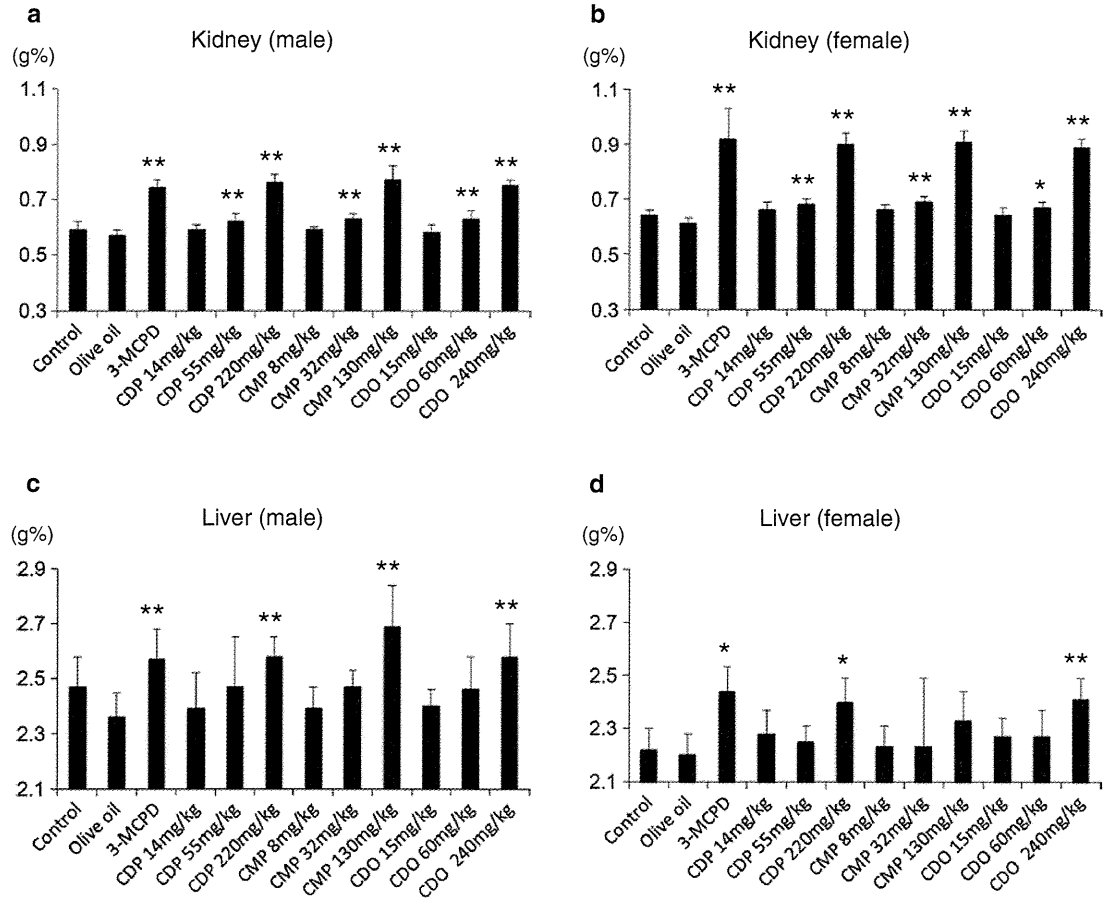

Fig. 2 Relative kidney and liver weights for F344 rats treated with 3-MCPD fatty acid esters for 13 weeks: **a** relative kidney weights for males; **b** relative kidney weights for females; **c** relative liver weights for males; **d** relative liver weights for females. *, **Significantly different from the olive oil group at $p < 0.05$ and $p < 0.01$, respectively

Histopathology

Severe tubular necrosis of the kidney was observed in the dead or moribund rats treated with 3-MCPD (Supplementary Fig. S2). The dead rats in 3-MCPD fatty acid ester groups did not show such changes in the kidney (Supplementary Table S7) but exhibited lesions including hemorrhage of the esophagus and thoracic cavity. The histopathological findings in the rats treated with high-dose test chemicals and controls for 13 weeks are summarized in Table 1. Additional examination of all dose groups was performed for the testis, epididymis and kidney. In the testis, focal granuloma, unilateral or bilateral seminiferous tubular atrophy and aspermatogenesis were observed without clear relation to the treatment and/or dose dependence. No treatment-related changes in Leydig cells were observed. In the epididymis, apoptotic cell death (Fig. 4A) was noted, limited to the columnar epithelium of the initial segment, the degree being apparently related to the dose. For quantitative evaluation, the rates of ducts with apoptotic cells in the initial segment of the epididymis were analyzed and that in male 3-MCPD and high-dose 3-MCPD fatty acid esters groups was significantly increased as compared to the vehicle control group (Fig. 4B). In the kidneys, neither proliferative lesions nor fibrosis were observed in the treated groups. The labeling index of Ki-67 in the proximal tubules was not altered in the treated groups (Supplementary Fig. S3). The incidence of renal mineralization in female 3-MCPD fatty acid ester groups tended to be increased (Supplementary Fig. S4). In the brain, small spongiotic lesions with slight gliosis were observed in the bilateral nucleus of the diencephalon of one female treated with high-dose CDO (Supplementary Fig. S5). This lesion was not found in other rats, although additional sections for evaluation were carefully examined. In other tissues, there were no significant changes in the incidences of lesions between the vehicle control and treatment groups.

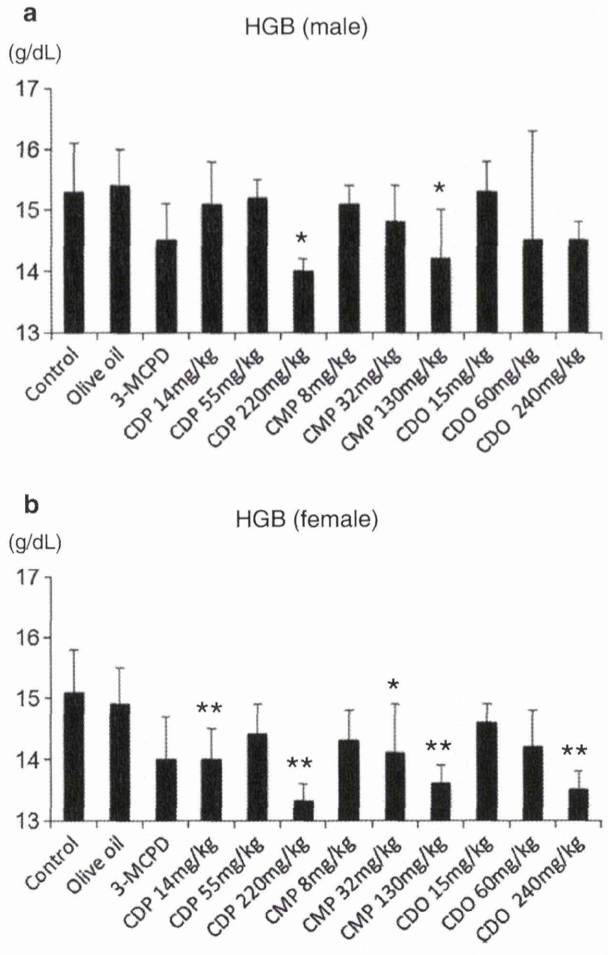

Fig. 3 Concentration of HGB for F344 rats treated with 3-MCPD fatty acid esters for 13 weeks: **a** males; **b** females. *, **Significantly different from the olive oil group at $p < 0.05$ and $p < 0.01$, respectively

Discussion

In the present experiment, for comparison of toxicity of three 3-MCPD fatty acid esters and 3-MCPD in rats, we decided a dose level of 3-MCPD close to that of the two-year rat carcinogenesis study performed by Cho et al. (2008b) in which 400 ppm in drinking water (equivalent to 29.50 and 37.03 mg/kg B.W./day for male and female, respectively) was defined as the high-dose level (Cho et al. 2008b). The mortality in our study was higher than that earlier at 13 weeks. In our experiment, five out of 10 females and one out of 10 males treated with 3-MCPD died or became moribund by the end of week four. Barocelli et al. (2011) also reported that single oral gavage administration of 3-MCPD resulted in similar mortality (50 %) but lower mortality (20 %) when 3-MCPD was given twice a day at half the dose (Barocelli et al. 2011). Therefore, it was suggested that peak level of blood 3-MCPD concentration might be critical for acute toxicity of 3-MCPD by oral gavage, apparently more in female than in male rats. Indeed, similar to Barocelli's study, histopathological examination revealed tubular necrosis in all six dead rats treated with 3-MCPD. On the other hand, histological evidence of damage such as hemorrhage of the esophagus ~ thoracic cavity and/or bacterial colony of the lung were noted without any kidney lesions in all dead rats treated with 3-MCPD fatty acid esters, thus suggesting accidental death related to gavage.

Abraham et al. (2013) reported oral gavage application of free 3-MCPD with corn oil was followed by rapid absorption resulting in high peak levels in the blood within 30 min. In contrast, elevation of the blood 3-MCPD concentration after administration of CDP was considerably slower and resulted in correspondingly lower peak levels at 2–3 h (Abraham et al. 2013). Considering that the mortality of rats given 3-MCPD in the drinking water (Cho et al. 2008b; Sunahara et al. 1993) was also low, our study supports the hypothesis that differences in peak 3-MCPD blood levels affect the acute renal toxicity and the survival rate.

In serum biochemistry, none of the changes observed in this study were considered as toxicity-related, because they were opposite to those expected for toxic change or non dose-dependent. Surprisingly, no change related to renal toxicity was noted.

In hematological data, a proclivity for hypochromic anemia was observed in all 3-MCPD fatty acid ester groups and more prominent in females than males with dose dependence, although histopathological changes in the hematopoietic organs related to hematopoesis or severe hemorrhage resulting in anemia were not observed. In the study conducted by Barocelli et al. (2011), a tendency for anemia was observed in males and females treated with either 3-MCPD or CDP (Barocelli et al. 2011).

In organ weight data, the absolute and relative kidney weights in males and females were significantly increased in 3-MCPD and in all 3-MCPD fatty acid ester groups given at medium and high dose. Furthermore, significant increase in relative liver weights was noted in 3-MCPD and high-dose 3-MCPD fatty acid esters groups, except for female CMP group, without any related changes in histology or serum chemistry. Cho et al. (2008a) reported similar increase in relative kidney weights with 200 and 400 ppm (equivalent to 36.97 and 76.79 mg/kg B.W./day for male, 30.23 and 61.34 mg/kg B.W./day for female, respectively) 3-MCPD administration and increase in relative liver weights in females with 400 ppm 3-MCPD administration for 13 weeks in B6C3F1 mice (Cho et al. 2008a). Similar effects were also observed at high doses of either 3-MCPD or CDP in Wistar rats (Barocelli et al. 2011). Other

Table 1 Histopathological findings for F344 rats treated with 3-MCPD fatty acid esters for 13 weeks

Organs and findings	Males						Females					
Treatment:	Control	Olive oil	3-MCPD 40 mg/kg	CDP 220 mg/kg	CMP 130 mg/kg	CDO 240 mg/kg	Control	Olive oil	3-MCPD 40 mg/kg	CDP 220 mg/kg	CMP 130 mg/kg	CDO 240 mg/kg
No. of animals:	10	10	9	9	10	10	10	10	5	9	10	10
Liver												
Microgranuloma	8	5	3	3	2	7	9	5	4	4	4	4
Bile duct hyperplasia, focal	2	2	0	0	2	2	0	0	0	0	1	0
Kidney												
Karyomegaly, renal tubles, focal	0	0	0	0	0	0	0	0	1	0	0	0
Renal cyst, focul	2	2	0	0	1	0	3	1	3	1	0	1
Mineralization	2	7	4	5	5	6	6	5	5	9	10	10
Heart												
Mononuclear cell infiltration, focal	7	2	2	1	3	3	2	1	0	1	1	0
Lung												
Foam cell infiltration, focal	0	2	3	1	1	0	0	0	0	0	0	0
Osseous metaplasia	1	0	0	0	0	0	0	0	0	0	0	0
Stomach												
Erosion, pyloric gland	0	0	0	0	0	1	0	1	0	0	0	0
Testis												
Seminiferous tubule atrophy, bilateral	0	0	0	1	2	1	–	–	–	–	–	–
Seminiferous tubule atrophy, unilateral	0	1	2	0	0	0	–	–	–	–	–	–
Aspermatogenesis, bilateral	0	0	0	1	1	1	–	–	–	–	–	–
Aspermatogenesis, unilateral	0	1	1	0	0	0	–	–	–	–	–	–
Epididymis												
Epithelial cell apoptosis, initial segment[1]	0	0	8**	8**	10**	6	–	–	–	–	–	–
Prostate												
Mononuclear cell infiltration, focal	2	1	0	2	2	2	–	–	–	–	–	–
Brain												
Spongiosis, focal	0	0	0	0	0	0	0	0	0	0	0	1

-: not evaluated

** Significantly different from the olive oil-treated group at $p < 0.01$

[1] Cases with more than 10 % of tubules featuring apoptotic cells

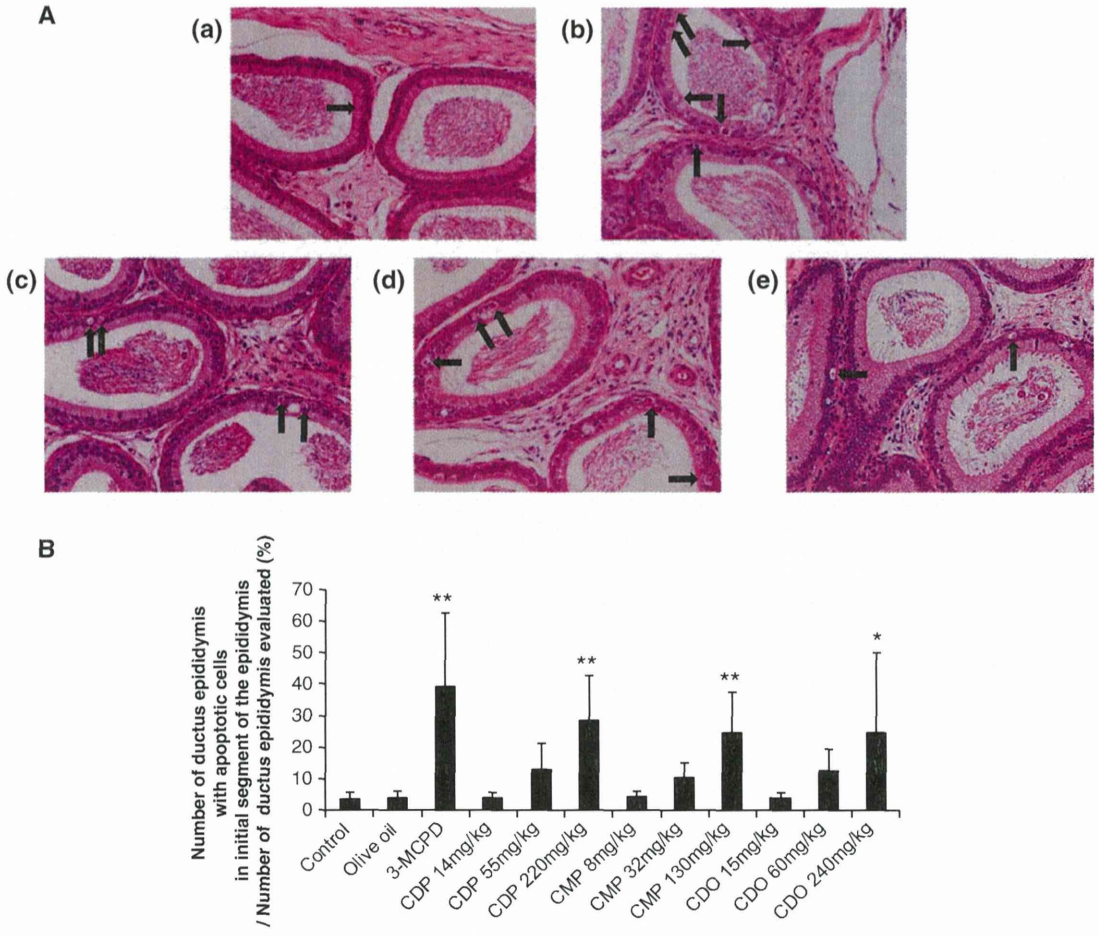

Fig. 4 **A** Representative photographs of initial segments of the epididymis from the olive oil (**a**), 3-MCPD (**b**), CDP (**c**), CMP (**d**) and CDO (**e**) male rat groups. *Black arrows* indicate apoptotic cells. **B** Rates of ductus epididymis with apoptotic cells in the initial segment of the epididymis treated with 3-MCPD fatty acid esters for 13 weeks

sporadic changes were not considered toxicity-related, since they were supported by neither histological nor serum biochemical alteration.

On histopathological analysis, significant changes were acute renal tubular necrosis in the 3-MCPD group and apoptosis in the initial segment of the epididymis observed in all three 3-MCPD fatty acid ester groups as well as 3-MCPD groups at week 13. Liu et al. (2012) reported that renal tubular necrosis and protein casts in the kidneys were the major histopathological changes caused by acute oral toxicity of both CMP and CDP in Swiss mice (Liu et al. 2012). The 90-day toxicology study of 3-MCPD and CDP administered by daily oral gavage conducted by Barocelli et al. (2011) revealed tubular epithelial hyperplasia or proliferation, basophilic or hyaline material and karyomegaly of tubular epithelial cells in CDP-treated rat kidneys (Barocelli et al. 2011). However, we did not observe significant renal lesion at the final kill in any of the treated animals, despite careful observation including analysis of Ki-67-labeling indices. Regarding the testis, decreased spermatids was reported in the seminiferous tubules in Swiss mice given single oral administrations of CMP and CDP. Barocelli et al. (2011) also scored testicular lesions such as decrease of spermatids and degeneration of seminiferous tubules and documented that statistically significant changes were observed only at the high doses but were three times more severe in rats treated with 3-MCPD (29.5 mg/kg B.W./day) than with the equimolar amount of CDP. In our study including a vehicle control group, a few unilateral or bilateral atrophic testes were noticed with a tendency for association with sperm granuloma. Treatment-related changes in the testis including Leydig cells were not observed. More characteristically, quantitative increase in epithelial apoptosis in the initial segment of the epididymis was observed with clear dose dependence and statistical significance in the rats treated with equimolar amounts