The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Investigation of DNA damage of glycidol and glycidol fatty acid esters using Fpg-modified comet assay

Ryo Inagaki, Kohei Uchino, Yuko Shimamura and Shuichi Masuda

Laboratory of Food hygiene, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan

(Received December 28, 2018; Accepted January 9, 2019)

ABSTRACT — Glycidol fatty acid esters (GEs) are food process contaminants detected in edible oils. It has been thought that glycidol is released from GEs by lipase *in vivo*, and shows genotoxicity. While DNA damage from glycidol has been reported, there is very little information on the DNA damaging potency of GEs *in vivo*. Therefore, we estimated DNA damage of glycidol and glycidyl oleate, which is one type of GEs, using the standard comet assay and a formamidopyrimidine glycosylase(Fpg)-modified comet assay. ICR male mice were orally administrated glycidol and glycidyl oleate (1.0 and 2.0 mmol/kg body weight) at 24 and 3 hr prior to dissection. In the standard comet assay, DNA damage (tail length and % tail DNA) in liver, kidney and blood samples of glycidol-treated groups were increased in a concentration-dependent manner. In Fpg-modified comet assay, glycidol showed DNA damage with higher sensitivity compared with the standard comet assay. DNA damage was not observed in the administration group of glycidyloleate in the standard comet assay. However, in Fpg-modified comet assay, glycidyl oleate showed significant DNA damage in the liver, kidney and blood samples compared with the standard comet assay. In this study, it was revealed that glycidol and glycidyl oleate induce DNA damage, such as oxidative and alkylation damage, recognized by Fpg protein.

Key words: Glycidol, Glycidol fatty acid ester, Comet assay, Formamidopyrimidine glycosylase

INTRODUCTION

Glycidol fatty acid esters (GEs) have been reported as food contaminants at high concentrations in edible oils (Craft *et al*., 2013). GEs are formed in high temperature conditions during the deodorization step in the oil refining process (Weisshaar *et al*., 2010). GEs are broken down equimolalglycidol *in vivo* by hydrolysis of the action of lipase (Wakabayashi *et al*., 2012). Glycidol has epoxides and alcohols in the structure and can be used as stabilizers (natural fats and oils, agrichemicals, vinyl resins etc.), modifiers (epoxy resins, reaction diluents of alkyd resins, cotton, wool) (National Toxicology Program, 2007). However, since glycidol is highly reactive with biological components *in vivo* due to the epoxy structure, it has been reported that glycidol exhibits some genotoxicities (International Agency for Research on Cancer, 2000).

Several reports on genetic toxicity of glycidol have been reported. In *in vitro* tests, Canter *et al.* clarified that glycidol showed mutagenicity in the presence and absence of a metabolic activation using the Ames test (TA 98, TA 100, TA 1535, TA 1537) (Canter *et al*., 1986). Kim *et al.* reported that glycidol showed DNA damage in the comet assay using L5178Y cells with or without metabolic activation (Kim *et al*., 2005). In the *in vivo* test system, micronucleus induction has been confirmed in the bone marrow cells of ICR mice exposed to glycidol using micronucleus test (Ikeda *et al*., 2012). Significant micronucleus inducibility in bone marrow is also recognized even when glycidol was intraperitoneally administered to

Correspondence: Shuichi Masuda (E-mail: masudas@u-shizuoka-ken.ac.jp)

B6C3F1 mice (Irwin *et al*., 1996). In carcinogenesis studies, it was confirmed that oral administration of glycidol to F344 rat for 103 weeks resulted in increased mortality and induction of mesothelioma or mammary tumors of the peritoneum/peritoneum (National Toxicology Program, 2007). Based on these results and reports, the International Cancer Institute (IARC) has classified glycidol as Group 2A (possibly carcinogenic) (IARC, 2000).

The genotoxicity of GEs have also been reported. The mutagenicity of the glycidyl linoleate was examined using the Ames test (TA 98, TA 100, TA 1535, TA 1537). It was confirmed that glycidyl linoleate showed mutagenicity in TA 100 and TA 1535 in the presence and absence of the metabolic activation system (Ikeda *et al*., 2012). The induction of chromosomal aberrant by treatment with glycidyl linoleate was not observed regardless of metabolic activation (Ikeda *et al*., 2012). In the percutaneous carcinogenesis study, sarcoma was observed when glycidyl stearate acid ester was subcutaneously administered to rats, but it was not confirmed in the case of exposing glycidyl oleate (Walpole, 1958; Swern *et al*., 1970).

There are some research reports on the genotoxicity of glycidol and GEs, but their judgment is various depending on the end point of the genotoxicity methods. The risk assessment of genotoxicity *in vivo* has recommended first using *in vivo* a bone marrow micronucleus test, and second, an *in vivo* test for single-cell gel electrophoresis (comet assay). Comet assay is a rapid method used in genotoxicity test *in vitro* and *in vivo*. Generally, the standard comet assay can detect DNA double strand breaks (DSB), DNA single strand breaks (SSB) and alkali-labile sites (ALS) as low level DNA damage in individual cells (Møller, 2018). Some researchers have developed a modified comet assay to detect the specific classes of DNA damage. After lysis, cellular DNA can be reacted with lesion-specific repair endonucleases to exhibit specific base damage as single strand breaks (Azqueta *et al*., 2013). The formamido pyrimidine glycosylase (Fpg) protein is recommended for the detection of oxidative DNA base damage. Because Fpg protein recognizes 8-oxoG (7, 8-dihydro-8-oxoguanine), FapyG (2,6- diamino-4-oxo-5-formamidopyrimidine) and Fapy A (4,6- diamino-5-formamido), and remove mutations occurring in purine bases such as pyrimidine, it is possible to detect oxidative DNA damage with high sensitivity (Shukla *et al*., 2011). It is also known that Fpg protein can detect basic sites (AP sites) and ring-opened N-7 guanine adducts (Lomax *et al*., 2005). As yet, only the result of performing ordinary comet assay on L5178Y cells has been reported for glycidol, but there are few reports using experimental animals or modified comet assay (Kim *et al*., 2005). In this experiment, we evaluated the DNA damaging potency of glycidol and glycidyl oleate (GO), which is a type of GEs,using the standard comet assay and Fpg-modified comet assay to estimate the mechanisum of genotoxicity induction of their compounds.

MATERIALS AND METHODS

Chemicals

Glycidol (purity 98.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Glycidyl oleate (GO, purity 98.0%), soy oil, potassium chloride (KCl), sodium hydrogen phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), tris(hydroxymethyl)aminomethane (Tris), sodium lauroylsalcosinate, dimethyl sulfoxide and TritonX-100 were purchased from Wako Chemicals (Osaka, Japan). Sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl) and ethanol were purchased from Kanto Chemical (Tokyo, Japan). Albumin, from Bovine Serum and low melting point agarose were purchased from Sigma Aldrich (Tokyo, Japan). Ethylenediaminetetraacetic acid disodium salt, 2-hydrate (EDTA-2Na) was purchased from DOJINDO LABORATORIES (Kumamoto, Japan). Ethidium bromide was purchased from MERCK japan (Tokyo, Japan). Formamidopyrimidine-DNA glycosylase (Fpg) protein (8000 units/mL) was purchased from New England Biolabs Japan (Tokyo, Japan).

Animal treatment

6-week-old mare ICR mice were purchased from Japan SLC (Tokyo, Japan). The mice were bred at a temperature 24.5 ± 0.5°C and humidity 55 ± 5°C, light-dark change 12 hr. There were five mice in each group, and five groups were formed. Feed was CE-2 (CLEA Japan, Tokyo, Japan) free feeding. Glycidol and GO were dissolved in soy oil at 0.1 mmol/mL and 0.2 mmol/mL, was administered (10 mL/kg body weight) at 24 and 3 hr prior to dissection. Soy oil was used as control. All animal experiments were approved by the Animal Care and Use Committee of the University of Shizuoka (nos.185182), and were conducted in accordance with the Guidelines and Regulation for the Care and Use of Experiment Animals by the University of Shizuoka.

Single cell gel electrophoresis assay (Comet assay)

Comet assay were performed according to OECD test guideline 489 and added Fpg treatment (OECD Guidelines for the Testing of Chemicals, 2016). River, kidney, blood sample were nucleated from mice administered G

or GO. River and kidney picked 200 mg into 2 mL of 30 mM EDTA-0.9% KCl solution. The liver and kidney samples were conducted mincing in solution. After still standing, 75 µL of supernatant mixed with 75 µL of 1.4% low melting point agarose. The blood sample was mixed with equal parts of 30 mM EDTA-0.9%KCl solution. 75 µL of the blood mixture was amalgamated to 75 µL of 1.4% low melting point agarose. In sequence, 0.7% agar-gel solution and sample mixtures gel solution, 0.7% low melting point agarose solution were placed on the slide, each sample containing 75 µL of material. After gel fixation, the slide was immersed into cold Lysing solution (2M NaOH, 2.5M NaCl, 100 mM EDTA-2Na, 10 mM Tris, 1.1% Sodium N-lauroylsalcosinate, 1% TritonX-100, 10% DMSO, pH 10, 4°C) for 1 hr. A cell sample from the cell lysate was immersed in Fpg buffer (100 mM NaCl, 0.01% BSA, 12.5 mM tris-buffer, 1 mM EDTA). Fpg buffer was replaced three times every 5 minutes with a new one. 80 µL of Fpg solution (1 µg/mL, using Fpg buffer as a control) was added dropwise, covered with a cover glass and reacted in an incubator at 37°C for 15 min. All slides had the cover glass removed and were placed in electrophoresis tank and immersed in migration buffer solution (300 mM NaOH, 1 mM EDTA-2Na, pH 13, 4°C) to perform unwinding for 20 min, subsequently, electrophoresed (25 V, 300 mA, 20 min, 4°C). After electrophoresis, slides were immersed in an excess amount of 0.4M tris-buffer (pH 7.5) to neutralize the alkali, and then fixed in 100% ethanol for 15 min.

Measurement of the slide was performed by fluorescence microscope (Nikon, Tokyo, Japan) with an image analysis device (Comet Analyzer, YOU WORKS, Tokyo, Japan). The slide was stained with 20 µg/mL ethidium bromide and cover glass was mounted on the slide. Tail length and % tail DNA calculated from the distance between the center of gravity of the DNA distribution in the tail and the center of gravity of the DNA distribution in the head × the fraction of DNA in the tail) in 100 cell per slide was measured at 200 × magnification.

Statistical analysis

Experimental data are presented as mean ± SE. Statistical analyses of the comet assay data about comparison between control and administration group were performed by Dunnett test. The influence of addition and non-addition of FPG was carried out using Mann-Whitney U test. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Tail length and % tail DNA in liver samples of groups treated by glycidol and GO using the standard comet assay and Fpg-modified comet assay were shown in Figs. 1 and 2. In a standard comet assay, glycidol increased tail length and % tail DNA in a concentration-dependent manner, and a significant increase was confirmed at 2.0 mmol/kg body weight. In a Fpg-modified comet assay, tail length was significantly increased by treating with glycidol at 1.0 and 2.0 mmol/kg body weight, and % tail DNA was significantly increased at 2.0 mmol/kg body weight. In a standard comet assay, GO significantly increased tail length at 2.0 mmol/kg body weight com-

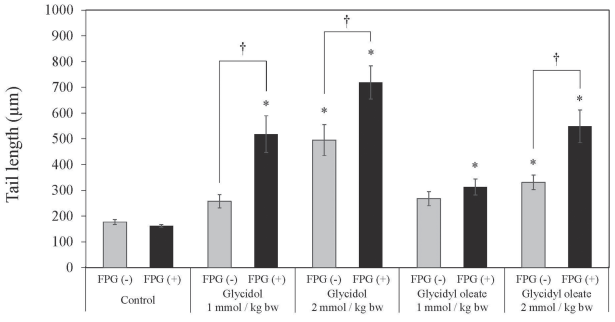

Fig. 1. Tail Length (the standard comet assay and Fpg-modified comet assay) of liver from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

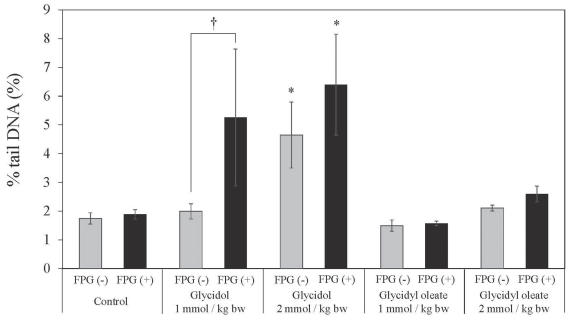

Fig. 2. Percent tail DNA (the standard comet assay and Fpg-modified comet assay) of liver from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

pared with control groups. In a Fpg-modified comet assay, tail length was significantly increased by treatment with GO in a concentration-dependent manner. In % tail DNA, GO showed a tendency to increase in a concentration-dependent manner. Post-treatment with Fpg protein strongly enhanced tail length and % tail DNA. The differences between the effects in the absence and the presence of the Fpg protein in liver samples by treatment with glycidol and GO were statistically significant.

Tail length and % tail DNA in kidney samples of groups treated by glycidol and GO in the standard comet assay and Fpg-modified comet assay were shown in Figs. 3 and 4. In a standard comet assay and Fpg-modified comet assay, glycidol significantly increased tail length and % tail DNA in a concentration-dependent manner. The values of tail length and % tail DNA in Fpg-modified comet assay were higher than that of the standard comet assay. In kidney samples, GO did not result in an increase of tail length and % tail DNA in a standard comet assay. However, in a Fpg-modified comet assay, significant increases in tail length and % tail DNA were confirmed in GO-treated groups. Post-treatment with Fpg protein significantly enhanced glycidol and GO-induced DNA damage.

Tail length and % tail DNA in blood samples of groups treated by glycidol and GO in the standard comet assay and Fpg-modified comet assay were shown in Figs. 5 and 6. In blood samples, glycidol and GO showed significant increases in tail length and % tail DNA at 2.0 mmol/kg body weight only in Fpg-modified comet assay. From these results, the sensitivity of the Fpg-modified comet assay was higher than that of the standard comet assay.

There are few reports about DNA damage of glycidol and GEs using comet assay. In addition, it is almost unclear that induction mechanism of DNA damage of glycidol and GEs *in vivo*. In this study, we investigated the DNA damaging potency of glycidol and GO using the standard comet assay and Fpg-modified comet assay *in vivo*. Fpg is used for the detection of oxidative DNA damage, in particular oxidative damage. Fpg recognizes 8-oxoG (7,8-dihydro-8-oxoguanine), FapyG (2,6-diamino-4-oxo-5-formamidopyrimidine), FapyA (4,6- diamino-5-formamido), and remove mutations occurring in purine bases such as pyrimidine (Shukla *et al*., 2011). It is possible to detect oxidative DNA damage with high sensitivity by Fpg treatment. Additionally, it is known that Fpg also detects AP sites and various kinds of ring-opened N-7 guanine adducts (Lomax *et al*., 2005). We clearly indicated that glycidol and GO showed an increase in comet assay parameters of DNA damage by post-treatment with Fpg. It was also confirmed that the differences between the effects in the absence and the presence of the Fpg protein were statistically significant. Glycidol is a direct-alkylating epoxide and have the ability to react with nucleophilic sites forming DNA adducts (Solomon, 1999). Because it was suggested that epoxide of glycidol reacts with DNA with a similar mechanism as that of ethylene oxide, glycidol could form N-7 guanine adducts (Melnick, 2002). Because Fpg can also detect various kinds of N-7 guanine adducts, Fpg proteins are used in the comet assay for the detection of DNA-damaging effect of alkylating agents.

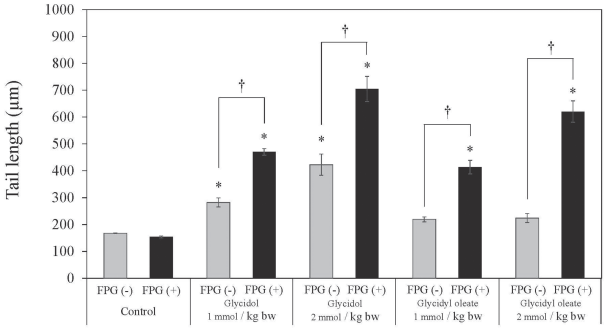

Fig. 3. Tail Length (the standard comet assay and Fpg-modified comet assay) of kidney from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

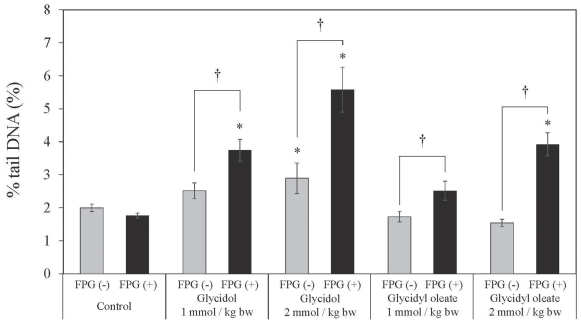

Fig. 4. Percent tail DNA (the standard comet assay and Fpg-modified comet assay) of kidney from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

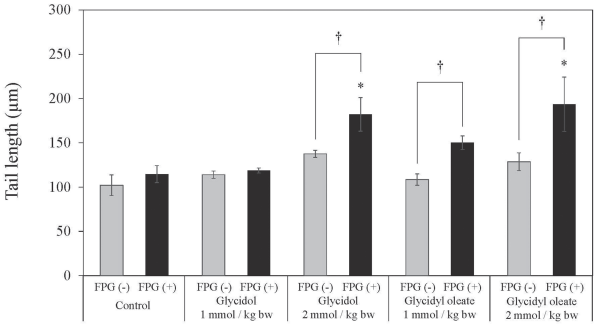

Fig. 5. Tail Length (the standard comet assay and Fpg-modified comet assay) of blood from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

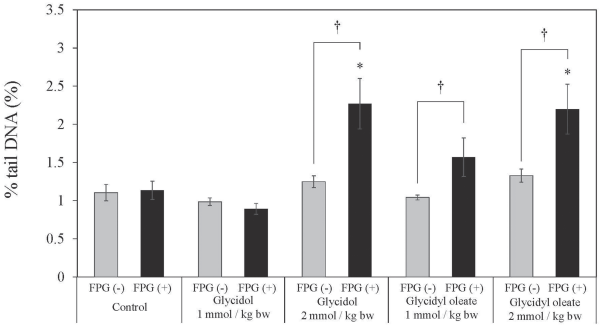

Fig. 6. Percent tail DNA (the standard comet assay and Fpg-modified comet assay) of blood from ICR mice administered with glycidol and GE. The values presented are the mean ± SEM of at least four independent experiments. The asterisk (*) indicate significantly different between control and administration group. The dagger (†) indicate significantly different between without FPG and with FPG ($p < 0.05$).

Therefore, glycidol and GO increased DNA damaging parameters in Fpg-modified comet assay with high sensitivity. The Fpg-modified comet assay is a useful and high sensitive method for detecting various genotoxic chemicals that show the oxidative damage and DNA damaging potency of alkylating agents. In addition, the DNA damaging mechanism of glycidol and GEs was able to estimate by the treatment with Fpg protein in comet assay.

In the present study, the DNA damaging potency of GO tended to be weaker than that of glycidol. The reason is that GEs are not completely decomposed to the glycidol state by lipase *in vivo*. However, the conversion rate from glycidol to GEs *in vivo* is thought to be remarkably different between rodents and humans (Wakabayashi *et al*., 2012). It is necessary for doing risk assessment for humans that metabolic behaviors in humans and the endpoints of each genotoxicity test should be considered. Furthermore, there is the possibility that a high pH in rat stomachs might degrade from GEs to glycidol, and low pH in human stomach, containing high concentration of hydrochloric acid, might shift fromglycidol to 3-monochloropropane-1,2-diol (Jones, 1975).

In the present study, we estimated DNA damaging potency of glycidol and GEs using the standard comet assay and Fpg-modified comet assay. In particular, DNA damage induced by GEs was able to be confirmed using Fpg-modified comet assay. Therefore, the modified comet assay using Fpg protein is a useful and highly sensitive genotoxicity test for initial screening of risk assessment of various chemicals. In the future, it is necessary to estimate the types of DNA damage induction by glycidol and GEs using modified comet assays, such as another repair enzymes-modified comet assay or a cellular comet assay.

ACKNOWLEDGMENT

This work was supported by Grant-in-Aid for JSPS Research Fellow Number JP 17J11445.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Azqueta, A., Arbillaga, L., López, C.A. and Collins, A. (2013): Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG. Mutagenesis, **28**, 271-277.

Canter, D.A., Zeiger, E., Haworth, S., Lawlor, T., Mortelmans, K. and Speck, W. (1986): Comparative mutagenicity of aliphatic epoxides in Salmonella. Mutat. Res., **172**, 105-138.

Craft, B.D., Chiodini, A., Garst, J. and Granvogl, M. (2013): Fatty acid esters of monochloropropanediol (MCPD) and glycidol in refined edible oils. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess., **30**, 46-51.

Ikeda, N., Fujii, K., Sarada, M., Saito, H., Kawabata, M., Naruse, K., Yuki, K., Nakagiri, H., Honda, H., Tamaki, Y., Nishiyama, N. and Kasamatsu, T. (2012): Genotoxicity studies of glycidol fatty acid ester (glycidol linoleate) and glycidol. Food Chem. Toxicol., **50**, 3927-3933.

International Agency for Research on Cancer. (2000): Glycidol. Monogr Eval Carcinog Risks Hum, **77**, 469-486.

Irwin, R.D., Eustis, S.L., Stefanski, S. and Haseman, J.K. (1996): Carcinogenicity of glycidol in F344 rats and B6C3F1 mice. J.

Appl. Toxicol., **16**, 201-209.
Jones, A.R. (1975): The metabolism of 3-chloro-, 3-bromo- and 3-iodopropan-1,2-diol in 21 rats and mice. Xenobiotica, **5**, 155-165.
Kim, J.Y., Kwon, J., Kim, J.E., Koh, W.S., Chung, M.K., Yoon, S., Song, C.W. and Lee, M. (2005): Identification of potential biomarkers of genotoxicity and carcinogenicity in L5178Y mouse lymphoma cells by cDNA microarray analysis. Environ. Mol. Mutagen., **45**, 80-89.
Lomax, M.E., Salje, H., Cunniffe, S. and O'Neill, P. (2005): 8-OxoA inhibits the incision of an AP site by the DNA glycosylases Fpg, Nth and the AP endonuclease HAP1. Radiat. Res., **163**, 79-84.
Melnick, R.L. (2002): Carcinogenicity and mechanistic insights on the behavior of epoxides and epoxide-forming chemicals. Ann. N. Y. Acad. Sci., **982**, 177-189.
Møller, P. (2018): The comet assay: ready for 30 more years. Mutagenesis, **33**, 1-7.
National Toxicology Program. (2007): Toxicology and carcinogenesis study of glycidol (CAS No. 556-52-5) in genetically modified haploinsufficient p16(Ink4a)/p19(Arf) mice (gavage study). Natl. Toxicol. Program Genet. Modif. Model Rep., **13**, 1-81.
Organisation for Economic Co-operation and Development. (2016): Test No. 489: In Vivo Mammalian Alkaline Comet Assay. *In series:OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects*.
Shukla, A.K., Pragya, P. and Chowdhuri, D.K. (2011): A modified alkaline Comet assay for *in vivo* detection of oxidative DNA damage in Drosophila melanogaster. Mutat. Res., **726**, 222-226.
Solomon, J.J. (1999): Cyclic adducts and intermediates induced by simple epoxides. IARC Sci. Publ., **982**, 123-135.
Swern, D., Wieder, R., McDonough, M., Meranze, D.R. and Shimkin, M.B. (1970): Investigation of fatty acids and derivatives for carcinogenic activity. Cancer Res., **30**, 1037-1046.
Wakabayashi, K., Kurata, Y., Harada, T., Tamaki, Y., Nishiyama, N. and Kasamatsu, T. (2012): Species differences in toxicokinetic parameters of glycidol after a single dose of glycidol or glycidol linoleate in rats and monkeys. J. Toxicol. Sci., **37**, 691-698.
Walpole, A.L. (1958): Carcinogenic action of alkylating agents. Ann. N. Y. Acad. Sci., **68**, 750-761.
Weisshaar, R. and Perz, R. (2010): Fatty acid esters of glycidol in refined fats and oils. Eur. J. Lipid Sci. Technol., **112**, 158-165.