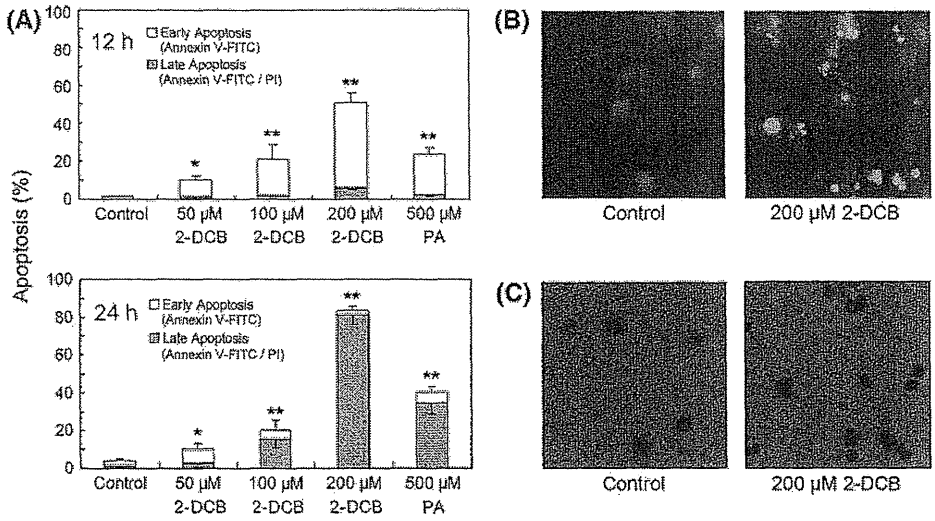

Fig. 3 Apoptosis induced by 2-DCB and PA in U937 cells. **a** U937 cells were treated with 50, 100 and 200 μM 2-DCB and 500 μM PA. The cells were stained with Annexin V-FITC and PI for flow cytometry after 12 and 24 h. Where indicated, values for the treated cells are significantly different to the untreated cells at the same time point at $^{*}p < 0.05$; $^{**}p < 0.01$. The results are expressed as means ± SD ($n = 3$). **b** Apoptosis of U937 cells in response to treatment with 2-DCB for 24 h. The cells subjected to Annexin V-FITC and PI staining were detected by microscopy at a magnification of 400×. One representative photomicrograph from three independent experiments is shown. **c** Morphological changes. Signs of apoptosis were detected by Hoechst 33258 staining and then examined by microscopy at 400× magnification. One representative photomicrograph from three independent experiments is shown

enhanced intracellular superoxide formation at 3 and 6 h (Fig. 4a) ($p < 0.05$). We also evaluated the intracellular levels of peroxides using DCFH-DA staining. The results showed that 2-DCB and PA induced peroxide generation at 6 h (Fig. 4b) ($p < 0.05$), and that the 2-DCB- and PA-treated U937 cells exhibited an increase in DCF fluorescence compared with the control cells (Fig. 4c). Next, we investigated whether this increase in ROS production may play a role in the apoptosis induced by 2-DCB and PA. Our results showed that 10 mM NAC (an antioxidant, *N*-acetyl-L-cysteine) significantly inhibited apoptosis induced by 2-DCB and PA ($p < 0.05$) (Fig. 4d).

Detection of caspase-dependent mitochondrial apoptosis pathway

Caspases are an important executors of apoptosis caused by various apoptotic stimuli. To determine whether the caspase-dependent mitochondrial pathway for apoptosis is related to apoptosis induced by 2-DCB and PA, we measured the MMP change and intracellular caspase-8 and caspase-3 activities at 6 h. The loss of MMP (i.e., an increase in percent subpopulation with low MMP) was observed in the 2-DCB- and PA-treated cells, as determined by TMRM staining and flow cytometry (Fig. 5a) ($p < 0.05$). The treatment of the U937 cells with 2-DCB and PA resulted in a significant increase in both caspase-8 (Fig. 5b) and caspase-3 (Fig. 5c) activities compared with that of the control cells ($p < 0.05$). To confirm the role of caspase on apoptosis induced by 2-DCB and PA in U937 cells, apoptosis (Annexin V-FITC and PI staining) was examined in the presence of 2-DCB and PA with or without z-VAD. Pretreatment with 10 μM z-VAD significantly reduced the extent of 2-DCB- and PA-induced apoptosis ($p < 0.01$) (Fig. 5d), which revealed that 2-DCB- and PA-induced apoptosis was mediated through a caspase-dependent pathway in U937 cells.

Bcl-2 family proteins with anti- or pro-apoptotic functions can control the release of mitochondrial apoptosis factors including cytochrome *c* and the apoptosis-inducing factor (AIF) [27]. Western blot analysis revealed that Bid was cleaved into tBid by 2-DCB and PA at 24 h (Fig. 6). Moreover the treatment with 2-DCB and PA induced Bax up-regulation and Bcl-2 down-regulation (Fig. 6). In addition, we found that the release of cytochrome *c* from the mitochondria to the cytosol was induced by 2-DCB and PA at 24 h (Fig. 6). These results suggest that the enhancement of apoptosis induced by 2-DCB and PA is associated with mitochondrial dysfunction, Bid activation, and the subsequent activation of caspases.

Fas activation by 2-DCB and PA

We investigated Fas activation in relation to the 2-DCB- and PA-induced apoptosis. The treatment with 2-DCB and PA significantly suppressed Fas activation compared with that in the control cells at 3 h and 6 h ($p < 0.05$) (Fig. 7).

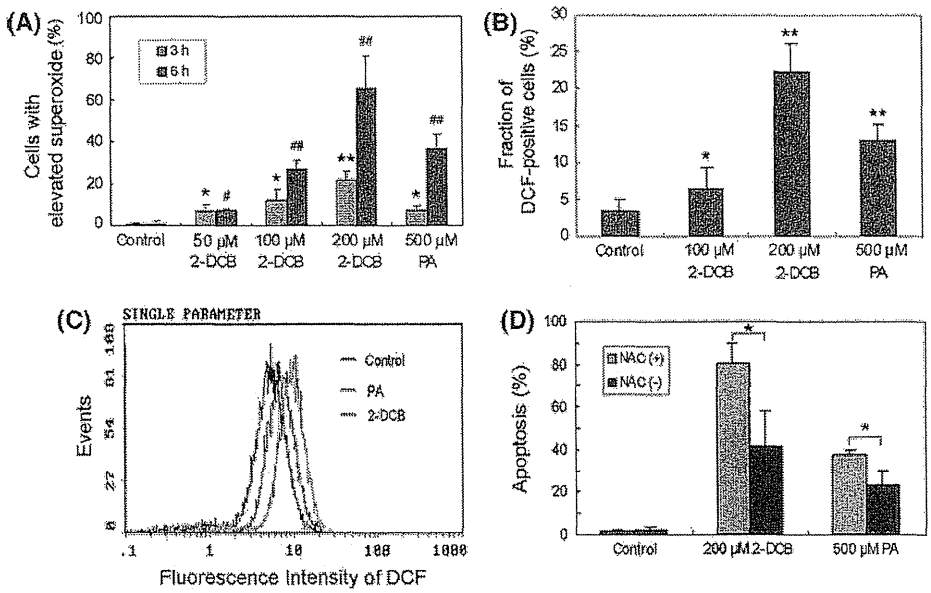

Fig. 4 Effects of 2-DCB and PA on generation of intracellular superoxide and peroxides in U937 cells. **a** Induction of superoxide by 2-DCB and PA in U937 cells. The cells were treated with 2-DCB and PA for 3 and 6 h, followed by the measurement of superoxide formation by flow cytometry. Where indicated, values for the treated cells are significantly different to the untreated cells at the same time point at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. The results are expressed as means ± SD ($n = 3$). **b** Induction of intracellular peroxides by 2-DCB and PA. U937 cells were treated with 2-DCB and PA for 6 h. The cells subjected to 5 μM H_2DCF-DA staining were incubated at 37°C for 30 min before measuring of the intracellular peroxides by flow cytometry. $^{*}p < 0.05$; $^{**}p < 0.01$. The results are expressed as means ± SD ($n = 3$). **c** U937 cells were treated with 200 μM 2-DCB and 500 μM PA for 6 h. 2-DCB- and PA-treated U937 cells exhibited an increase in DCF fluorescence compared with the control cells. One representative photomicrograph from three independent experiments is shown. **d** The cells were pretreated with or without 10 mM NAC for 1 h and stimulated with 200 μM 2-DCB and 500 μM PA for 24 h followed by the measurement of apoptosis by flow cytometry. $^{*}p < 0.05$. The results are expressed as means ± SD ($n = 3$)

Analysis of Ca^{2+}-dependent pathway

We carried out digital imaging of Fura-2 fluorescence to examine the change in $[Ca^{2+}]_i$ after the treatment with 2-DCB and PA. The ratio of fluorescence at 340 nm excitation to that at 380 nm excitation, F (340/380), was used as an indicator of $[Ca^{2+}]_i$ in single cells. The order of the pseudocolors of blue, green, yellow and red indicates the changes in fluorescence ratio from low to high in Fig. 8. Therefore, the blue, green and yellow pseudocolor images of individual cells also indicate the changes in $[Ca^{2+}]_i$ from low to high. The treatment of 2-DCB and PA induced an increase in the number of cells showing a higher $[Ca^{2+}]_i$ in a time-dependent manner. As shown in Fig. 8, the cells treated with 2-DCB and PA exhibited a much higher $[Ca^{2+}]_i$ than the control cells.

Discussion

Food irradiation is gaining interest in light of the increasing incidence of foodborne diseases as it efficiently reduces pathogen population. The process has been approved by FDA and WHO, and also endorsed by many countries and organizations [28]. Irradiation can be performed either with γ-rays produced from a radioactive nuclide such as ^{60}Co or with an electron beam or X-rays generated from machine sources, and the maximum absorbed dose delivered to a food subjected to radiation processing should not exceed 10 kGy [29, 30]. Previous studies have detected 2-ACBs in irradiated fat-containing food such as chicken, pork, lamb, and beef and mechanically recovered meat, and also in irradiated liquid whole eggs [1]. 2-ACBs are not formed by cooking or any other heat-processing method, and were therefore of increasing interest for research in food toxicology. Results of previous in vitro studies of primary human colon cells and in vivo experiments in rats fed 2-DCB suggested that these radiolytic derivatives are genotoxic and enhance the progression of colon tumors [4, 5]. 2-DCB was also shown to induce single-strand DNA damage in LT97 and HT 29 human colon adenoma cells [6, 7]. Additionally, some studies showed that PA can induce apoptosis in various cells such as human lung carcinoma cells, hepatocytes, and renal cells [8, 10, 11, 14, 31]. However, the effect of 2-DCB on apoptosis is not known. Therefore, we elucidate the apoptotic potential of 2-DCB,

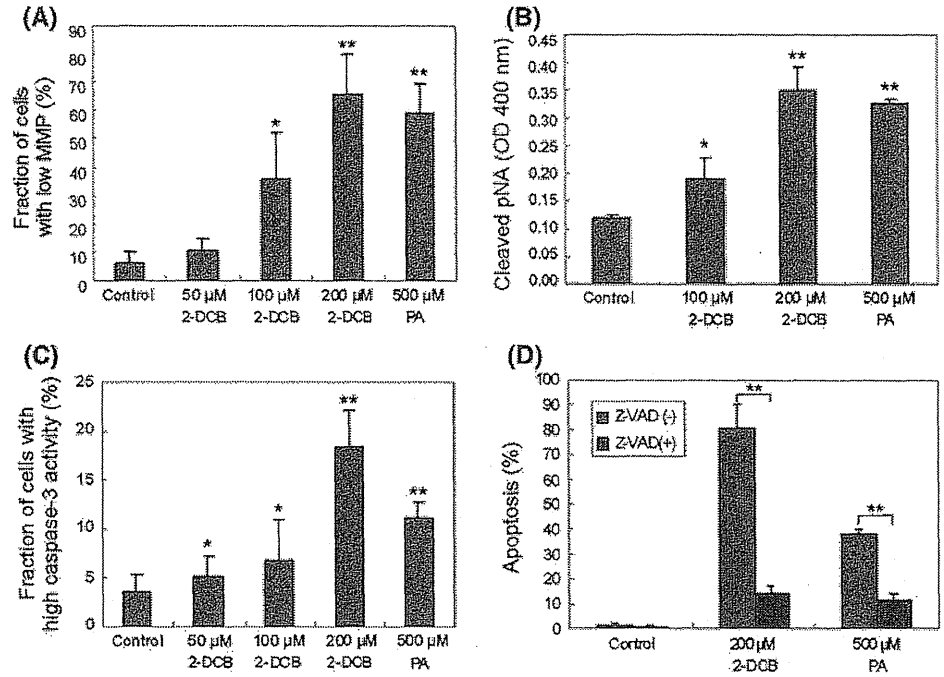

Fig. 5 Effects of 2-DCB and PA on loss of MMP and activation of caspase-8 and caspase-3. **a** The loss of MMP was observed in the cells treated with 2-DCB and PA for 6 h, as determined by flow cytometry using TMRM staining. $*p < 0.05$; $**p < 0.01$. **b** Caspase-8 activation induced by 2-DCB and PA was evaluated by IETD-pNA-cleavage assay. $*p < 0.05$; $**p < 0.01$. **c** Caspase-3 activation induced by 2-DCB and PA was measured by flow cytometry using FITC-DEVD-FMK staining. $*p < 0.05$; $**p < 0.01$. **d** The cells were pretreated with or without z-VAD for 1 h and then coincubated with 2-DCB and PA for 24 h, and apoptosis was measured by flow cytometry. $**p < 0.01$. The results are expressed as means ± SD ($n = 3$)

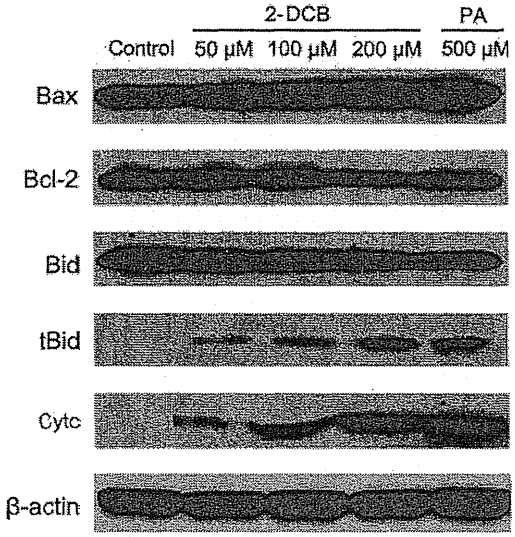

Fig. 6 Effects of 2-DCB and PA on expressions of Bcl-2 family proteins and release of cytochrome *c*. U937 cells were treated with 2-DCB and PA for 24 h. The cell lysates were harvested for Western blot analysis. Changes in expressions of Bax, Bcl-2, Bid, tBid proteins and release of cytochrome *c* (Cytc) in cytosolic fraction induced by 2-DCB and PA. One representative picture from three independent experiments is shown

generated from PA and demonstrate the underlying mechanisms of apoptosis induced by 2-DCB in U937 cells.

ROSs, such as hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2), play a key role in apoptosis induction [32, 33]. These free radicals can induce nucleic acid damage, i.e., oxidative base damage (e.g. 8-oxo-guanine), secondary (due to repair processes) single- and double-strand DNA breaks, DNA–protein cross-links, lipid peroxidation, and protein degradation [34, 35]. PA has been reported to induce mitochondrial superoxide and peroxide production in various cells [31, 36, 37] Previous results showed that 2-DCB genotoxicity occurs mainly by the induction of DNA base damage recognized by Fpg. Fpg recognizes some ring-opened purines and apurinic/apyrimidinic (AP) sites, stet 8-oxo-guanine, which is the most biologically relevant substrate of Fpg. An oxidative endpoint marker of DNA, 8-oxo-guanine has attracted particular attention since it is mutagenic it causes G-to-T transversions upon replication and has been suggested to play an important role in carcinogenesis [38]. In the current study, 2-DCB enhanced intracellular ROS generation in a time-dependent manner and the pretreatment of cells with NAC significantly prevented 2-DCB-induced apoptosis, indicating that

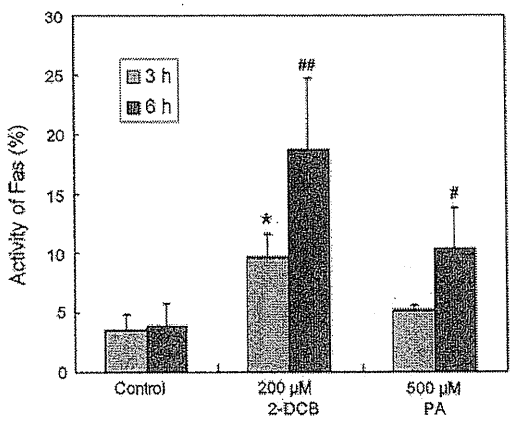

Fig. 7 Effects of 2-DCB and PA on Fas activation. U937 cells were treated with 2-DCB and PA for 3 and 6 h. Then Fas activation was determined by flow cytometry using anti-Fas FITC-conjugated antibody. Where indicated, values for the treated cells are significantly different to the untreated cells at the same time point at $^{*}p < 0.05$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. The results are expressed as means ± SD ($n = 3$)

ROS-dependent pathways were involved in the 2-DCB-mediated cell death.

Two major pathways, the extrinsic and intrinsic pathways, leading to caspase activation have been found [10, 15]. Our data showed the activation of Fas and caspase-8 after the treatment with 2-DCB and PA. The extrinsic pathway is due to the direct interaction of cell surface receptors, such as Fas, with caspase-8, that in turn activates downstream effector caspases. Bcl-2 family proteins are involved in pro- or anti-apoptotic processes by interacting with the mitochondria [27]. In this study, the Bcl-2 down-regulation and Bax up-regulation were shown by Western blot analysis. Moreover, Bid was cleaved by 2-DCB and PA. Bid can be cleaved by caspase-8, and the cleaved Bid as the carboxyl-terminal fragment translocates to the mitochondria to induce the release of cytochrome *c* [4]. Cytochrome *c* functions with Apaf-1 to induce caspase-9 activation, thereby initiating the apoptotic caspase cascade [15]. These two apoptotic pathways may be interconnected

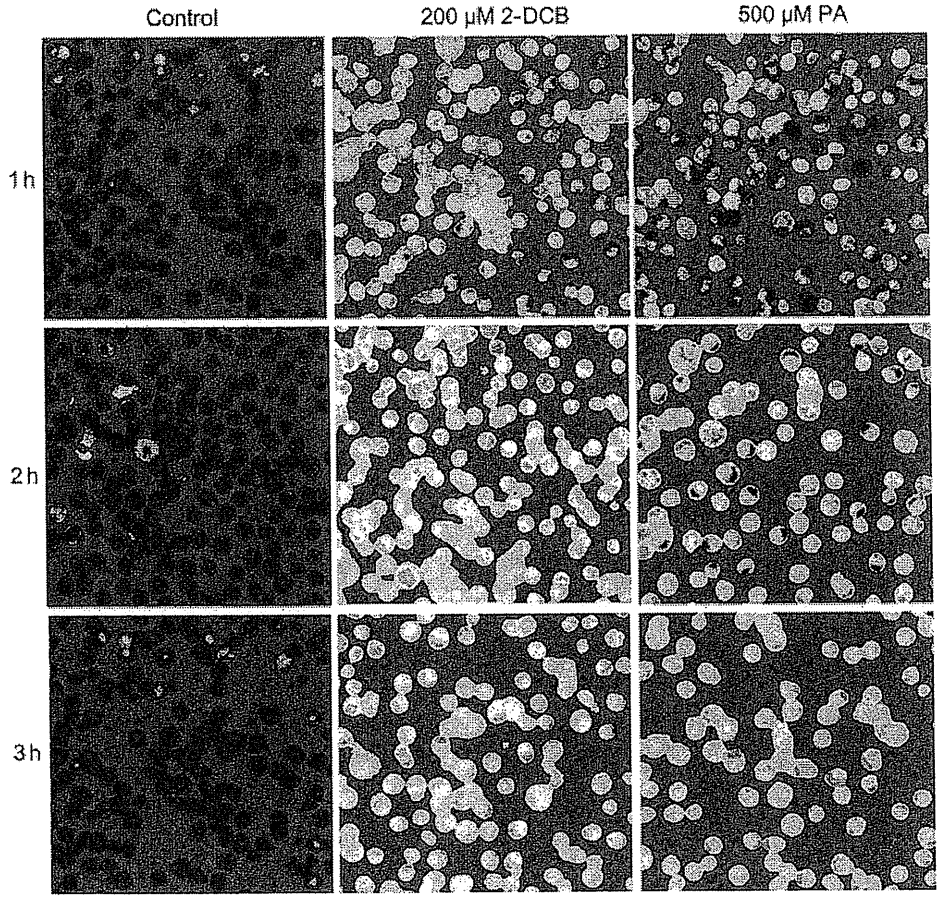

Fig. 8 Effects of 2-DCB and PA on $[Ca^{2+}]_i$ in U937 cells. The cells were stained with Fura-2-AM as described in "Materials and methods" after treatment with 2-DCB and PA, and $[Ca^{2+}]_i$ was then measured. One representative digital images of Fura-2 fluorescence from three independent experiments is shown here

by the caspase-8-mediated cleavage of Bid, which triggers the activation of the mitochondrial pathway. In this study, an increase in the fraction of cells showing the loss of MMP, the release of cytochrome *c* to the cytosol, and the activation of capase-8 and caspase-3 were observed. These results indicate that the apoptosis induced by 2-DCB is involved in the Fas-caspase-8-mediated mitochondrial pathway in U937 cells.

Some studies have shown that Fas activation can trigger apoptosis via ROS induction, a direct means of activating the caspase cascade, or via mitochondria by activating caspase-8 and Bid [39–41]. In this study, we found an enhancement of ROS generation, Fas activation, caspase-8 activation and Bid cleavage after the treatment with 2-DCB and PA in U937 cells. These findings indicate that mitochondrial dysfunction is responsible for the contribution of Fas to apoptosis induction.

It has been reported that oxidative stress induced by peroxides causes an increase in $[Ca^{2+}]_i$ in chondrocytes [42]. It has also been suggested that the loss of MMP preceding nuclear apoptosis is mediated by the opening of permeability transition (PT) pores [43]. Many effectors that can induce apoptosis, including oxidative stress, can induce PT pore opening [44]. We have shown that an increase in $[Ca^{2+}]_i$ is involved in apoptosis induction induced by radiation, hyperthermia and chemicals (such as 6-formylpterin, macrosphelides, and sanazole), which are associated with the enhancement of ROS formation [20, 22, 23, 25, 26]. In this study, ROS generation and an increase in $[Ca^{2+}]_i$ were observed after the treatment with 2-DCB and PA. These results indicate that the apoptosis induced by 2-DCB and PA is associated with the increase in $[Ca^{2+}]_i$ in U937 cells.

Taken together, our preliminary data first showed that 2-DCB has the potential to induce apoptosis mainly via the Fas-caspase-8-mediated mitochondrial pathway, resulting from oxidative stress in human lymphoma U937 cells. The evaluation of $[Ca^{2+}]_i$ also contributes to apoptosis induction by 2-DCB.

Acknowledgment This work was supported in part by grants provided by Food Safety Commission, the Cabinet Office, Japan.

References

1. Stevenson MH, Hamilton JT (1990) Irradiation detection. Nature 344:202–203
2. Crone AVJ, Hamilton JTG, Stevenson MH (1992) Effect of storage and cooking on the dose response of 2-dodecylcyclobutanone, a potential marker for irradiated chicken. J Sci Food Agric 58:249–252
3. Gadgil P, Hachmeister KA, Smith JS, Kropf DH (2002) 2-Alkylcyclobutanones as irradiation dose indicators in irradiated ground beef patties. J Agric Food Chem 50:5746–5750
4. Delincée H, Pool-Zobel B (1998) Genotoxic properties of 2-dodecyl-cyclobutanone, a compound formed on irradiation of food containing fat. Radiat Phys chem 52:39–42
5. Sommers CH, Mackay WJ (2006) DNA damage-inducible gene expression and formation of 5-fluorouracil-resistant mutants in *Escherichia coli* exposed to 2-dodecylcyclobutanone. J Food Sci 70:C254–C257
6. Knoll N, Weise A, Claussen U et al (2006) 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary human colon cells and in cells from preneoplastic lesions. Mutat Res 594:10–19
7. Hartwig A, Pelzer A, Burnouf D et al (2007) Toxicological potential of 2-alkylcyclobutanones—specific radiolytic products in irradiated fat-containing food—in bacteria and human cell lines. Food Chem Toxicol 45:2581–2591
8. Abe A, Yamane M, Yamada H, Sugawara I (2002) The omega-hydroxy palmitic acid induced apoptosis in human lung carcinoma cell lines H596 and A549. J Biochem Mol Biol Biophys 6:37–43
9. Artwohl M, Lindenmair A, Roden M et al (2009) Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure. Atherosclerosis 202:351–362
10. Katsoulieris E, Mabley JG, Samai M, Green IC, Chatterjee PK (2009) α-Linolenic acid protects renal cells against palmitic acid lipotoxicity via inhibition of endoplasmic reticulum stress. Eur J Pharmacol 623:107–112
11. Ricchi M, Odoardi MR, Carulli L et al (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J Gastroenterol Hepatol 24:830–840
12. Jiang H, Liang C, Liu X et al (2010) Palmitic acid promotes endothelial progenitor cells apoptosis via p38 and JNK mitogen-activated protein kinase pathways. Atherosclerosis 210:71–77
13. Hennige AM, Ranta F, Heinzelmann I et al (2010) Overexpression of kinase-negative protein kinase Cdelta in pancreatic beta-cells protects mice from diet-induced glucose intolerance and beta-cell dysfunction. Diabetes 59:119–127
14. Rachek LI, Musiyenko SI, LeDoux SP, Wilson GL (2007) Palmitate induced mitochondrial deoxyribonucleic acid damage and apoptosis in l6 rat skeletal muscle cells. Endocrinology 148:293–299
15. Peterson JM, Wang Y, Bryner RW, Williamson DL, Alway SE (2008) Bax signaling regulates palmitate-mediated apoptosis in C_2C_{12} myotubes. Am J Physiol Endocrinol Metab 295:E1307–E1314
16. Belosludtsev KN, Saris NE, Belosludtseva NV, Trudovishnikov AS, Lukyanova LD, Mironova GD (2009) Physiological aspects of the mitochondrial cyclosporin A-insensitive palmitate/Ca^{2+}-induced pore: tissue specificity, age profile and dependence on the animal's adaptation to hypoxia. J Bioenerg Biomembr 41:395–401
17. Ghosh P, Besra SE, Tripathi G, Mitra S, Vedasiromoni JR (2006) Cytotoxic and apoptogenic effect of tea (*Camellia sinensis* var. *assamica*) root extract (TRE) and two of its steroidal saponins TS1 and TS2 on human leukemic cell lines K562 and U937 and on cells of CML and ALL patients. Leuk Res 30:459–468
18. Shin GC, Kim C, Lee JM et al (2009) Apigenin-induced apoptosis is mediated by reactive oxygen species and activation of ERK1/2 in rheumatoid fibroblast-like synoviocytes. Chem Biol Interact 182:29–36
19. Gorman A, McGowan A, Cotter TG (1997) Role of peroxide and superoxide anion during tumour cell apoptosis. FEBS Lett 404:27–33
20. Zhao QL, Fujiwara Y, Kondo T (2006) Mechanism of cell death induction by nitroxide and hyperthermia. Free Radic Biol Med 40:1131–1143

21. Royall JA, Ischiropoulos H (1993) Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 302:348–355
22. Yu DY, Zhao QL, Wei ZL et al (2009) Enhancement of radiation-induced apoptosis of human lymphoma U937 cells by sanazole. Apoptosis 14:655–664
23. Cui ZG, Kondo T, Ogawa R et al (2004) Enhancement of radiation-induced apoptosis by 6-formylpterin. Free Radic Res 38:363–373
24. Cui ZG, Kondo T, Matsumoto H (2006) Enhancement of apoptosis by nitric oxide released from α-phenyl-tert-butyl nitrone under hyperthermic conditions. J Cell Physiol 206:468–476
25. Ahmed K, Zhao QL, Matsuya Y et al (2007) Rapid and transient intracellular oxidative stress due to novel macrosphelides trigger apoptosis via Fas/caspase-8-dependent pathway in human lymphoma U937 cells. Chem Biol Interact 170:86–99
26. Yu DY, Zhao QL, Wei ZL, Shehata M, Kondo T (2009) Enhancement of hyperthermia-induced apoptosis by sanazole in human lymphoma U937 cells. Int J Hyperthermia 25:364–373
27. Tsujimoto Y, Shimizu S (2000) Bcl-2 family: life-or-death switch. FEBS Lett 466:6–10
28. Kaferstein FK, Moy GG (1993) Public health aspects of food irradiation. J Public Health Policy 14:149–163
29. Alsayyed AMI, Dale KJ, Diehl JF, Karlsruhe et al (1990) Food safety aspects relating to the application of X-ray surveillance equipment: memorandum from a WHO meeting. Bull World Health Organ 68:297–301
30. Swallow AJ (1991) Wholesomeness and safety of irradiated foods. Adv Exp Med Biol 289:11–31
31. Yuzefovych L, Wilson G, Rachek L (2010) Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress. Am J Physiol Endocrinol Metab 299:E1096–E1105
32. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12:913–922
33. Singh SV, Srivastava SK, Choi S et al (2005) Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J Biol Chem 280:19911–19924
34. Riley PA (1994) Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol 65:27–33
35. Wallace SS (1998) Enzymatic processing of radiation-induced free radical damage in DNA. Radiat Res 150:S60–S79
36. Almaguel FG, Liu JW, Pacheco FJ, Casiano CA, De Leon M (2009) Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid. J Neurosci Res 87:1207–1218
37. Kim JE, Song SE, Kim YW et al (2010) Adiponectin inhibits palmitate-induced apoptosis through suppression of reactive oxygen species in endothelial cells: involvement of cAMP/protein kinase A and AMP-activated protein kinase. J Endocrinol 207:35–44
38. Floyd RA (1990) The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis 11:1447–1450
39. Nitobe J, Yamaguchi S, Okuyama M et al (2003) Reactive oxygen species regulate FLICE inhibitory protein (FLIP) and susceptibility to Fas-mediated apoptosis in cardiac myocytes. Cardiovasc Res 57:119–128
40. Strasser A, Newton K (1999) FADD/MORT1, a signal transducer that can promote cell death or cell growth. Int J Biochem Cell Biol 31:533–537
41. Yin XM (2000) Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. Cell Res 10:161–167
42. Asada S, Fukuda K, Nishisaka F, Matsukawa M, Hamanisi C (2001) Hydrogen peroxide induces apoptosis of chondrocytes; involvement of calcium ion and extracellular signal-regulated protein kinase. Inflamm Res 50:19–23
43. Macho A, Hirsch T, Marzo I et al (1997) Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. J Immunol 158:4612–4619
44. Costantini P, Chernyak BV, Petronilli V, Bernardi P (1996) Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 271:6746–6751

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Short communication

Tissue Sample Preparation for *In Vivo* Rodent Alkaline Comet Assay

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The Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study group conducted a collaborative study to investigate whether cell nuclei or whole cells might be more suitably used to correctly detect genotoxic chemicals in the *in vivo* rodent alkaline Comet assay. Four participating laboratories applied four sample processing methods, i.e., three homogenization methods using the usual Potter-type shaft, a customized (loose) Potter-type shaft, or a Downs-loose-type shaft, for preparing cell nuclei, and the mesh membrane method for preparing whole cells, to the male rat liver. Homogenization with the usual Potter-type shaft clearly produced damage of the cell nuclei and DNA, while the other three methods seemed to provide similar conditions of the tissue samples. The proportion of cell nuclei: whole cells was 80–90%: 10–20% in all laboratories when the samples were prepared by homogenization using a Downs-loose-type shaft or by the mesh membrane method. The %DNA in tail were comparable in both samples among the negative control groups (single oral administration with physiological saline) of all laboratories, and showed an equal degree of increase in both samples of the ethyl methanesulfonate groups (single oral administration at 250 mg/kg) in all laboratories. In conclusion, the homogenization method using a loosely customized Potter-type shaft or a Downs-loose-type shaft, and the mesh membrane method would be equally acceptable for the *in vivo* rodent alkaline Comet assay.

Key words: *in vivo* Comet assay, tissue sample preparation, cell nuclei, whole cells

Introduction

Although the *in vivo* rodent alkaline Comet assay is widely used for detecting genotoxic chemicals, the standard protocol and the assay procedure, especially the method of preparation of free cells/nuclei, are still under debated. The International Workshop on Genotoxicity Testing (IWGT; 1,2) and the 4th International Comet Assay Workshop (3) proposed recommended protocols. Currently, the Japanese Center for the Validation of Alternative Methods (JaCVAM) has been coordinating an international validation study of the *in vivo* Comet assay to evaluate the assay capability for detecting genotoxic chemicals, and a standardized study protocol is already established based on the above recommendations and the pre-validation study results (4).

The largest data sets on the assay predictivity for chemical carcinogenicity have been established by Sasaki *et al.* (5,6), which are based on the analysis results of isolated cell nuclei prepared by a tissue homogenization method for many organs. The question has been raised about whether isolated cell nuclei or isolated whole cells may be more suitable to use in order to detect the genotoxic potential of test chemicals. The background for this question is the discrepant *in vivo* rodent Comet assay results published about *ortho*-phenyl phenol (OPP, CAS No. 90-43-7). Sasaki *et al.* reported that OPP showed positive results in the mouse using cell nuclei of the liver, kidney, lung and urinary bladder (7). On the other hand, Bomhard *et al.* obtained negative results in the same species (8). The only difference in the procedure used between Sasaki *et al.* and Bomhard *et al.* was in relation to the object isolated as

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the target for the Comet assay using the liver and kidney (8). One possible explanation for the difference in results was based on how the tissues were processed. Cell death is associated with increased levels of DNA strand breaks, and many researchers in this field feel that isolated cell nuclei might be damaged to a greater degree than isolated whole cells because of the homogenization process, resulting in increased false-positive results. Although there was much discussion on this subject in the 4th IWGT meeting, it was decided that more data were needed before a conclusion could be arrived at and that any international validation study should consider both processing methods (2).

Under this complex circumstance, the Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study group (JEMS/MMS) planned and coordinated a collaborative study to investigate which might be more suitable, cell nuclei or whole cells, for the *in vivo* rat alkaline Comet assay. Four laboratories took part in the collaborative study, applied both sample processing methods to the liver after oral administration to male rats of ethyl methanesulfonate at 250 mg/kg or physiological saline as the negative control, and then compared the assay data. Here, we report results of the collaborative study and discuss which might be more suitable, cell nuclei or whole cells, for the *in vivo* rat alkaline Comet assay.

Materials and Methods

Test chemicals: Ethyl methanesulfonate (EMS, CAS No. 62-50-0) was purchased from Sigma-Aldrich (St. Louis, MO, USA). EMS at 250 mg was dissolved in 10 mL of physiological saline immediately before administration. Physiological saline was also used as the negative control material.

Animals: Crj:CD(SD) male rats were obtained from Charles River Laboratories Japan (Kanagawa). The rats were housed in polycarbonate or stainless-steel cages under air-conditioned circumstances (20–24°C room temperature, 30–70% humidity, and 12 h-light/dark cycle), fed with rodent chow *ad libitum*, and given free access to tap water. The animals were quarantined and acclimated for at least 5 days, and then used at 8-week-old for the experiments.

Animal treatment: First, one non-treated rat was used for comparison of the isolation process of the cell nuclei and whole cells in each laboratory. Then, for the comparison between the isolated cell nuclei and the isolated whole cells, rats (three animals/group) were orally administered one dose of EMS at 250 mg/kg (10 mL/kg) or physiological saline at 10 mL/kg. Four laboratories participated in the former examination, and three in the latter.

Homogenizer shafts: Three types of homogenizer shafts were used for the isolation of the cell nuclei of the liver, i.e., a Potter-type shaft (Shaft A), a loosely customized Potter-type shaft (Shaft B), and a Downs-loose-type shaft (Shaft C). Shaft B was prepared by the following procedures: the Teflon part of Shaft A was ground with sand-paper and adjusted so as to not damage the cell nuclei and/or DNA (i.e., so as to allow loose contact with the homogenizer glass tube).

Isolation of cell nuclei and whole cells: Isolation of the cell nuclei with Shafts A and B was conducted by the method reported by Sasaki *et al.* (9), and that with Shaft C was done by the method reported by Miyamae *et al.* (10). The whole cells were isolated by the method reported by Tice (11). Animals were sacrificed by carbon dioxide inhalation or by cutting the abdominal aorta/vein under anesthesia (e.g., ethyl ether), and the liver was removed. A small piece of the liver (about 5 mm cube fragment) was minced with a pair of scissors and put into a glass tube with a chilled 75 mM sodium chloride solution containing 24 mM EDTA disodium, homogenized once (just one stroke, with the head of the shaft never reaching the bottom of the tube) with Shaft A or B at 600 rpm, and then centrifuged at 700 g for 10 min at 4°C to collect the cell nuclei. Accordingly, two samples of isolated cell nuclei were obtained as Sample A with Shaft A, and Sample B with Shaft B. Another piece of the liver was put into a glass tube with chilled phosphate-buffered saline [Ca^{2+} and Mg^{2+} free: PBS(−)] containing 54 mM EDTA, and then homogenized once (just one stroke) mildly by hand, and the suspension was passed through a nylon mesh (150 μm pore size, Tokyo Rikakikai, Tokyo), and a sample of isolated cell nuclei was obtained as Sample C. For the whole-cell isolation, the mesh membrane method was used. The liver fraction (about 5 mm cube fragment) was minced with a pair of scissors in chilled PBS(−) containing 54 mM EDTA, and the suspension was passed through a metal or nylon mesh (150 μm pore size), and Sample D was obtained as isolated whole cells.

Microscopic observation of the cell nuclei and whole cells: An aliquot of each sample was used for the Comet analysis described below. The remaining Samples C and D were centrifuged at 1500 rpm for 5 min in 4°C. The pellet was suspended and fixed with 5 mL of 10% neutral buffered formalin solution, and the suspension was mixed with Acridine orange (A.O.) solution at the ratio of 1:1. The mixture was immediately examined under a fluorescent microscope, and the ratio of cell nuclei and whole cells was calculated for 500 nuclei.

Comet analysis: The procedure has been described in previous reports (1–6). Briefly, an aliquot of each sample was embedded in 0.5% (w/v) low-melting agarose gel (Lonza, Rockland, ME, USA) on a slide. Two slides were prepared for each animal. The slides were immersed overnight in chilled lysing solution con-

taining 100 mM EDTA disodium, 2.5 M sodium chloride, 10 mM tris hydroxymethyl aminomethane, 1% (v/v) Triton-X100, and 10% (v/v) DMSO in purified water at pH 10, and then rinsed in purified water. The slides were covered with an alkaline solution consisting of 300 mM of sodium chloride and 1 mM of EDTA disodium in purified water at pH > 13 for 10 min, applied for electrophoresis at 1 V/cm and approximately 300 mA for 15 min below 4°C, and immersed for 10–20 min in neutralization buffer consisting of 0.4 M tris hydroxymethyl aminomethane in purified water at pH 7.5. Slides were dehydrated with absolute ethanol for at least 5 min, dried, and then stained with 20 μg/mL of ethidium bromide solution. Nuclei were scored with an image analysis system [Comet Assay IV (Perceptive Instrument Ltd., Suffolk, UK) or Rainbow Star System (Komet 4; Kinetic Imaging Ltd., Liverpool, UK)], and the %DNA in tail were calculated.

Results

In microscopic examination of the two homogenized samples prepared with Potter-type Shafts A and B, it seemed that both Samples A and B consisted of a lot of cell nuclei and few whole cells, and many more damaged cell nuclei and whole cells were observed in Sample A than in Sample B. In another homogenized sample C prepared with the Downs-loose-type Shaft C, there seemed to be a number of cell nuclei, but few whole cells. The sample condition of Sample C seemed similar to that of Sample B, i.e., containing lower numbers of damaged cell nuclei and whole cells. In Sample D, which was prepared with the mesh membrane method, the sample condition seemed to be the same as those of Samples B and C. In the Comet analysis performed with four samples, Sample A clearly showed DNA migration, even samples obtained from non-treated rats (Fig. 1a), and there was no obvious difference between Samples B, C and D prepared from the non-treated rats (Figs. 1b, 1c and 1d). Consequently, the % DNA in tail was increased in Sample A as compared with those in Samples B, C and D (Fig. 2). The number of cell nuclei and whole cells for Samples C and D was scored independently in three laboratories, and the ratio was determined to be 80–90% cell nuclei: 10–20% whole cells for both samples in all laboratories (Fig. 3). The Comet responses in the liver after *in vivo* treatment with EMS were also examined using Samples C and D. The %DNA in tail in both Samples C and D were comparable among the negative control groups in the three laboratories (Fig. 4). The average of these parameters increased similarly in both Samples C and D of the EMS group as compared with the finding in the negative con-

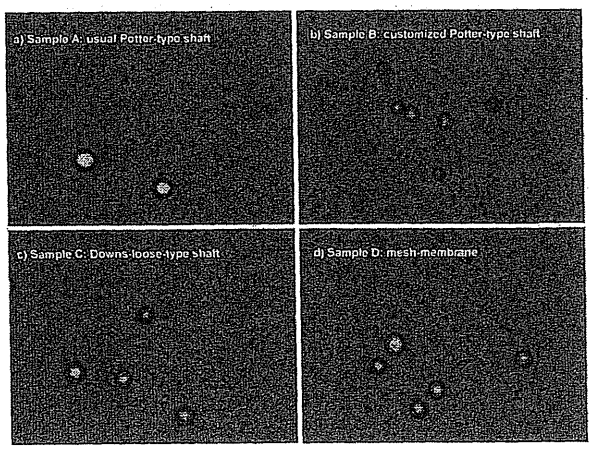

Fig. 1. Comet images obtained using the four sample preparation methods for the non-treated rat liver. Nuclei were stained with ethidium bromide solution. Sample A clearly showed DNA migration, and Samples B, C and D seemed to show no damage to the nuclei.

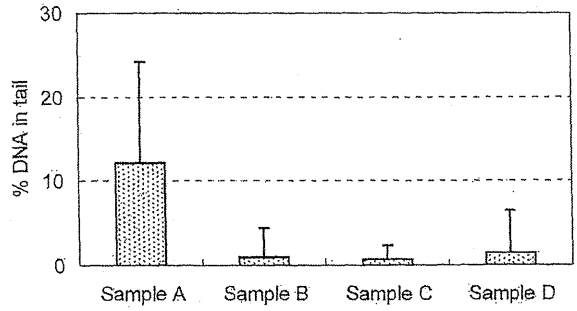

Fig. 2. %DNA in tail in Samples A, B, C and D. All samples were prepared from the liver of one non-treated rat, and analyzed with an image analysis system of Comet Assay IV. Each column shows the mean ± S.D. ($n = 100$ nuclei). The %DNA in tail of Sample A was higher than those of Samples B, C and D, indicating that the sample preparation procedure using the usual Potter-type shaft was associated with increased DNA migration.

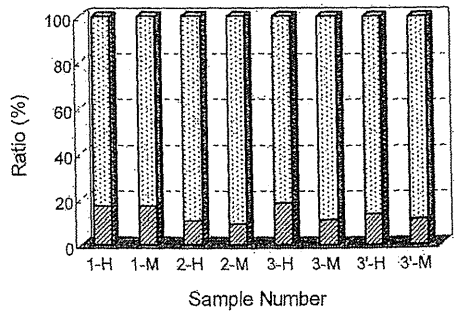

Fig. 3. Ratio of the cell nuclei (dot column) and whole cells (shadow column) in Samples C and D in three laboratories. The sample number shows the laboratory code: 1, 2 or 3, and H: homogenized sample obtained using a Downs-loose-type shaft, and M: samples obtained by the mesh membrane method. Laboratory 3 repeated the experiments, and the results are shown as 3′-H or 3′-M. The resultant ratio obtained was 80–90% cell nuclei: 10–20% whole cells for both samples in all laboratories.

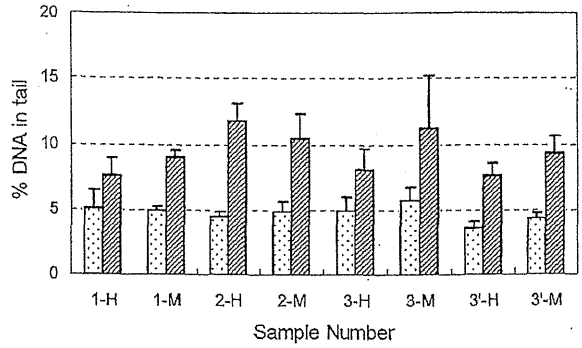

Fig. 4. %DNA in tail in Sample C prepared using a Downs-loose-type shaft and Sample D obtained using the mesh membrane method. Both samples were prepared from the liver of EMS-treated rats (shadow column) or saline-treated rats (dot column), and analyzed with an image analyzer of Rainbow Star System. Fifty nuclei/slide and two slides/animal were scored, and the mean of each animal was calculated. Each column shows the mean±S.D. ($n=3$ animals). The sample number shows the laboratory code: 1, 2 or 3, and H: homogenized sample prepared using a Downs-loose-type shaft, or M: samples prepared using the mesh membrane method. Laboratory 3 repeated the experiments, and the results are shown as 3'-H or 3'-M. The average values of the parameters increased similarly in both Samples C and D of the EMS group as compared with those in the negative control groups in all three laboratories.

trol groups in the three laboratories, although the magnitude of responses in the EMS groups differed slightly among laboratories (Fig. 4).

Discussion

Three homogenizing procedures to prepare cell nuclei were used in the present study. In general, to prepare cell nuclei, a Downs-loose-type homogenizer shaft (Shaft C in this report) is suitable and useful for preventing damage of cell nuclei and/or DNA. However, Sasaki *et al.* who reported a huge database about *in vivo* rodent Comet assay (5,6) selected and used a Potter-type shaft and a homogenizer machine. Since the usual Potter-type homogenizer shaft (Shaft A in this report) is generally used to fragment tissue samples, Sasaki *et al.* slimmed the homogenizer shaft for use, described in this report as Shaft B (Sasaki, personal communication). In this study, Shaft A clearly produced damage of the cell nuclei and DNA. However, Shaft B yielded samples with a lower degree of damage of the cell nuclei and DNA (Figs. 1a, 1b and 2). The condition of the sample obtained using Shaft B seemed to be comparable to that obtained using Shaft C (Figs. 1b, 1c and 2). Therefore, it was considered that Shaft B (loosely customized Potter-type) and shaft C (Downs-loose-type) might be equally acceptable for preparation of the cell nuclei for the *in vivo* rodent Comet assay.

For the preparation of whole cells, the mesh membrane method was used in this study, which is the simplest and most popular method to prepare samples for the *in vivo* rodent Comet assay and is thus used in the JaCVAM initiative international validation study of the *in vivo* rodent Comet assay (4). However, surprisingly, the sample yielded by the mesh membrane method (Sample D in this report) contained numerous cell nuclei, but fewer whole cells, and the ratio of the cell nuclei and whole cells was similar to that in Sample C prepared using the Downs-loose type homogenizer (Fig. 3). In each of the three laboratories, the % DNA in tail in both Samples C and D were comparable in the negative control groups and the EMS-treated groups (Fig. 4). These data indicate that both sample preparation methods mainly provide cell nuclei and have no significant effect on the Comet assay results. The magnitude of the responses in the EMS groups seemed to be slightly different among laboratories, and these variations may raise some issues for our future validation study. The main purpose of this study, however, was not to obtain comparably positive results with EMS in all laboratories, but to examine whether comparable samples were obtained using different sample preparation methods, and thus these issues will be discussed after this study.

We could not obtain whole-cell samples using the mesh-membrane method. Another option to obtain intact whole cells is the enzyme digestion (e.g., collagenase-perfusion) method. Actually, some investigators have used this technique and the data on OPP by Bomhard *et al.* (8) were provided using an *in situ* perfusion technique for the liver and kidney. Therefore, the enzyme digestion method also needs to be examined in order to understand whether or not the discrepant results for OPP are truly related to the sample condition. However, the enzyme digestion methods require additional and complicated steps of cell preparation. When considering the actual status that the mesh membrane method has been the most widely and popularly used, additional investigation of the enzyme digestion method would seem to be less significant.

In conclusion, the homogenization method using a loosely customized Potter-type shaft or a Downs-loose-type shaft, and the mesh membrane method provided similar tissue sample conditions, with the samples mainly consisting of cell nuclei under the conditions used in this collaborative study. Thus, we consider that all of these methods might be equally acceptable for *in vivo* rodent alkaline Comet assay.

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References

1 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J-C, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro*

and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000; 35: 206–21.

2 Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: Results of the *in vivo* Comet assay workgroup. Mutat Res. 2007; 627: 31–5.

3 Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis. 2003; 18: 45–51.

4 JaCVAM protocol for international validation study. Available at: http://www.cometassay.com/index.htm (accessed 7 February 2012).

5 Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The Comet assay with multiple mouse organs: comparison of Comet assay results and carcinogenicity with 208 chemicals selected from the IARC Monographs and U.S. NTP Carcinogenicity Database. Crit Rev Toxicol. 2000; 30: 629–799.

6 Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie E, Knasmüller S, Tsuda S, Sasaki YF. Comparative investigation of multiple organs of mice and rats in the Comet assay. Mutat Res. 2002; 517: 53–75.

7 Sasaki YF, Saga A, Akasaka M, Yoshida K, Nishidate E, Su YQ, Matsusaka N, Tsuda S. *In vivo* genotoxicity of ortho-phenylphenol, bisphenyl, and thiabendazole detected in multiple mouse organs by the alkaline single cell gel electrophoresis assay. Mutat Res. 1997; 395: 189–98.

8 Bomhard EM, Brendler-Schwaab SY, Freyberger A, Herbold BA, Leser KH, Richter M. O-phenylphenol and its sodium and potassium salts: a toxicological assessment. Crit Rev Toxicol. 2002; 32: 551–626.

9 Sasaki YF, Tsuda S, Izumiyama F, Nishidate E. Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (comet) assay. Mutat Res. 1997; 388: 34–44.

10 Miyamae Y, Yamamoto M, Sasaki YF, Kobayashi H, Igarashi-Soga M, Shimoi K, Hayashi M. Evaluation of a tissue homogenization technique that isolates nuclei for the *in vivo* single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories. Mutat Res. 1998; 418: 131–40.

11 Tice RR. The single cell gel/comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Phillips DH, Venitt S, editors. Environmental mutagenesis. Oxford: Bios Scientific Publishers; 1995. p. 315–39.

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Usefulness of combined *in vivo* skin comet assay and *in vivo* skin micronucleus test

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ABSTRACT

We have already found that the *in vivo* skin comet assay is useful for the evaluation of primary DNA damage induced by genotoxic chemicals in epidermal skin cells. The aim of the present study was to evaluate the sensitivity and specificity of the combined *in vivo* skin comet assay and *in vivo* skin micronucleus (MN) test using the same animal to explore the usefulness of the new test method.

The combined alkaline comet assay and MN test was carried out with three chemicals: 4-nitroquinoline-1-oxide (4NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and benzo[a]pyrene (B[a]P). In the first experiment, we compared DNA- and chromosome-damaging effects of 3 [72, 24 and 3 hours (h) before sacrifice] and 4 applications (72, 48, 24 and 3 h before sacrifice) of 4NQO, which induces dermal irritancy. The animals were euthanized and their skin was sampled for the combination test. As a result, the 4-application method was able to detect both DNA- and chromosome-damaging potential with a lower concentration; therefore, in the second experiment, MNNG and B[a]P were topically applied four times, respectively. The animals were euthanized, and then their skins were sampled for combination tests. In the alkaline comet assay, significant differences in the percent of DNA (%DNA) in the tail were observed in epidermal skin cells treated with MNNG and B[a]P. In the MN test, an increased frequency of MN cells (%MN) cells was observed by treatment with MNNG; however, there were no significant increases. In contrast, significant differences in %MN were observed by treatment with B[a]P.

From these results, we conclude that the combined *in vivo* skin comet assay and *in vivo* MN test was useful because it can detect different genotoxicity with the same sampling time and reduce the number of animals used.

1. Introduction

Recently, reduction, refinement and replacement (3Rs) have been the focus of regulatory testing using animal experiments [1–3]. Combined gentoxicity tests have been used to reduce the number of animals used [4,5]. There are various *in vivo* genotoxicity tests, such as the micronucleus (MN) test [6], alkaline comet assay [7], unscheduled DNA synthesis (UDS) test [8] and gene mutation assay that require transgenic animals [9]. In these methods, a combined alkaline comet assay and MN test has been more commonly used to simplify experimental methodology [10].

The alkaline comet assay is a technically simple and rapid test to detect alkali-labile sites and DNA strand breaks in various organs; these types of DNA damage can be initial damage induced by genotoxic chemicals [11]. In particular, the liver is often used as the target organ due to its high metabolic activity [12]. Also, the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) guidelines propose the use of a second *in vivo* genotoxicity test (alkaline comet assay) as a follow-up to *in vitro* positive results [13]. The MN test is also a technically simple and rapid test to detect chromosome damage, such as chromosome breaks [14,15].

We have been interested in the skin as a target organ, and have evaluated various chemicals using the alkaline comet assay [16]. Also, the skin can be highly exposed, both acutely and chronically, to various chemicals. For instance, there are highly concentrated risks of occupational exposure [17,18]. For this reason, it is important to evaluate skin tissue as a target organ; however, there are few reports of *in vivo* genotoxicity studies (alkaline comet assay and MN test) of the skin compared to other organs. There have been a few reports of combination tests of the alkaline comet assay and gene mutation assay using skin; however, a combination test of the alkaline comet assay and MN test has not reported. Therefore, it is important to explore the usefulness of this combination test

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 4NQO, 4-nitroquinoline-1-oxide; B[a]P, benzo[a]pyrene; MN, micronucleus.

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using skin. Additionally, this approach also reduces the number of animals required.

In the present study, we examined the combined alkaline comet assay and MN test in the epidermis of hairless mice treated with skin carcinogens, 4-nitroquinoline-1-oxide (4NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and benzo[a]pyrene (B[a]P). Furthermore, we evaluated DNA-damaging potential in the liver, which has high metabolic activity.

2. Materials and methods

2.1. Chemicals

MNNG was purchased from Kanto Chemical (Tokyo). 4NQO, B[a]P, acetone, olive oil, ethylendiaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), acetic acid, methanol and potassium chloride (KCl) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Low melting point (LMP) agarose was purchased from Lonza Rockland (Rockland, USA). Normal melting point (NMP) agarose, Trizma base and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO). Trypsin 250 was purchased from Becton, Dickinson and Company (NJ, USA).

2.2. Animal and treatments

Six-week-old male Hos:HR-1 hairless mice were purchased from Hoshino Laboratory Animals (Bando, Japan) and used at 7 weeks old. They were individually housed in TPX synthetic resin cages with bedding, PAPER CLEAN (Japan SLC), and were maintained on a 12-h light-dark cycle using a white fluorescent lamp at 21–25 °C and 40–75% relative humidity. Commercial pellets (CE-2; Clea Japan, Tokyo) and tap water were provided *ad libitum*, and animals were acclimated for at least one week before the experiment. Three mice were assigned to each experimental group.

All test chemicals were dissolved in acetone-olive oil (AOO, 4:1) just before use. AOO was used as the negative control in this study and was prepared just before use. A 0.2 mL amount of each dosing formulation was applied to an area of approximately 3 cm × 4 cm on the dorsal skin. The concentrations of each chemical were selected according to the reports of Toyoizumi et al. [16], Nishikawa et al. [19,20] and Mori et al. [8].

Mice were topically administered three times [72, 24 and 3 hours (h) before sacrifice (only 4NQO group)] or four times (72, 48, 24 and 3 h before sacrifice). Mice treated with 4NQO and MNNG were sacrificed by cervical dislocation 3 h after the last administration. Mice treated with B[a]P were sacrificed by exsanguination after intraperitoneal administration of pentobarbital sodium 3 h after the last administration. We used exsanguination as the sacrifice method in the B[a]P group to prevent contamination of the blood cells. The animal experiments in this study were conducted in accordance with the "Guidance for Animal Experiments in Hatano Research Institute Food and Drug Safety Center".

2.3. Evaluation of skin reaction using the Draize scoring system

Skin reactions of the applied area were evaluated visually according to the Draize scoring system 3 h after the 1st application, and just before and 3 h after the 2nd, 3rd and 4th applications [21].

2.4. Isolation of epidermal skin cells for the combination assay

Epidermal skin cells were isolated according to the method of the skin MN test [6], with some modifications. First, the dorsal tissue was removed from the skin and rinsed in cold, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(−)). The dorsal skin was placed on filter paper and excess PBS(−) was removed with a paper towel. The dorsal skin, along with the filter paper, was sliced into approximately 1–3 mm wide strips with a razor. The strips were immersed in cold 0.02 w/v% EDTA/PBS(−) solution for 10 minutes (min) and then in 0.25 w/v% trypsin/PBS(−) solution overnight at 4 °C. The strips were again rinsed in cold PBS(−). The epidermis was peeled from the dermis and transferred to cold 10 mL minimum essential medium supplemented with 10 v/v% bovine serum. The epidermal strips were gently stirred for approximately 1 h using a rotator at 4 °C to prepare a cell suspension, which was then passed through a 70 μm cell strainer. For the skin comet assay, the cell numbers were counted to prepare the cell suspension of about 1.5×10^6 cells/mL for 40 μL (see Section 2.6). For the skin micronucleus test, the remaining cell suspension was centrifuged at 310 × *g*, 5 min and the supernatant was removed (see Section 2.7).

2.5. Isolation of liver cells (hepatocytes) for the alkaline comet assay

A liver sample was also taken from mice treated with B[a]P. The tissue (approximately 5 mm × 5 mm × 5 mm) obtained from the left lateral liver lobe was washed in cold mincing buffer (20 mM Na_2EDTA, 10 v/v% DMSO in Hanks' Balanced Salt Solution, pH 7.5). It was then minced with a pair of fine scissors to release the cells. The minced cells were suspended in 1 mL cold mincing buffer by gentle pipetting. This cell suspension was passed through a 35 μm cell strainer to remove lumps and then placed on ice until needed for comet slide preparation [22].

2.6. In vivo skin and liver comet assay

The alkaline comet assay was performed according to the method of the "Fourth International Workgroup on Genotoxicity testing: results of the *in vivo* Comet assay workgroup" [23] and "International validation of the *in vivo* rodent alkaline comet assay for detection of genotoxic carcinogens (Draft version 14)" [22].

The slide glasses were coated with 1.0 (w/v%) NMP agarose solution the day before preparation of the specimens for the alkaline comet assay. A 40 μL sample was mixed with 360 μL of 0.5 (w/v%) LMP agarose solution. The 150 μL mixture was layered on 1.0 (w/v%) NMP agarose and covered with cover glass. This slide was placed on ice and refrigerated for more than 20 min. Each cover glass was removed from the slide glass, and the slide was immersed in 150 mL chilled lysing solution (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Trizma base, 1 (v/v%) of Triton-X 100 and 10 (v/v%) DMSO, pH 10) overnight at 4 °C. After lysis, the slide was washed with chilled water. The slide was then placed in alkaline electrophoresis buffer for 20 min. Electrophoresis was performed at 26 V (0.7 V/cm), 0.3 A for 20 min at 4 °C. After completion of electrophoresis, the slide was immersed in neutralization buffer for 20 min. The slide was dehydrated by immersion into absolute ethanol (99.6%) for 10 min. The cells were stained with 50 μL SYBR Gold (1:10,000 dilution of liquid concentrate). Slides were analyzed using a fluorescence microscope (magnification: 200×) equipped with a digital camera. For each sample (tissue), 50 comets per slide were analyzed, with two slides scored per sample. The percent of %DNA in the tail was measured according to the DNA damage degree using computer software (Comet Assay IV; Perceptive Instruments). Furthermore, comets with a distinct head and tail due to highly fragmented DNA were counted as "hedgehog" comets [15]. The frequency of hedgehogs was determined per sample based on the visual scoring of 50 cells per slide.

In comet analyses, the %DNA in the tail (average of each animal) in the 4NQO (0.05–0.2 w/v%), MNNG (0.02–0.1 w/v%) and B[a]P (0.2–1 w/v%) groups was compared with that in the negative control group (treated with AOO) by Dunnett's test (two-tailed) at 1 and 5% significance levels. Differences between the average %DNA in the tail value of 2 (w/v%) B[a]P-treated group and control group was compared using Student's *t*-test (two-tailed) when the variances were homogeneous based on the *F* test at 1 and 5% significance levels. In hedgehogs analyses, the %hedgehogs (average of each animal) in the 4NQO (0.05–0.2 w/v%), MNNG (0.02–0.1 w/v%) and B[a]P (0.2–1 w/v%) groups were compared with that in the negative control group by Dunnett's test (one-tailed) at 1 and 5% significance levels. Differences between the average %hedgehogs in the 2 (w/v%) B[a]P-treated group and control group were compared using Student's *t*-test (one-tailed) when the variances were homogeneous based on the *F* test at 1 and 5% significance levels.

2.7. In vivo skin micronucleus test

Isolated epidermal skin cells were incubated in 10 mL of 75 mM KCl for 15 min at 37 °C, and immediately were fixed with 1.25 mL of cold 1:3 acetic alcohol (Carnoy's fixative solution). Isolated epidermal skin cells were collected by centrifugation (310 × *g*, 5 min) and treated with 10 mL of 75 mM KCl hypotonic solution for 15 min at 37 °C. Next, 1.25 mL of cold Carnoy's fixative solution was added to the cell suspension, and the mixture was agitated. The cell suspension was centrifuged at 310 × *g* for 5 min and the supernatant was removed. Furthermore, 10 mL of cold Carnoy's fixative solution was added to the cell suspension, which was centrifuged at 310 × *g* for 5 min and the supernatant was removed. Again, the cell suspension was fixed using 10 mL of cold Carnoy's fixative solution, which was centrifuged at 310 × *g* for 5 min and the supernatant was removed. The suspension was added to 10 mL of cold 1:99 acetic acid and methanol (fixative solution) was added to the cell suspension. The supernatant was removed and the epidermal skin cells were re-fixed with the fixative solution. Two drops of the cell suspension were placed on a slide glass, which was dried at room temperature and stained with 40 μg/mL acridine orange solution. Slides were observed using a fluorescence microscope (magnification 400×). For each sample (tissue), 1000 epidermal skin cells per slide were observed [6], with 2 slides scored per sample. The frequency of micronucleate epidermal skin cells in the negative control and treated groups was statistically analyzed by the Kastenbaum and Bowman's table [24] at 5% and 1% significance levels.

3. Results

3.1. Skin reaction and genotoxic effects in epidermal skin cells by 3 and 4 4NQO treatments

Table 1 shows the skin reaction in the treated groups. Three treatments with 4NQO induced slight edema of dorsal skin at 0.1 (w/v%) and severe reddening and edema of dorsal skin at 0.2 (w/v%) 3 h after the final application. Four treatments with 4NQO induced slight reddening and edema at 0.05 (w/v%), moderate reddening

Table 1
Skin reaction in male HR-1 (hairless) mice by 3 or 4 applications of 4NQO in the combined skin comet assay and skin micronucleus test.

Compound	No. of treatment	Concentration (w/v%)	Animals No.	General condition						
				1st application	2nd application		3rd application		4th application	
				3 h	Pre	3 h	Pre	3 h	Pre	3 h
4NQO	3	0	1	–	–	–	–	–		
			2	–	–	–	–	–		
			3	–	–	–	–	–		
		0.05	1	–	–	–	–	–		
			2	–	–	–	–	–		
			3	–	–	–	–	–		
		0.1	1	–	–	a	–	–		
			2	–	–	a,b	b	b		
			3	–	–	a,b	b	b		
		0.2	1	a^{+}	a^{++},b	a^{++},b^{+}	a^{++},b^{+}	a^{++},b^{+}		
			2	a	–	a,b	b^{+}	a^{++},b^{++}		
			3	a	a,b	a,b^{+}	a,b^{+}	a^{++},b^{++}		
	4	0	1	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–
			3	–	–	–	–	–	–	–
		0.05	1	–	–	–	–	–	–	–
			2	–	–	–	–	–	–	–
			3	–	–	b	–	b	–	a,b
		0.1	1	a	–	–	–	a+,b	a,b	a^{++},b^{+}
			2	a,b	–	a,b	b	a,b	a,b	a,b
			3	a	–	–	–	a,b	a,b	a,b^{+}
		0.2	1	a,b	b	a,b^{+}	b	a^{++},b^{++},c	a^{++},b^{++},c	a^{++},b^{++},c
			2	a,b	b	a,b^{+}	b	a^{++},b^{++},c	a^{++},b^{++},c	a^{++},b^{++},c
			3	a,b	b	a,b	b	a^{++},b^{++}	a^{++},b^{++}	a^{++},b^{++}

4-Nitroquinoline 1-oxide (4NQO).
–, No abnormality.
a, Reddening of dorsal skin (slight: Draize score 1–2 points).
a^{+}, Edema of dorsal skin (moderate: Draize score 3 points).
a^{++}, Reddening of dorsal skin (severe: Draize score 4 points).
b, Edema of dorsal skin (slight: Draize score 1–2 points).
b^{+}, Edema of dorsal skin (moderate: Draize score 3 points).
b^{++}, Edema of dorsal skin (severe: Draize score 4 points).
c, Eschar.

and edema at 0.1 (w/v%) and severe reddening and edema at 0.2 (w/v%). Furthermore, eschar was observed 3 h after the 3rd application in the 0.2 (w/v%) 4NQO-treated group.

Table 2 shows %DNA in the tail values, %hedgehog and frequency of MN (%MN) by 3 and 4 treatments with 4NQO. Fig. 1 shows a histogram of the alkaline comet assay, which was obtained from epidermal skin cells pooled from 3 animals. DNA-damaging potential and cytotoxicity induced by 3 treatments with 0.2 (w/v%) 4NQO were detected through significant increases of %DNA in the tail and %hedgehog, while chromosomal damage induced with 0.1 and 0.2 (w/v%) 4NQO was detected through significant increases of %MN. DNA-damaging potential induced by 4 treatments with 0.1 and 0.2 (w/v%) was detected through a significant increase of %DNA in the tail. A significant increase of %hedgehog was induced by 4 treatments with 0.2 (w/v%) 4NQO, while chromosomal damage induced by 0.05 and 0.1 (w/v%) was detected through a significant increase of %MN.

3.2. Skin reaction and genotoxic effects in epidermal skin cells by MNNG application

Table 3 shows the skin reaction in the treated groups. Slight edema of dorsal skin was observed by 0.1 (w/v%) MNNG application.

Table 4 shows the %DNA in the tail values, %hedgehog and %MN by 4 treatments of MNNG. Fig. 2 shows a histogram of the alkaline comet assay, which was obtained from epidermal skin cells pooled from 3 animals. DNA-damaging potential induced by 0.05 and 0.1 (w/v%) MNNG application was detected through a significant increase of %DNA in the tail; however, there were no significant increases of %hedgehog in any dosing group, while there were no significant increases of %MN in any dosing group.

3.3. Skin reaction and genotoxic effects in epidermal skin cells by B[a]P application

Tables 5 and 6 show the skin reaction with 4 treatments with B[a]P. Slight reddening and edema of dorsal skin were observed with 1 and 2 (w/v%) B[a]P applications.

Tables 7 and 8 show the %DNA in the tail values, %hedgehog and %MN by 4 treatments with B[a]P. Fig. 3 shows a histogram of treated groups, which was obtained from epidermal cells pooled from 3 animals. DNA-damaging potential induced by 2 (w/v%) B[a]P application was detected through a significant increase of %DNA in the tail; however, there were no significant increases of %hedgehog in any dosing group. On the other hand, B[a]P showed significant increases of %MN by applications of 0.5, 1 and 2 (w/v%).

3.4. DNA-damaging potential in liver cells by B[a]P application

Tables 9 and 10 show the %DNA in the tail values and %hedgehog in the B[a]P group. There were no significant increases of %DNA in the tail and %hedgehog in any dosing group.

4. Discussion

Animals' welfare is recently concerned in regulatory testing using animal experiments and such as, animal number reduction and animal testing alternatives are required [2,4]. Combined alkaline comet assay and MN test is often used because simply method, and use of its combination test also leads to aim to reduce animal

Table 2
DNA-damaging potential, hedgehog frequency and chromosome-damaging potential of epidermal cells by 4NQO application.

No. of treatment	Group	No. of animals	Skin comet assay				Skin micronucleus test	
			% DNA in tail		Frequency of hedgehogs		Observed cells	No. of MN cells
			Observed cells	Average (S.D.) of 100 cells	Observed cells	% of hedgehogs		
3	Negative control AOO	1	100	7.0 (14.6)	100	5	2000	3
		2	100	6.7 (15.9)	100	6	2000	1
		3	100	13.0 (19.2)	100	8	2000	1
		Total	300	–	300	19	6000	5
		Average ± S.D.	–	8.9 ± 3.5	–	6.3 ± 1.5	–	0.08 ± 0.06
	4NQO	1	100	11.7 (19.8)	100	14	2000	1
	0.05 (w/v%)	2	100	7.6 (13.2)	100	10	2000	2
		3	100	8.8 (16.5)	100	5	2000	2
		Total	300	–	300	29	6000	5
		Average ± S.D.	–	9.4 ± 2.1	–	9.7 ± 4.5	–	0.08 ± 0.03
	4NQO	1	100	18.3 (21.6)	100	12	2000	2
	0.1 (w/v%)	2	100	18.7 (18.9)	100	11	2000	7
		3	100	12.2 (16.9)	100	17	2000	4
		Total	–	–	–	40	6000	13†
		Average ± S.D.	300	16.4 ± 3.7	300	13.3 ± 3.2	–	0.22 ± 0.13
	4NQO	1	100	45.1 (22.6)	100	25	2000	28
	0.2 (w/v%)	2	100	36.9 (26.4)	100	31	2000	10
		3	100	30.9 (26.3)	100	20	2000	13
		Total	–	–	–	76	–	51††
		Average ± S.D.	300	37.6 ± 7.1**	300	25.3 ± 5.5##	6000	0.85 ± 0.48
4	Negative control AOO	1	100	14.9 (18.4)	100	10	2000	1
		2	100	16.8 (19.8)	100	27	2000	0
		3	100	16.3 (18.2)	100	16	2000	2
		Total	–	–	–	53	–	3
		Average ± S.D.	300	16.0 ± 0.9	300	17.7 ± 8.6	6000	0.05 ± 0.05
	4NQO	1	100	25.0 (18.1)	100	16	2000	2
	0.05 (w/v%)	2	100	20.3 (17.5)	100	15	2000	9
		3	100	23.6 (21.6)	100	15	2000	5
		Total	–	–	–	46	6000	16††
		Average ± S.D.	300	22.9 ± 2.3	300	15.3 ± 0.6	–	0.27 ± 0.18
	4NQO	1	100	38.3 (21.4)	100	25	2000	21
	0.1 (w/v%)	2	100	36.1 (21.9)	100	16	2000	18
		3	100	35.9 (24.3)	100	20	2000	16
		Total	–	–	–	61	6000	55††
		Average ± S.D.	300	36.8 ± 1.5**	300	20.3 ± 4.5	–	0.92 ± 0.13
	4NQO	1	100	21.0 (23.1)	100	65		
	0.2 (w/v%)	2	100	34.8 (26.3)	76	82		Toxicity
		3	100	38.4 (23.8)	80	63		
		Total	–	–	–	210		
		Average ± S.D.	300	31.4 ± 1.7*	256	70.0 ± 10.4##		

AOO, acetone:olive oil (4:1), 4-nitroquinoline 1-oxide (4NQO).
* Significantly higher than the negative control at 5% level.
** Significantly higher than the negative control at 1% level (Dunnett' s test, two-tailed).
Significantly higher than the negative control at 1% level (Dunnett's test, one-tailed).
† Significantly different from the negative control at 5% level.
†† Significantly different from the negative control at 1% level (Kastenbaum and Bowman' table).

numbers. However, there are no reports of combination test (alkaline comet assay and MN test) using skin. We aimed to explore that the usefulness of combination test using skin.

The DNA-damaging potential of many genotoxic chemicals toward various organs (stomach, liver, lung et al.) was evaluated using the alkaline comet assay [11,23]. Some genotoxic chemicals showed DNA-damaging potential with the alkaline comet assay 3 h after treatment, while other genotoxic chemicals showed DNA-damaging potential 24 h after treatment [25]. The chromosome-damaging potential of many genotoxic chemicals toward bone marrow [26], liver [27], skin [6,19,20] and testes [28] was evaluated using the MN test. According to MN test results using skin [6,19,20], MMC and methylmethane sulfonate showed the highest %MN 72 h after a single application; furthermore, these results showed that the skin MN test could detect the chromosome-damaging potential of skin carcinogens by 3 applications (72, 48 and 24 h before sacrifice); therefore, we set two application schedules for the combination test, which were determined by reference to Nishikawa's report [6,19,20]: application 72, 24 and 3 h before sacrifice; and application 72, 48, 24 and 3 h before sacrifice [19,20]. As a result, 4 applications could detect DNA- and chromosome-damaging potential at a lower concentration; therefore, in the second experiment, we evaluated two chemicals using 4 applications. However, skin irritation by the Draize score was stronger with 4 than 3 applications (Table 1, Fig. 1); therefore, with strong irritant chemicals, 3 applications may be appropriate to avoid cytotoxic effects on the skin.

We selected a direct mutagen, MNNG [29,30], and an indirect mutagen, B[a]P [31–33], which are both skin carcinogens [34,33]. The average %DNA in the tail in the MNNG groups was significantly higher than in the negative control group and increased dose dependently (Table 4, Fig. 2). On the other hand, significant MN induction was not observed (Table 4). Similar positive comet results were observed in our previous study [16]. According to Nishikawa's report, significant MN induction was observed in the skin cells of ICR male mice [19], whereas it was not observed in our

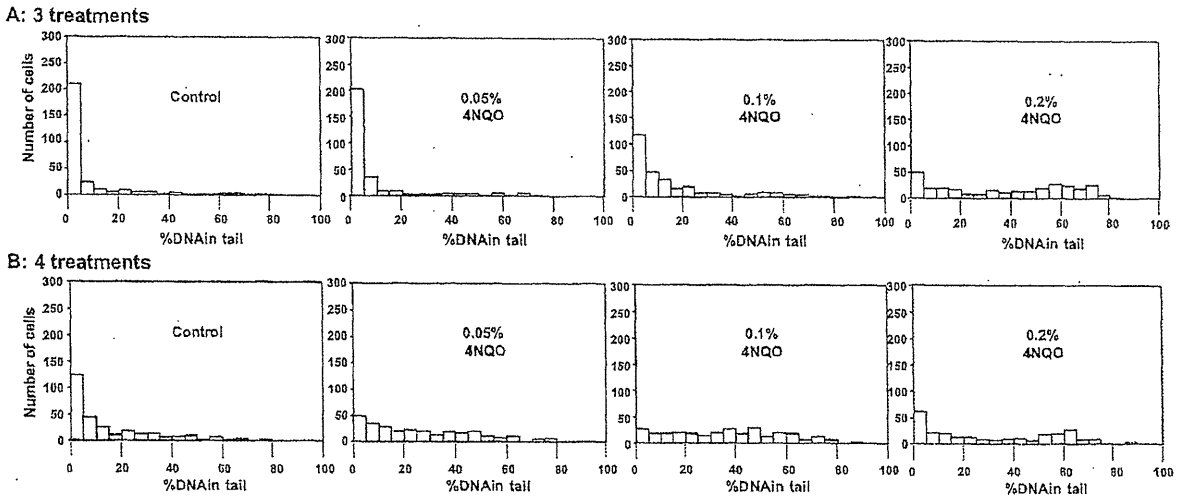

Fig. 1. Distribution of %DNA in the tail in the skin comet assay of epidermal cells pooled from 3 animals. A and B show the results of 3 and 4 applications using 4NQO, respectively.

results using Hos:HR-1 mice. The differences between Nishikawa's report [19] and our results might be related to strain specificity and mechanical damage by shaving the hair of ICR mice. In the future, we need to evaluate genotoxicity in ICR mice using the combination test. B[a]P is a carcinogen [33] whose carcinogenic actions are thought to be initiated by P450 [35]. The average %DNA in the tail in the 2 (w/v%) B[a]P group was significantly higher than in the negative control group (Table 8, Fig. 3). On the other hand, %MN were also significantly higher than in the control group and increased dose dependently (Tables 7 and 8). Similar positive results were observed in the skin comet assay [16] and skin MN test to Nishikawa's reports [19,20].

In the present study, we found that the alkaline comet assay and MN test were nearly sensitive to 4NQO (the MN test was more sensitive than the alkaline comet assay). In regard to MNNG, the alkaline comet assay was more sensitive, but B[a]P was not (Table 11). These results suggested that each assay had strong and weak points. A stable DNA adduct such as metabolized B[a]P would be difficult to detect using the alkaline comet assay [35,36]; however, this weak point of the alkaline comet assay could be supported by the MN test. Also, some studies showed that the alkaline comet assay detected carcinogens that were negative or equivocal in the MN test [37,38]; therefore, alkaline comet assay is important to compensate for the lack of sensitivity of the MN test.

Some reports have indicated that indirect mechanisms related to cytotoxicity did not affect comet results [5,39,40]. Despite observations of inflammation and necrosis in the liver, the alkaline comet assay could not detect these secondary DNA fragmentations [41]; however, the relationship between the skin comet assay and cytotoxic effects which can lead to a false-positive comet result is unknown. Therefore, we have to carefully consider cytotoxicity when the *in vivo* skin comet assay is added to repeated dose toxicity studies. In addition, we need to evaluate the effects of irritating and caustic materials which have no genotoxicity.

Metabolic activity in the liver is higher than in other organs. In the present study, DNA-damaging potential was not detected in the liver using this application route (Tables 9 and 10). We confirmed that it is important to evaluate DNA-damaging potential according to the administration route. Generally, the genotoxic response is affected by mouse phylesis and the administration route [36,42].

Table 3
Skin reaction in male HR-1 (hairless) mice by application of MNNG in the combined skin comet assay and skin micronucleus test.

Compound	Concentration (w/v%)	Animals No.	General condition						
			1st application	2nd application		3rd application		4th application	
			3 h	Pre	3 h	Pre	3 h	Pre	3 h
MNNG	0	1	–	–	–	–	–	–	–
		2	–	–	–	–	–	–	
		3	–	–	–	–	–	–	–
	0.01	1	–	–		–	–	–	–
		2	–	–	–	–	–	–	–
		3	–	–	–	–	–	–	–
	0.02	1	–	–	–	–	–	–	–
		2	–	–	–	–	–	–	–
		3	–	–	–	–	–	–	–
	0.05	1	–	–	–	–	–	–	–
		2	–	–	–	–	–	–	–
		3	–	–	–	–	–	–	–
	0.1	1	–	–	–	–	b	b	b
		2	–	–	–	–	b	b	b
		3	–	–	–	–	b	–	b

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).
–, No abnormality.
b, Edema of dorsal skin (slight: Draize score 1–2 points).

Table 4
DNA-damaging potential, hedgehog frequency and chromosome-damaging potential of epidermal cells by MNNG application.

Group	No. of animals	Skin comet assay				Skin micronucleus test	
		% DNA in tail		Frequency of hedgehogs		Observed cells	No. of MN cells
		Observed cells	Average (S.D.) of 100 cells	Observed cells	% of hedgehogs		
Negative control AOO	1	100	12.0 (21.0)	100	10	2000	1
	2	100	16.8 (24.0)	100	11	2000	0
	3	100	9.3 (15.1)	100	9	2000	3
	Total	300	–	300	30	6000	4
	Average ± S.D.	–	12.7 ± 3.8	–	10.0 ± 1.0	–	0.07 ± 0.08
MNNG 0.01%	1	100	12.2 (17.1)	100	28	2000	0
	2	100	12.1 (18.6)	100	10	2000	0
	3	100	9.3 (15.6)	100	11	2000	0
	Total	300	–	300	49	6000	0
	Average ± S.D.	–	11.2 ± 1.7	–	16.3 ± 10.1	–	0.00 ± 0.00
MNNG 0.02%	1	100	21.8 (15.5)	100	10	2000	3
	2	100	17.5 (19.1)	100	16	2000	2
	3	100	8.8 (13.4)	100	4	2000	2
	Total	–	–	300	30	6000	7
	Average ± S.D.	300	16.0 ± 6.6	–	10.0 ± 6.0	–	0.12 ± 0.03
MNNG 0.05%	1	100	25.1 (18.7)	100	10	2000	6
	2	100	20.5 (16.8)	100	12	2000	1
	3	100	28.2 (15.8)	100	9	2000	0
	Total	300	–	300	31	6000	7
	Average ± S.D.	–	24.6 ± 3.9		10.3 ± 1.5	–	0.12 ± 0.16
MNNG 0.1%	1	100	39.4 (16.3)	100	14	2000	4
	2	100	48.0 (15.0)	100	17	2000	3
	3	100	45.7 (13.7)	100	7	2000	4
	Total	300	–	300	38	6000	11
	Average ± S.D.	–	44.3 ± 4.5	**	12.7 ± 5.1	–	0.18 ± 0.03

AOO, acetone:olive oil (4:1).
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG).
* Significantly higher than the negative control at 5% level (Dunnett's test).
** Significantly higher than the negative control at 1% level (Dunnett's test).

In particular, the administration route is an important factor that is well-known to affect the results of *in vivo* genotoxicity tests [42]. In genotoxicity evaluation of chemical products, genotoxic potential has been evaluated *via* oral and intraperitoneal administration routes for this purpose. The onset of toxicity by accidental ingestion and oral ingestion can be assumed; however, if the actual high concentration exposure is assumed, it is thought that the skin has the highest chemical exposure risk. This means that it is important to evaluate the genotoxic effects on skin using a single genotoxicity test or combination test using skin, and these genotoxic dates can offer very productive information for considering the safety of work exposure. Hence, we must evaluate other chemicals using this combination method to confirm its usefulness.

Lowest effective concentrations of each genotoxicity test using skin are summarized in Table 11. From these results, we found that each assay had strong and weak points because of differences in detection principle of each test; however, these could be compensated for in combination. Also, this combination test can detect a skin specific response based on chemicals applied to mouse dorsal skin. In addition, it can detect genotoxicity with the same sampling

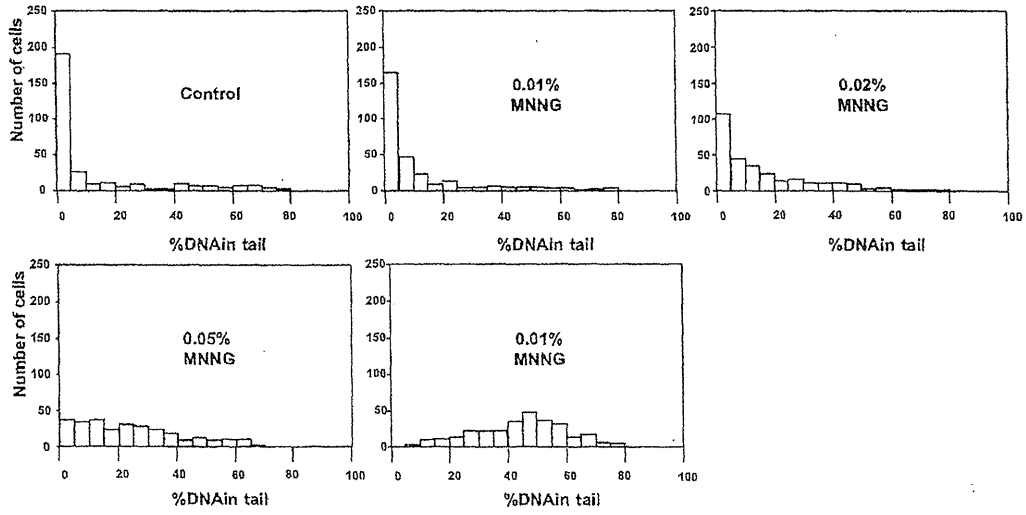

Fig. 2. Distribution of % DNA in the tail in the skin comet assay of epidermal cells pooled from 3 animals. These data show the results of the MNNG-treated group.

Table 5
Skin reaction in male HR-1 (hairless) mice by B[a]P application in the combined skin comet assay and skin micronucleus test.

Compound	Concentrate (w/v%)	Animals No.	General condition						
			1st application	2nd application		3rd application		4th application	
			3 h	Pre	3 h	Pre	3 h	Pre	3 h
B[a]P	0	1	–	–	–	–	–	–	–
		2	–	–	–	–	–		
		3		–	–	–	–	–	–
	0.2	1	–	–	–	–	–	–	–
		2	–	–	–	–	–	–	–
		3		–	–	–	–	–	–
	0.5	1	–	–					
		2	–	–	–	–	–	–	–
		3	–	–	–	–	–	–	–
	1	1	–	–	b	b	b	–	b
		2	–	–	–	–	b	b	–
		3	–	–	–	b	b	b	b

Benzo[a]pyrene (B[a]P).
–, No abnormality.
b, Edema of dorsal skin (slight: Draize score 1–2 points).

Table 6
DNA-damaging potential, hedgehog frequency and chromosome-damaging potential of epidermal cells by B[a]P application.

Group	No. of animals	Skin comet assay				Skin micronucleus test	
		% DNA in tail		Frequency of hedgehogs		Observed cells	No. of MN cells
		Observed cells	Average (S.D.) of 100 cells	Observed cells	% of hedgehogs		
Negative control AOO	1	100	12.0 (21.0)	100	10	2000	1
	2	100	16.8 (24.0)	100	11	2000	0
	3	100	9.3 (15.1)	100	9	2000	3
	Total	300	–	300	30	6000	4
	Average ± S.D.	–	12.7 ± 3.8	–	10.0 ± 1.0	–	0.07 ± 0.08
B[a]P 0.2 (w/v%)	1	100	10.8 (18.0)	100	7	2000	2
	2	100	12.1 (19.4)	100	10	2000	3
	3	100	13.6 (18.4)	100	9	2000	4
	Total	300	–	300	26	6000	9
	Average ± S.D.	–	12.2 ± 1.4	–	8.7 ± 1.5	–	0.15 ± 0.05
B[a]P 0.5 (w/v%)	1	100	8.4 (15.1)	100	11	2000	11
	2	100	18.3 (23.4)	100	6	2000	5
	3	100	9.5 (12.9)	100	11	2000	5
	Total	300	–	300	28	6000	21[††]
	Average ± S.D.	–	12.1 ± 5.4	–	9.3 ± 2.9	–	0.35 ± 0.17
B[a]P 1 (w/v%)	1	100	16.4 (23.2)	100	13	2000	5
	2	100	16.4 (18.6)	100	9	2000	16
	3	100	9.4 (17.1)	100	13	2000	13
	Total	300	–	300	35	6000	34[††]
	Average ± S.D.	–	14.1 ± 4.1	–	11.7 ± 2.3	–	0.57 ± 0.28

AOO, acetone:olive oil (4:1).
Benzo[a]pyrene (B[a]P).
†† Significantly different from the negative control at 1% level (Kastenbaum and Bowman's table).

Table 7
Skin reaction in male HR-1 (hairless) mice by 2 (w/v%) B[a]P application in the combined skin comet assay and skin micronucleus test.

Compound	Concentration (w/v%)	Animals No.	General condition						
			1st application	2nd application		3rd application		4th application	
			3 h	Pre	3 h	Pre	3 h	Pre	3 h
B[a]P	0	1	–	–	–	–	–	–	–
		2	–	–	–	–	–	–	–
		3	–	–	–	–	–	–	–
	2	1	–	–	b	a,b	a,b	b	a,b
		2	–	–	–	–	a,b	b	a,b
		3	–	–	b	a,b	a,b	b	a,b

Benzo[a]pyrene (B[a]P).
–, No abnormality.
a, Reddening of dorsal skin (slight: Draize score 1–2 points).
b, Edema of dorsal skin (slight: Draize score 1–2 points).

Table 8
DNA-damaging potential, hedgehog frequency and chromosome-damaging potential of epidermal cells by 2 (w/v%) B[a]P application.

Group	No. of animals	Skin comet assay				Skin micronucleus test	
		% DNA in tail		Frequency of hedgehogs		Observed cells	No. of MN cells
		Observed cells	Average (S.D. of 100 cells	Observed cells	% of hedgehogs		
Negative control AOO	1	100	9.9 (14.6)	100	16	2000	1
	2	100	11.8 (19.3)	100	11	2000	0
	3	100	9.9 (14.6)	100	4	2000	2
	Total	300	–	300	31	6000	3
	Average ± S.D.	–	10.2 ± 1.4	–	10.3 ± 6.0	–	0.05 ± 0.05
B[a]P 2 (w/v%)	1	100	19.2 (22.8)	100	10	2000	16
	2	100	20.7 (24.9)	100	22	2000	8
	3	100	19.3 (21.5)	100	16	2000	11
	Total	300	–	300	48	6000	35[¤]
	Average ± S.D.	–	19.7 ± 0.8**	–	16.0 ± 6.0	–	0.58 ± 0.20

AOO, acetone:olive oil (4:1).
Benzo[a]pyrene (B[a]P).
** Significantly higher than the negative control at 1% level (Student's *t*-test, two-tailed).
¤ Significantly different from the negative control at 1% level (Kastenbaum and Bowman's table).

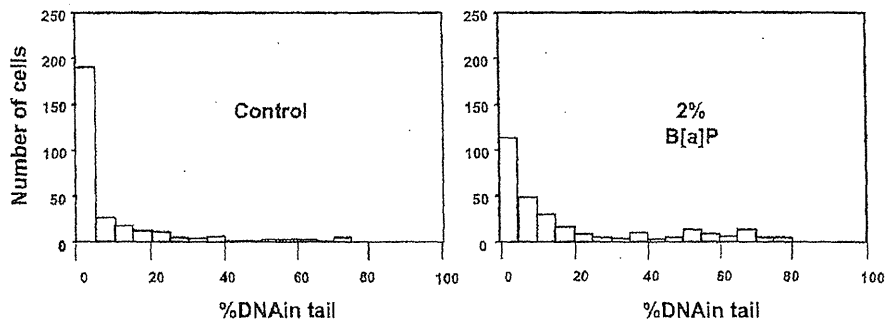

Fig. 3. Distribution of % DNA in the tail in the skin comet assay of epidermal cells pooled from 3 animals. These data show the results of the B[a]P-treated group.

Table 9
DNA-damaging potential and hedgehog frequency of liver cells by B[a]P application.

Group	No. of animals	Liver comet assay			
		% DNA in tail		Frequency of hedgehogs	
		Observed cells	Average (S.D.) of 100 cells	Observed cells	% of hedgehogs
Negative control AOO	1	100	7.4 (11.3)	100	4
	2	100	6.7 (11.4)	100	10
	3	100	3.4 (4.8)	100	4
	Total	300	–	300	18
	Average ± S.D.	–	5.8 ± 2.1	–	6.0 ± 3.5
B[a]P 0.2 (w/v%)	1	100	3.6 (7.5)	100	5
	2	100	6.2 (10.3)	100	5
	3	100	5.5 (10.4)	100	11
	Total	300	–	300	21
	Average ± S.D.	–	5.1 ± 1.3	–	7.0 ± 3.5
B[a]P 0.5 (w/v%)	1	100	7.2 (10.5)	100	9
	2	100	5.6 (8.7)	100	7
	3	100	3.4 (7.1)	100	3
	Total	300	–	300	19
	Average ± S.D.	–	5.4 ± 1.9	–	6.3 ± 3.1
B[a]P 1 (w/v%)	1	100	5.0 (9.0)	100	8
	2	100	4.8 (7.1)	100	9
	3	100	5.9 (6.5)	100	5
	Total	300	–	300	22
	Average ± S.D.	–	5.2 ± 0.6	–	7.3 ± 2.1

AOO, acetone:olive oil (4:1).
Benzo[a]pyrene (B[a]P).

Table 10
DNA-damaging potential and hedgehog frequency of liver cells by 2 (w/v%) B[a]P application.

Group	No. of animals	Liver comet assay			
		%DNA in tail		Frequency of hedgehogs	
		Observed cells	Average (S.D.) of 100 cells	Observed cells	% of hedgehogs
Negative control AOO	1	100	10.2 (10.8)	100	10
	2	100	9.5 (11.3)	100	8
	3	100	4.9 (9.4)	100	4
	Total	300	–	300	22
	Average ± S.D.	–	8.2 ± 2.9	–	7.3 ± 3.1
B[a]P 2 (w/v%)	1	100	7.2 (10.7)	100	14
	2	100	7.3 (9.1)	100	2
	3	100	8.7 (10.5)	100	2
	Total	300	–	–	18
	Average ± S.D.	–	7.7 ± 0.8	300	6.0 ± 6.9

AOO, acetone:olive oil (4:1).
Benzo[a]pyrene (B[a]P).

Table 11
Lowest effective concentration (w/v) of each genotoxicity test.

Compound	No. of treatment	Skin comet assay	Skin MN test
4NQO	3	0.2%	0.1%
	4	0.1%	0.05%
MNNG	4	0.05%	>0.1%
B[a]P	4	2%	0.5%

time and reduce the number of animals required. Therefore, we conclude that the combination of the *in vivo* skin comet assay and *in vivo* MN test is a promising method and more chemicals need to be evaluated.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

[1] A.M. Holmes, S. Creton, K. Chapman, Working in partnership to advance the 3Rs in toxicity testing, Toxicology 267 (2010) 14–19.
[2] E.L. Dahl, R. Curren, B.C. Barnett, Z. Khambatta, K. Reisinger, G. Ouedraogo, B. Faquet, A.C. Ginestet, G. Mun, N.J. Hewitt, G. Carr, S. Pfuhler, M.J. Aardema, The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: detailed protocol and harmonized scoring atlas, Mutat. Res. 720 (2011) 42–52.
[3] S. Pfuhler, M. Fellows, J. van Benthem, R. Corvi, R. Curren, K. Dearfield, P. Fowler, R. Frötschl, A. Elhajouji, L. Le Hégarat, T. Kasamatsu, H. Kojima, G. Ouédraogo, A. Scott, G. Speit, In vitro genotoxicity test approaches with better predictivity: summary of an IWGT workshop, Mutat. Res. 723 (2011) 101–107.
[4] S. Pfuhler, D. Kirkland, P. Kasper, M. Hayashi, P. Vanparys, P. Carmichael, S. Dertinger, D. Eastmond, A. Elhajouji, C. Krul, A. Rothfuss, G. Schoening, A. Smith, G. Speit, C. Thomas, J. van Benthem, R. Corvi, Reduction of use of animals in regulatory genotoxicity testing: identification and implementation opportunities—report from an ECVAM workshop, Mutat. Res. 680 (2009) 31–42.
[5] A. Rothfuss, M. Honma, A. Czich, M.J. Aardema, B. Burlinson, S. Galloway, S. Hamada, D. Kirkland, R.H. Heflich, J. Howe, M. Nakajima, M. O'Donovan, U. Plappert-Helbig, C. Priestley, L. Recio, M. Schuler, Y. Uno, H.J. Martus, Improvement of in vivo genotoxicity assessment: combination of acute tests and integration into standard toxicity testing, Mutat. Res. 723 (2011) 108–120.
[6] T. Nishikawa, M. Haresaku, K. Adachi, M. Masuda, M. Hayashi, Study of a rat skin in vivo micronucleus test: data generated by mitomycin C and methyl methanesulfonate, Mutat. Res. 444 (1999) 159–166.
[7] U. Wirnitzer, N. Gross-Tholl, B. Herbold, E. von Keutz, Photo-chemically induced DNA effects in the comet assay with epidermal cells of SKH-1 mice after a single oral administration of different fluoroquinolones and 8-methoxypsoralen in combination with exposure to UVA, Mutat. Res. 609 (2006) 1–10.
[8] M. Mori, H. Kobayashi, C. Sugiyama, Y. Katsumura, C. Furihata, Induction of unscheduled DNA synthesis in hairless mouse epidermis by skin carcinogens, J. Toxicol. Sci. 24 (1999) 217–226.
[9] N. Thein, P. Møller, H. Arotoft, U. Vogel, B. Korsholm, H. Autrup, H. Wallin, A strong genotoxic effect in mouse skin of a single painting of coal tar in hairless mice and in MutaMouse, Mutat. Res. 468 (2000) 117–124.
[10] L. Recio, C. Hobbs, W. Caspary, K.L. Witt, Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol, J. Toxicol. Sci. 35 (2010) 149–162.
[11] Y.F. Sasaki, K. Sekihashi, F. Izumiyama, E. Nishidate, A. Saga, K. Ishida, S. Tsuda, The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database, Crit. Rev. Toxicol. 30 (2000) 629–799 (Review).
[12] M. Struwe, A. Zeller, T. Singer, E. Gocke, Possibility of methodical bias in analysis of comet assay studies: Re: DNA damage detected by the alkaline comet assay in the liver of mice after oral administration of tetrachloroethylene (Mutagenesis, 25, 133–138, 2010), Mutagenesis 26 (2011) 473–474.
[13] ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S2(R1): guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, 2008.
[14] M. Hayashi, T. Sofuni, M. Ishidate Jr., An application of Acridine Orange fluorescent staining to the micronucleus test, Mutat. Res. 120 (1983) 241–247.
[15] M. Yasui, N. Koyama, T. Koizumi, K. Senda-Murata, Y. Takashima, M. Hayashi, K. Sugimoto, M. Honma, Live cell imaging of micronucleus formation and development, Mutat. Res. 692 (2010) 12–18.
[16] T. Toyoizumi, R. Ohta, Y. Nakagawa, Y. Tazura, M. Kuwagata, S. Noguchi, K. Yamakage, Use of the in vivo skin comet assay to evaluate the DNA-damaging potential of chemicals applied to the skin, Mutat. Res. 726 (2011) 175–180.
[17] M.D. McClean, R.D. Rinehart, L. Ngo, E.A. Eisen, K.T. Kelsey, J.K. Wiencke, R.F. Herrick, Urinary 1-hydroxypyrene and polycyclic aromatic hydrocarbon exposure among asphalt paving workers, Ann. Occup. Hyg. 48 (2004) 565–578.
[18] P.J. Tsai, H.Y. Shieh, W.J. Lee, S.O. Lai, Health-risk assessment for workers exposed to polycyclic aromatic hydrocarbons (PAHs) in a carbon black manufacturing industry, Sci. Total Environ. 278 (2001) 137–150.
[19] T. Nishikawa, M. Haresaku, A. Fukushima, T. Nakamura, K. Adachi, M. Masuda, M. Hayashi, Further evaluation of an in vivo micronucleus test on rat and mouse skin: results with five skin carcinogens, Mutat. Res. 513 (2002) 93–102.
[20] T. Nishikawa, T. Nakamura, A. Fukushima, Y. Takagi, Further evaluation of the skin micronucleus test: results obtained using 10 polycyclic aromatic hydrocarbons, Mutat. Res. 588 (2005) 58–63.
[21] ISO 10993: Biological Evaluation of Medical Devices-Part10: Tests for Irritation and Delayed-type Hypersensitivity (September 1, 2002).
[22] International validation of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens (Draft version 14).
[23] B. Burlinson, R.R. Tice, G. Speit, E. Agurell, S.Y. Brendler-Schwaab, A.R. Collins, P. Escobar, M. Honma, T.S. Kumaravel, M. Nakajima, Y.F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, In Vivo Comet Assay Workgroup, part of the Fourth International Workgroup on Genotoxicity Testing, Mutat. Res. 627 (2007) 31–35.
[24] M.A. Kastenbaum, K.O. Bowman, Tables for determining the statistical significance of mutation frequencies, Mutat. Res. 9 (1970) 527–549.
[25] K. Ohsawa, S.Y. Nakagawa, M. Kimura, C. Shimada, S. Tsuda, K. Kabasawa, S. Kawaguchi, Y.F. Sasaki, Detection of in vivo genotoxicity of endogenously formed N-nitroso compounds and suppression by ascorbic acid, teas and fruit juices, Mutat. Res. 539 (2003) 65–76.
[26] D.E. Bowen, J.H. Whitwell, L. Lillford, D. Henderson, D. Kidd, S. Mc Garry, G. Pearce, C. Beevers, D.J. Kirkland, Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test, Mutat. Res. 722 (2011) 7–19.
[27] M. Takasawa, H. Suzuki, I. Ogawa, Y. Shimada, K. Kobayashi, Y. Terashima, H. Matsumoto, K. Oshida, R. Ohta, T. Imamura, A. Miyazaki, M. Kawabara, S. Minowa, A. Maeda, M. Hayashi, Evaluation of a liver micronucleus assay in young rats (IV): a study using a double-dosing/single-sampling method by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese