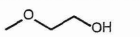
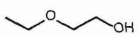
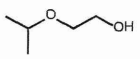
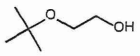
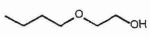
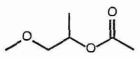
Table 2
A toxicity data matrix for EGME and related chemicals from HESS for forming a category.

Chemical structure	Chemical name, CAS no.	Alkoxyacetic acid formation	Testicular toxic effects, levels	Strain of rats, duration, route, dose	Data source
	EGME 109-86-4	+ Methoxyacetic acid (*in vivo*)	Degeneration of germinal epithelium in the seminiferous tubules, ⩾750 ppm (0.93 mmol/kg/d) weight↓, ⩾1500 ppm (2.17 mmol/kg/d)	F344 rats, 13 weeks, drinking water, 750, 1500, 3000, 4500 ppm	NTP (1993)
	EGEE 110-80-5	+ Ethoxyacetic acid (*in vitro*) (*in vivo*)	Degeneration of germinal epithelium in the seminiferous tubules, ⩾5000 ppm (4.44 mmol/kg/d) weight↓, 10,000 ppm (8.79 mmol/kg/d)	F344 rats, 13 weeks, drinking water 1250, 2500, 5000, 10,000 ppm	NTP (1993)
	EGiPE 109-59-1	+ Isopropoxyacetic acid (*in vivo*)	No toxic effects	SD rats, 28 days, gavage, 30, 125, 500 mg/kg/d	MHLW (2002)
	EGtBE 7580-85-0	+ *tert*-Butoxyacetic acid (*in silico*)	No toxic effects	SD rats, 42 days, gavage, 4, 20, 100 mg/kg/d	MHLW (2001)
	EGBE 111-76-2	+ Butoxyacetic acid (*in vitro*) (*in vivo*)	No toxic effects	F344 rats, 13 weeks, drinking water, 750, 1500, 3000, 4500, 6000 ppm	NTP (1993)
	PGMEA 108-65-6	– (*in vivo*)	No toxic effects	SD rats, 44 days, gavage, 30, 100, 300, 1000 mg/kg/d	MHLW (1998)

study of hazard assessment of existing CSCL chemicals. The inventory was retrieved from the Toolbox and loaded into the HESS to generate potential metabolites using the Rat Cellular Metabolism Simulator, which proposes all the possible metabolites of a target chemical based on the observed reactions of metabolic maps of related chemicals in the HESS. Chemicals predicted to be metabolized to methoxy- or ethoxyacetic acid were then searched for, using a custom profiler which defines the structure of methoxy- or ethoxyacetic acid as a boundary. Both HESS and OECD QSAR Toolbox already have the profiler based on structures of EGME and EGEE. However, a new profiler was created in this study because careful evaluation of the AOP with mechanistic information from the current version of HESS DB strongly suggests that formation of their active metabolites, methoxy- or ethoxyacetic acid, is necessary for activating the AOP. The new mechanism-based profiler is more suitable to obtain relevant analogs since it is possible that several metabolic routes to methoxy- or ethoxyacetic acid are present. The modified profiler is updated in HESS and the Toolbox. As a result of inventory screening, about 40 chemicals were obtained. Some of the chemicals, such as those with atoms other than carbon, oxygen and hydrogen were then eliminated to simplify the current study: metabolism and toxicity data are lacking for such chemicals and their analogs. The remaining 22 chemicals were addressed for categorization by evaluating documented metabolism data of related chemicals in the HESS in Section 3.4.

3. Results

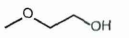
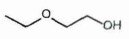
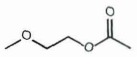
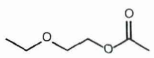
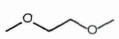
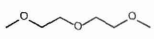
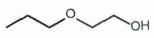
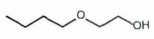
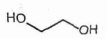
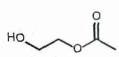
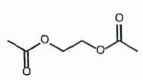
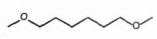
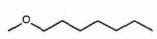
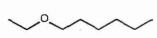
3.1. Building an AOP for the testicular toxicity of EGME

An AOP is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome (Ankley et al., 2010; Schultz, 2010). Using the publicly available literature on *in vitro* and *in vivo* metabolism and toxicological studies, the AOP for EGME-induced testicular toxicity was developed by considering the strength, consistency, and specificity of the association with the experimental evidence shown in Fig. 1.

There is strong evidence that formation of methoxyacetic acid in the liver is a prerequisite for EGME-induced testicular toxicity in rats. EGME undergoes oxidation in the rat liver to methoxyaldehyde, catalyzed by alcohol dehydrogenase, with subsequent oxidation by aldehyde dehydrogenase to methoxyacetic acid (Moss et al., 1985). Pretreatment with pyrazole, an alcohol dehydrogenase inhibitor, shows a protective effect against the formation of methoxyacetic acid and results in a significant decrease in testicular toxicity by EGME in rats (Foster et al., 1984; Moss et al., 1985). Administration of equimolar amounts of methoxyacetic acid to rats clearly induced testicular toxicity at similar levels to EGME (Foster et al., 1984). Furthermore, methoxyacetic acid caused cytotoxic effects in primary rat testicular cultures, but EGME itself did not (Gray et al., 1985).

A variety of studies has been performed to explore the mechanism of testicular toxicity of methoxyacetic acid. They include modulation of hormonal responses in the testis, induction of oxidative stress, perturbation of endogenous metabolism, and induction of Sertoli cell-mediated apoptosis (Ku et al., 1995; Rao and Shaha, 2002; Tirado et al., 2003; Barone et al., 2004; Yamamoto et al., 2007; Bagchi and Waxman, 2008; Bagchi et al., 2009; Takei et al., 2010). These biochemical events caused by methoxyacetic acid appear to be associated with cellular injury in the testis, leading to spermatocyte degeneration and cell death, and finally to atrophy and weight decrease of the testis. An AOP identifies a measurable or predictable key event linked to an adverse outcome for developing a category. In this AOP, the metabolic formation of methoxyacetic acid is the critical event associated with testicular toxicity. This AOP does not fulfill the full AOP requirements, since the molecular initiating event is not fully defined. However, generation of methoxyacetic acid seems to be a prerequisite for the molecular initiating event.

Table 3
A toxicity data matrix for EGME-related chemicals from different data sources for evaluating a category.

Chemical structure	Name, CAS no.	Formation of key metabolites[a]	Testicular toxicity (*in silico*)	Testicular toxic effects, levels	Strain of rats or mice, duration, route, dose	Data source
	EGME 109-86-4	+ (*in vitro*)	+	Atrophy of the seminiferous epithelium, weight↓, ⩾250 mg/kg/d (3.28 mmol/kg/d)	ICR mice, 5 weeks, gavage, 62.5, 125, 250, 500, 1000, 2000 mg/kg/d	Nagano et al. (1984)
	EGEE 110-80-5	+ (*in vitro*)	+	Atrophy of the seminiferous epithelium, weight↓, ⩾1000 mg/kg/d (11.09 mmol/kg/d)	ICR mice, 5 weeks, gavage, 500, 1000, 2000 mg/kg/d	Nagano et al. (1984)
	EGMEA 110-49-6	+ (*in vitro*)	+	Atrophy of the seminiferous epithelium, weight↓, ⩾500 mg/kg/d (4.23 mmol/kg/d)	ICR mice, 5 weeks, gavage, 500, 1000, 2000 mg/kg/d	Nagano et al. (1984)
	EGEEA 111-15-9	+ (*in vitro*)	+	Atrophy of the seminiferous epithelium, weight↓, ⩾1000 mg/kg/d (7.57 mmol/kg/d)	ICR mice, 5 weeks, gavage, 500, 1000, 2000, 4000 mg/kg/d	Nagano et al. (1984)
	EGDME 110-71-4	+ (*in silico*)	+	Atrophy of the seminiferous epithelium, weight↓, ⩾500 mg/kg/d (5.54 mmol/kg/d)	ICR mice, 5 weeks, gavage, 250, 500, 1000 mg/kg/d	Nagano et al. (1984)
	DEGDME 111-96-6	+ (*in vitro*) (*in vivo*)	+	Spermatocyte degeneration, weight↓, 684 mg/kg/d (5.10 mmol/kg/d)	SD rats, 20 days, gavage, 684 mg/kg/d	Cheever et al. (1989)
	EGPE 109-86-4	– (*in silico*)	–	No toxic effects	ICR mice, 5 weeks, gavage, 250, 500, 1000 mg/kg/d	Nagano et al. (1984)
	EGBE 111-76-2	– (*in vitro*) (*in vivo*)	–	No toxic effects	ICR mice, 5 weeks, gavage, 250, 500, 1000 mg/kg/d	Nagano et al. (1984)
	EG 107-21-1	– (*in vivo*)	–	No toxic effects	ICR mice, 5 weeks, gavage, 500, 1000, 2000, 4000 mg/kg/d	Nagano et al. (1984)
	EGA 542-59-6	– (*in silico*)	–	No toxic effects	ICR mice, 5 weeks, gavage, 250, 500, 1000 mg/kg/d	Nagano et al. (1984)
	EGDA 111-55-7	– (*in silico*)	–	No toxic effects	ICR mice, 5 weeks, gavage, 250, 500, 1000 mg/kg/d	Nagano et al. (1984)
	HGDE 13179-98-1	+ (*in vivo*)	+	Degeneration of seminiferous tubules↓, weight↓, 200 mg/kg/d (1.36 mmol/kg/d)	SD rats, 4 weeks, gavage, 2, 20, 200 mg/kg/d	Poon et al. (2005)
	MHE 629-32-3	– (*in vivo*)	–	No toxic effects	SD rats, 4 weeks, gavage, 2, 20, 200 mg/kg/d	Poon et al. (2005)
	EHE 5756-43-4	– (*in vivo*)	–	No toxic effects	SD rats, 4 weeks, gavage, 2, 20, 200 mg/kg/d	Poon et al. (2005)
	BE 142-96-1	– (*in vivo*)	–	No toxic effects	SD rats, 4 weeks, gavage, 2, 20, 200 mg/kg/d	Poon et al. (2005)

[a] EGME, EGEE, methoxyacetic acid or ethoxyacetic acid.

3.2. Grouping structural analogs metabolized to methoxy- or ethoxyacetic acid into a category for testicular toxicity

To develop a category, the data set of the following six chemicals were selected from HESS, based on variations in the alkyl chain: EGME, EGEE, EGiPE, EGtBE, EGBE and PGMEA. Table 2 shows a matrix that compares the metabolism and testicular toxic effects in rats of each of these chemicals. EGME, EGEE, EGiPE and EGBE were chiefly metabolized to their corresponding alkoxyacetic acids (Miller et al., 1984a; Sumner et al., 1995; Cheever et al., 1984; Hutson and Pickering, 1971; Ghanayem et al., 1987; Sabourin et al., 1992). Methoxyacetic acid and ethoxyacetic acid induced the degeneration of the dividing spermatocytes, whereas butoxyacetic acid showed no toxic effects in primary rat testicular cell

cultures (Gray et al., 1985). Repeated-dose toxicity studies have shown EGME to have greater testicular toxicity than EGEE (NTP, 1993). EGiPE, EGtBE and EGBE have no testicular toxicity (MHLW, 2001, 2002; NTP, 1993). There appears to be a negative correlation between the number of n-alkyl carbon substituent (n = 1–4) in EGAEs and the potency order of the testicular toxicity they induce. PGMEA was readily hydrolyzed to PGME, which underwent O-demethylation to form propylene glycol and conjugation to PGME glucuronide and sulfate. No methoxyacetic acid was detected in the rats (Miller et al., 1984b). Repeated administration of PGMEA caused no testicular toxic effects (MHLW, 1998).

Taken together, EGME and EGEE appear to be similar as to metabolism, mode of action and testicular toxic effects. EGiPE, EGtBE, EGBE and PGMEA are not included in this category due to the absence of metabolic formation of methoxy- or ethoxyacetic acid and of testicular toxic effects. Hence, the category we propose is the group of chemicals that are metabolized chiefly to methoxy- or ethoxyacetic acid. The category members produce testicular toxic effects by acting via the AOP of EGME.

3.3. Evaluation of the proposed category

To evaluate the proposed category, additional chemicals were selected from published literature reports outside of HESS which describe repeated-dose toxicity studies of EGME and related chemicals (Nagano et al., 1984; Cheever et al., 1989; Poon et al., 2005). They included the fifteen chemicals shown in Table 3. Testicular toxicity was then addressed for the tested analogs. The step is not carried out on the work flow of HESS. However, the present analysis showed that the expected results were obtained for the analogs by read-across, indicating the reliability of toxicity prediction with the category.

The testicular toxic effects of EGME and EGEE appeared after 5-week oral toxicity studies of ICR mice at 250 mg/kg/d (3.28 mmol/kg/d) and 1000 mg/kg/d (11.09 mmol/kg/d), respectively, and above (Nagano et al., 1984). Mice are less sensitive to testicular toxicity than rats, suggesting that the testicular toxicity of the related chemicals found in mice will appear more prominently in rats. EGMEA is an acetate ester of EGME. It has been shown to be readily hydrolyzed to generate EGME in mice (Stott and McKenna, 1985). It is thus reasonable to conclude that the substance falls into this category. Since EGMEA is highly susceptible to enzymatic ester hydrolysis, it appears that EGMEA has toxicokinetic features similar to EGME. The presumed LOAEL for testicular toxicity of these substances was estimated to be 0.93 mmol/kg/d in F344 rats and 6.95 mmol/kg/d in B6C3F1 mice, respectively, based on 13-week feeding studies on EGME (NTP, 1993). When EGMEA was given to mice by gavage for 5 weeks, atrophy of the seminiferous epithelium and testis weight decrease were observed at 500 mg/kg/d (4.23 mmol/kg/d) and over in ICR mice (Nagano et al., 1984).

EGEEA is similar to EGMEA in terms of structure, physicochemical properties and chemical reactivity. Stott and McKenna (1985) report that EGEEA is readily hydrolyzed to EGEE in mice. It is thus logical for the chemical to be included in our proposed category. The testicular toxic potency was predicted to be similar to that of EGEE. When EGEEA was administered by gavage to mice for 5 weeks, atrophy of the seminiferous epithelium and a decrease in testis weight were observed at doses of 1000 mg/kg/d (7.57 mmol/kg/d) and above (Nagano et al., 1984).

EGDME and DEGDME are not a class of EGAEs but have a substructure of EGME in the molecule. The literature on the metabolism of EGDME is not publicly available. However, it is strongly suggested that this chemical is metabolized to generate EGME (EPA, 2007). In rats, the major metabolic pathway of DEGDME produces 2-(2-methoxyethoxy)ethanol and the latter pathway produces EGME (Richards et al., 1993). Hence both belong to the category. In mice dosed with EGDME for 5 weeks, atrophy of the seminiferous epithelium and testicular weight loss were found at 5.54 mmol/kg/d and more (Nagano et al., 1984). In rats dosed with DEGDME for 20 days, spermatocyte degeneration and decreased testis weight were observed at 5.10 mmol/kg/d (Cheever et al., 1989).

EGPE has a straight alkyl chain with three carbons. No documented metabolism information was found, but it is reasonable to presume that EGPE is metabolized to propoxyacetic acid by analogy with the metabolism of EGEE and EGBE (Yamada et al., 2012). Propoxyacetic acid had very slight effect in primary rat testicular cell cultures at high concentrations (Gray et al., 1985), suggesting that EGPE is not a member of this category. Repeated administration of EGPE caused no testicular toxic effects in mice (Nagano et al., 1984) or rats (Katz et al., 1984).

EG is a minor metabolite of EGME (Sumner et al., 1995). The metabolic information showed no methoxy- or ethoxyacetic acid formation (Gessner et al., 1961; Lenk et al., 1989), indicating that the chemical does not fall into this category. It is reasonable to presume, additionally, that EGA and EGDA do not either. They showed no testicular toxic effects in mice (Nagano et al., 1984).

HGDE is structurally related to DEGDME. Methoxyacetic acid was found at high concentrations in the urine of HGDE-treated rats (Poon et al., 2005; Wade et al., 2006). Hence, the chemical is in the category, although its metabolic pathway remains unknown. In a 4-week gavage study, administration of HGDE to SD rats at 200 mg/kg/d (1.36 mmol/kg/d) induced spermatocyte

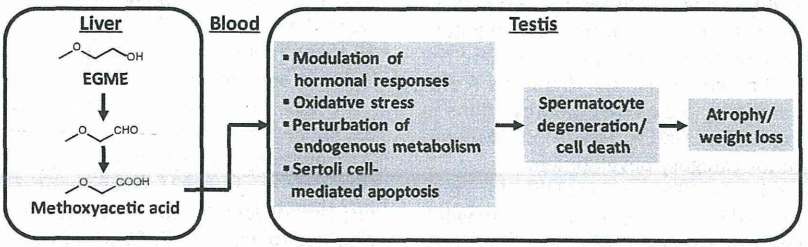


Fig. 1. An AOP of EGME-induced testicular toxicity in rats. EGME undergoes oxidation in the liver to methoxyaldehyde, catalyzed by alcohol dehydrogenase, with subsequent oxidation by aldehyde dehydrogenase to methoxyacetic acid (Moss et al., 1985). Methoxyacetic acid appears to be responsible for EGME-induced testicular toxicity (Gray et al., 1985; Foster et al., 1987; Li et al., 1996). Methoxyacetic acid is shown to modulate hormonal responses in the testis, including alteration of androgen-dependent transcriptional activity and changes in the balance between androgenic and estrogenic activity (Tirado et al., 2003; Jansen et al., 2004; Henley and Korach, 2006; Bagchi et al., 2009). Moreover, it was demonstrated that methoxyacetic acid induces oxidative stress in rat testicular tissues (Rao and Shaha, 2002; Yamamoto et al., 2007). Such oxidative stress may be associated with the apoptosis of spermatocytes via a mitochondrial pathway (Yan et al., 2000; Bagchi and Waxman, 2008). Endogenous metabolic disturbances, such as alterations in choline oxidation and beta-oxidation, were also observed in the testis of rats that was repeatedly exposed to EGME. Inhibition of the flavoprotein dehydrogenase enzyme family is proposed as one of the modes of action of EGME-induced testicular toxicity (Takei et al., 2010). Sertoli cells are the target of methoxyacetic acid. Germ cell apoptosis is produced via Sertoli cell-dependent pathway (Tirado et al., 2003; Barone et al., 2004). The multiple effects of methoxyacetic acid on the testis result in degeneration and cell death of the spermatocytes, leading to testicular atrophy and weight loss.

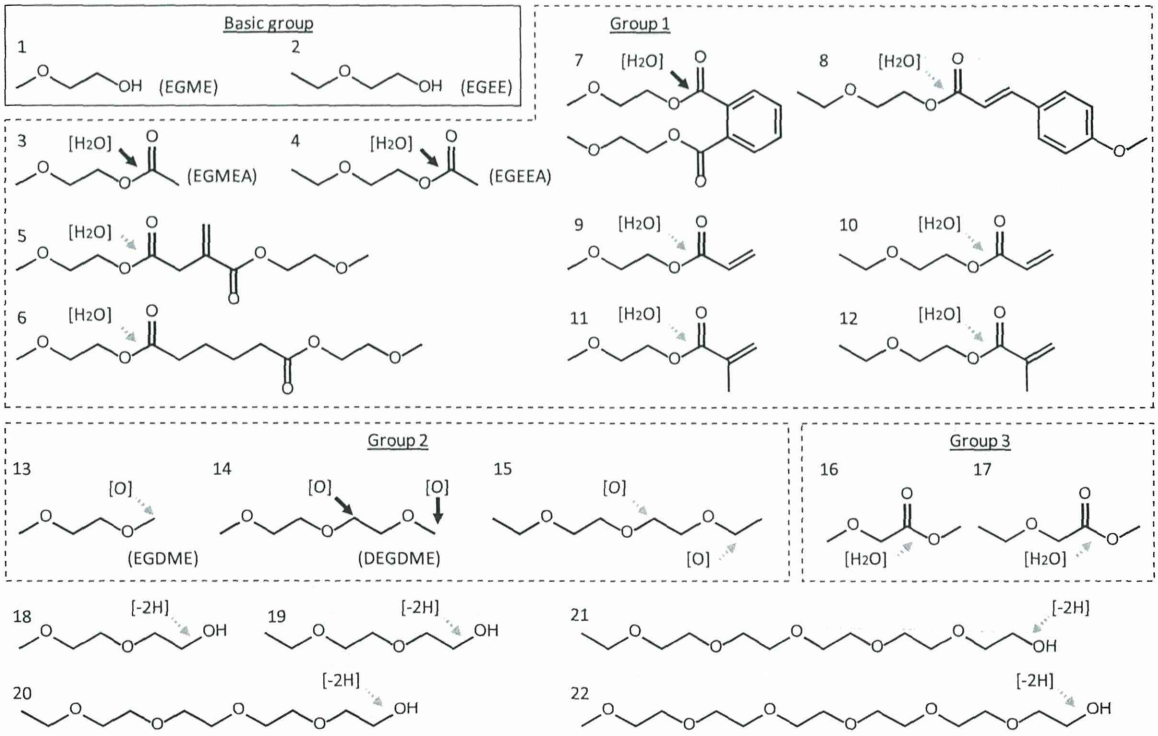


Fig. 2. Chemicals predicted to be metabolized to methoxy- or ethoxyacetic acid by the metabolism simulator of HESS from the MITI inventory. Chemical structures for which the metabolism simulator of HESS generated methoxy- or ethoxyacetic acid were arranged based on visual examination of 2D structural similarity (compounds **1–22**). Data sets of chemicals (tested chemicals) are indicated as their abbreviations shown in parentheses. Black and gray arrows respectively represent documented and predicted primary sites to be metabolized. See Section 3 for a detailed description of metabolism prediction. EGME and EGEE (compounds **1** and **2**) are defined as a basic group shown within a closed line. Chemicals enclosed with a dotted line are more likely to be converted to methoxy- or ethoxyacetic acid according to the related metabolism information. They can be classified as Groups 1–3 based on the possible metabolic routes. (See Section 4.)

degeneration in the seminiferous tubules (Poon et al., 2005). The structural analogs MHE, EHE and BE did not produce a significant increase in methoxyacetic acid in the urine of rats dosed at 200 mg/kg/d (1.53 mmol/kg/d). In the 4-week gavage studies, the substances did not cause testicular toxic effects, as was expected (Poon et al., 2005).

Given all the structural, metabolic and toxicological information in Tables 2 and 3, the category can be finally defined as chemicals which are metabolized to methoxy- or ethoxyacetic acid.

3.4. Categorization of chemicals forming methoxy- or ethoxyacetic acid from the MITI inventory

Chemical substances possibly being converted to methoxy- or ethoxyacetic acid were extracted from the Japanese MITI inventory using the HESS Rat Cellular Metabolism Simulator. Those are the compounds **1–22** in Fig. 2. They contained six chemicals (compounds **1–4**, **13**, **14**), which are indicated as their abbreviations in parentheses in Fig. 2 (EGME, EGEE, EGMEA, EGEEA, EGDME and DEGDME, respectively), used for category development.

Observed metabolic maps in the HESS are available for compounds **3**, **4**, and **14** (EGMEA, EGEEA and DEGDME, respectively), all of which have been shown to generate EGME or EGEE. Ritter et al. (1985) demonstrated that compound **7** was hydrolyzed to form EGME and the monophthalate ester. The remaining 15 substances (compounds **5**, **6**, **8–12**, and **15–22**) were addressed for categorization by predicting the primary metabolic reaction based on the *in vitro* and *in vivo* metabolism data of related chemicals in the HESS.

First, the data gaps for the metabolic hydrolysis of three substances (compounds **5**, **6** and **8**) were filled with the documented metabolism data of carboxylate esters with varied structures. Dimethyl succinate (CAS No. 106-65-0), bis(2-ethylhexyl) adipate (103-23-1) and allyl cinnamate (866-31-5) have been shown to be susceptible to metabolic hydrolysis (Ladrière et al., 1999; Takahashi et al., 1981; Silver and Murphy, 1978), suggesting that hydrolysis of compounds **5** and **6** to form EGME and compound **8** to form EGEE is likely.

Secondly, compounds **9–12** have an alpha–beta unsaturated double bond moiety in their molecules. Such an electrophilic substructure may react with nucleophilic groups of macromolecules. However, four metabolism studies in the HESS demonstrated that structurally diverse esters termed ethyl, butyl and 2-ethylhexyl acrylate, and 2-hydroxyethyl methacrylate (CAS No. 140-88-5, 141-32-2, 103-11-7 and 868-77-9, respectively) are readily hydrolyzed (Linhart et al., 1994a,b; Gut et al., 1988; Durner et al., 2009). The results suggest that compounds **9–12** may form EGME and EGEE through metabolic hydrolysis.

It has been revealed that compounds **14** (DEGDME) are oxidatively cleaved in the rat liver microsomes by P450 to generate 2-(2-methoxyetheoxy)ethanol (major) and EGME (minor) (Richards et al., 1993). Likewise, it is reasonable to presume that bis(2-ethoxyethyl) ether (compound **15**) undergoes oxidative cleavage to generate EGEE as a minor product.

Compounds **16** and **17** are methyl esters of methoxyacetic acid and ethoxyacetic acid, respectively. It is very likely that both simple esters are readily hydrolyzed to form the corresponding alkoxyacetic acids.

Compounds **18–22** consist of di- to oligo ethylene glycol methyl or ethyl ethers. It is possible that these substances undergo oxidative cleavage by P450, as does compound **14**. It is, however, reported that diethylene glycol butyl ether (CAS No. 112-34-5), a

prototype of the class of chemical, was mainly converted to the corresponding carboxylic acid through oxidation of the terminal alcohol group, presumably by alcohol dehydrogenase. The glucuronide was also a major metabolite. Butoxyacetic acid, an oxidative cleavage product, was a metabolite seen in trace amounts (Boatman et al., 1993). Given the notion that turnover of alcohol dehydrogenases is much higher than that of P450s, we assume that only trace, if any, amounts of methoxy- or ethoxyacetic acid are formed for this group of chemicals.

Taken together, we conclude that compounds **1–17** in Fig. 2 fall into the proposed category. Additionally, it is noted that HGDE, a category member as stated in Section 3.3, was not obtained in this screening, since the chemical is not present in the inventory.

4. Discussion

In the present study, we defined a new category of chemicals being metabolized to methoxy- or ethoxyacetic acid based on the AOP for the testicular toxic effects of EGME and based on data for repeated-dose toxicity studies of the related compounds. A Japanese chemical inventory was screened to obtain the category chemicals using the HESS rat metabolism simulator and other related metabolism information.

Repeated-dose toxicity has multiple endpoints. Oral administration of EGME resulted in severe organ toxicities associated with the hematopoietic system and immuno system (NTP, 1993). Here we focused on testicular toxicity, because this form of toxicity is more important from the regulatory aspect and the mechanism is better understood. The AOP for testicular toxicity in this case study is applicable for EGME and EGEE only and is not generalized for other chemicals. However, the AOP clearly identified formation of methoxy- or ethoxyacetic acid as a key event linked to the toxicity. Hence, we expanded the application of the AOP for category approach in a practical way by determining applicability of the AOP to other potential analogs based on formation of the active metabolites.

The metabolism simulator proposes all the possible metabolites of a query chemical based on the observed metabolic reactions of related chemicals. Thus, HESS can efficiently search for chemicals in an inventory possibly being metabolized to methoxy- or ethoxyacetic acid. However, false positive chemicals may be obtained, since the results of the metabolism simulation are qualitative. In the following step, chemicals producing methoxy- or ethoxyacetic acid more likely were categorized using documented quantitative metabolism information on the related chemicals. Consequently, data set chemicals known to cause testicular toxicity as a result of formation of methoxy- or ethoxyacetic acid or the precursors EGME or EGEE (compounds **1–4**, **13**, and **14**; EGME, EGEE, EGMEA, EGEEA, EGDME and DEGDME, respectively) have been successfully obtained (Fig. 2). The result confirms the validity of our approach.

We define Group 1 as esters of EGME and EGEE which are readily hydrolyzed to generate EGME and EGEE. It is possible that lipases and/or carboxylesterases participate in the primary hydrolytic reaction. Since both enzyme species have broad substrate specificity, it was concluded that compounds **3–12** fall into this group.

Group 2 is defined as chemicals that form EGME or EGEE via oxidative *O*-dealkylation. It was demonstrated in rats that compound **14** (DEGDME) was converted to methoxyethoxyacetic acid or methoxyacetic acid as final products through 2-(2-methoxyethoxy)ethanol or EGME. Methoxyacetic acid is a minor product but is associated with the testicular toxicity of compound **14** (Richards et al., 1993). As with compound **14**, it seems possible that compounds **13** and **15** are transformed to methoxy- and ethoxyacetic acid, respectively. A newly developed CYP2E1 metabolism prediction model (Yamazoe et al., 2011) estimates that 2-(2-methoxyethoxy)ethanol and EGME are metabolites of compound **14**. This result is consistent with the observed metabolism data (Richards et al., 1993), suggesting the validity of the prediction model for this class of chemicals. The CYP2E1 model predicts EGME to be a metabolite of compound **13** and EGEE to be a metabolite of compound **15**.

Esters of methoxy- and ethoxyacetic acid are defined as a different group (Group 3). It is logical to assume that the simple esters of compounds **16** and **17** are readily converted to methoxy- or ethoxyacetic acid.

HGDE was not obtained by screening of inventory since it is not present in the inventory. However, according to the literature on urinary methoxyacetic acid and testicular toxicity in rats (Poon et al., 2005), this chemical apparently falls into this category. Although the metabolic pathway remains unknown, it appears that HGDE is converted to 1-methoxyhexanol via oxidative demethylation by analogy to compound **14**. The oxidative demethylation is also suggested by the CYP2E1 metabolism prediction model to be a primary reaction (Yamazoe et al., 2011). It is likely that 1-methoxyhexanol readily oxidizes to 1-methoxyhexanoic acid, which subsequently undergoes beta-oxidation in the same way as straight-chain fatty acids, leading to the formation of methoxyacetic acid, possibly via the TCA cycle. We propose a new group (Group 4) for chemicals which generate methoxy- or ethoxyacetic acid through carboxylic acid formation followed by beta-oxidation. Previous studies demonstrated that repeated administration of 1,4-diethoxybutane at 200 mg/kg/d (1.53 mmol/kg/d) for 4 weeks did not cause testicular toxicity in rats. However, this result might be due to the low dose setting, because ethoxyacetic acid is less

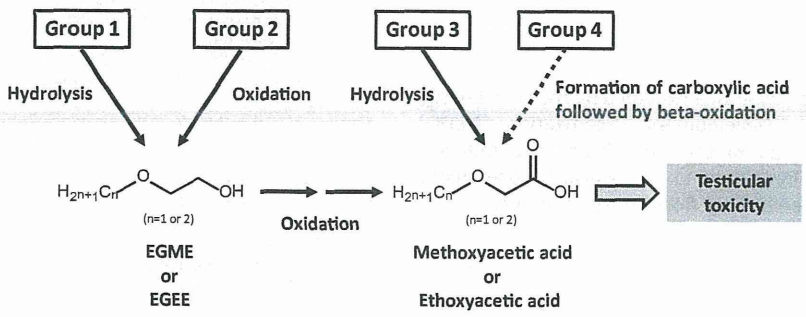


Fig. 3. Summary of the major metabolic routes generating methoxy- or ethoxyacetic acid. EGME or EGEE can be formed via hydrolysis of EGME or EGEE esters (Group 1) and oxidative cleavage of chemicals that have an EGME or EGEE substructure (Group 2). EGME or EGEE are readily oxidized to methoxy- or ethoxyacetic acid. The testicular toxicants can be also produced via different pathways: hydrolysis of esters of methoxy- or ethoxyacetic acid (Group 3) and possibly formation of methoxy- or ethoxycarboxylic acids followed by beta-oxidation (Group 4).

potent than methoxyacetic acid (Wade et al., 2006). It is possible that 1,4-diethoxybutane at higher doses, and the structural analogs of 1,4-dimethoxybutane and 1,8-dimethoxyoctane at similar doses, induce testicular toxicity.

Based on the analysis in this study, metabolic routes leading to the formation of methoxy- or ethoxyacetic acid include the following. (1) Hydrolysis and (2) oxidation followed by EGME or EGEE production, and (3) hydrolysis and (4) formation of carboxylic acid and the subsequent beta-oxidation followed by methoxy- or ethoxyacetic acid production. The scheme is summarized in Fig. 3.

It is likely that middle- or long-chain alcohols such as di- or oligoethylene glycol alkyl ether (compounds **18–22**) chiefly undergo oxidation of the terminal alcohol group, leading to formation of their corresponding carboxylic acids. The CYP2E1 metabolism prediction model suggests that oxidative dealkylation takes place in only trace amounts or not at all for this class of chemicals. However, one cannot exclude the possibility of the metabolic formation of small amounts of methoxy- or ethoxyacetic acid through oxidative dealkylation by other monooxygenases. At this point, the class of chemicals is not classified into Group 2 due to limited information on this type of metabolism. However, information from *in vitro* metabolism assays may further improve the reliability of prediction of its testicular toxicity.

Previous epidemiological studies suggest that testicular toxic effects are of concern for particular populations exposed to EGME (Cook et al., 1982; Welch et al., 1988). Most of the studies were on exposure to mixtures that included EGME. A recent risk assessment report (NITE, 2007) concluded that it is not clear whether the observed testicular effects are due to exposure to EGME. However, the changes appear to be related to animal findings from repeated-dose toxicity studies of EGME. Moreover, *in vitro* studies have demonstrated that methoxyacetic acid induces apoptosis and necrosis of germ cells in human primary testicular cultures (Li et al., 1996). $T_{1/2}$ of methoxyacetic acid in human urine is 77 h, which appears to be longer than in rats (Groeseneken et al., 1989; Moss et al., 1985). Hence further studies are needed before it will be possible to extrapolate the testicular toxicity of EGME in experimental animals to that in humans. The category chemicals proposed in this study are also of concern in connection with risk assessment.

In summary, we developed the AOP for testicular toxicity of EGME. Given the limited nature of the AOP, a study was undertaken to determine the applicability of the AOP to other potential analogs in an effort to develop a category approach. Insights derived could be incorporated into HESS and OECD QSAR Toolbox, which can be exploited by other users. Our results clearly demonstrate the practical utility of the AOP-based category approach to predict the repeated-dose toxicity of chemicals.

Conflict of interest

The authors declare there to be no conflicts of interest.

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Review Paper

Transition of Japan's statistical tools by decision tree for quantitative data obtained from the general repeated dose administration toxicity studies in rodents

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Abstract

Statistical significance is one of important criteria on judgment of regulatory toxicological testing. The decision tree for analysing quantitative data obtained from repeated dose administration studies in rodents has been in use in Japan around 1981. Since then, several authors proposed improved versions of the decision tree incorporating all possible situations of statistical analysis normally encountered in such studies. Recently, a decision tree, which traces a simple route, unlike the previously proposed ones which trace complex routes has been proposed by a few researchers in Japan. While tracing to the most appropriate statistical tool using a decision tree, we propose to consider following points which also play a significant role in selecting the most appropriate statistical tool: (1) statistical tools that fails to detect a significant difference in the low dose group, (2) use of the one-sided test with high power to detect a significant difference compared with two-sided, (3) as far as possible avoid carrying out statistical analysis on the transformed data, since the analytical result of such data is difficult to interpret, (4) it is important to mention what statistical tools of the decision tree are used for the analysis, (5) examine the data for both normality and homogeneity and (6) for testing homogeneity, use Levene's test. Selection of widely accepted statistical tools is usually preferred to less popular and complex statistical analysis. It has been observed that in recent years the preferred statistical tools for analyzing quantitative data obtained from toxicity studied are of simple in nature but with high power to detect a significant difference.

Keywords: *Decision Tree, Repeated Dose Administration Study, Statistical Significant Difference, Statistical Method.*

1. Introduction

Several attempts have been made to standardize statistical methodologies for the analysis of data obtained from the toxicological studies. One of the methodologies proposed by several authors is the decision trees. Decision trees are graphical representation of decision involved in the choice of the statistical procedure. In Japan, reports on toxicity studies related to medicines and pesticides are reviewed by Pharmaceutical and Medical Devices Agency and Food Safety Commission of Japan in Cabinet office, Government of Japan respectively, whereas reports on industrial general chemical substances by the Ministry of Health and Labour and Welfare (MHLW). However, statistics analytical method is not stated clearly definitely in each guideline on toxicity study. For instance, in repeated dose administration toxicity studies with pesticides to examine the significant difference among the groups (number of groups > 2) repeated *t*-test analysis is performed, whereas in the studies with medicine repeated *t*-test analysis or Dunnett's multiple comparison test (Dunnett's test) or other multiple range tests after analysis of variance (ANOVA) are performed. This paper reviews the changes of the decision tree for statistical analysis of the quantitative data obtained from general toxicity studies in Japan. Attempt was also made to compare the statistical methods used in Japan to analyse quantitative data obtained from repeated dose administration toxicity studies in rodents with other countries.

2. Survey items and results

2.1. History of decision tree for toxicity studies

Statistical decision tree (ketteiju in Japanese) did not exist earlier to 1980. During that period, the analysis of the data obtained from toxicity tests was carried out either by *t*-test or Dunnett's test (including few other tests) after the ANOVA. Since 1980, several decision trees have been proposed to analyse the toxicological data. A history of transition of the decision trees in Japan is briefed hereunder.

2.1.1. Before 1981

Most of the toxicity tests on pesticides were analyzed by repeated Student's *t*-test [1, 2] prior to 1981. The toxicity tests on pharmaceutical products were analysed using *t*-test [3, 4, 5], ANOVA [6], and Dunnett's test etc. [7, 8]. This Dunnett's test, Kobayashi [9] was introduced in Japanese for the first time in Japan.

2.1.2. In 1981

Yamazaki et al. [10] proposed a tree-type algorithm in 1981. This could be the first decision tree used in Japan for analyzing toxicology data. This decision tree (Fig. 1) traces Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Wallis's *H* test, nonparametric type Dunnett's test, and nonparametric type Scheffé. After 2005, this decision tree was seldom used; the reason for this could be Scheffé's test, nonparametric type Dunnett's test, and nonparametric type Scheffé's test with a low power to detect a significant difference, especially in the low dose group, that the decision tree contained.

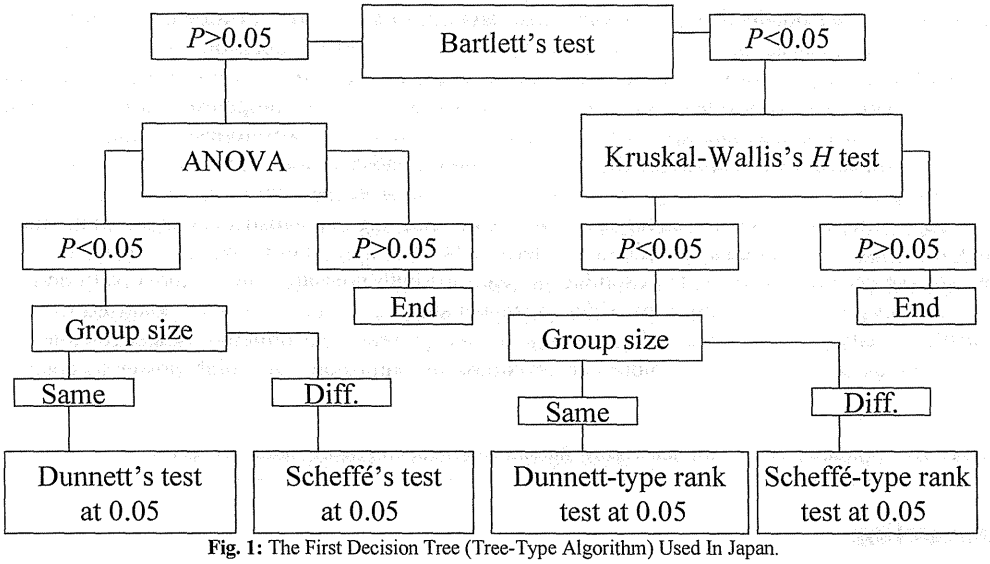

Fig. 1: The First Decision Tree (Tree-Type Algorithm) Used In Japan.

2.1.3. In 1986

A detailed decision tree (Fig. 2) was proposed by Gad and Weil [11] in 1986. This decision tree described the analysis of both quantitative and qualitative data. Statistical procedures for two groups, three or more than three groups and for multiple groups were described in this decision tree. This decision tree also contained the visual examination of data, parametric data/continuous data (body weight, blood cell counts, etc.), scatter gram, the ANOVA, group size, Duncan's multiple range test, Dunnett's test, *F*-test, Student's *t*-test, Cochran *t*-test, Wilcoxon rank sum test, Kruskal-Wallis *H* test, distribution free multiple comparison test, Fisher's exact test, and R×C Chi square test. This decision tree was extensively used in several toxicity test facilities including contract research organizations in Japan.

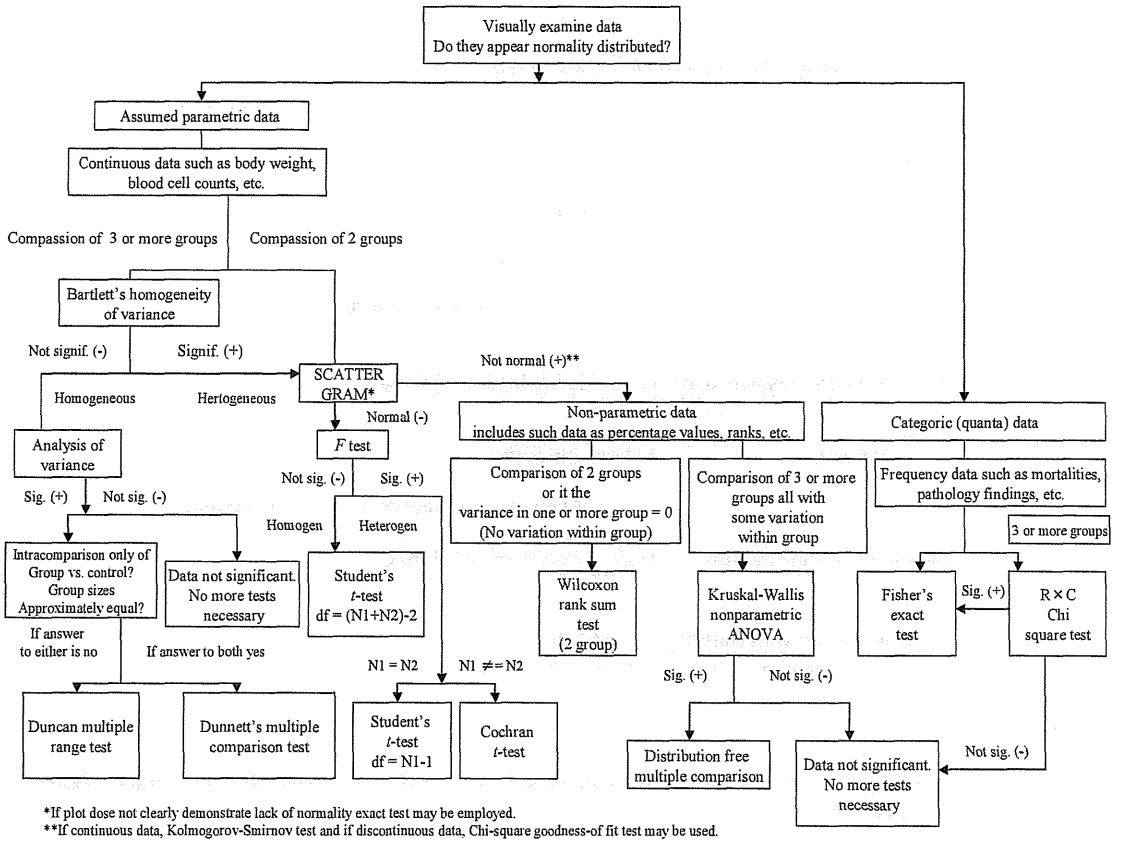

Fig. 2: Decision Tree for Selection of Hypothesis-Testing Procedures.

2.1.4. In 1990

The decision tree (Fig. 3) proposed by Sano and Okayama [12] was used until 2005. This decision tree can calculate the number of animals is changed. Bartlett's test, ANOVA, Dunnett's test, and Kruskal-Wallis's *H* test and nonparametric type Dunnett's test were included in this decision tree.

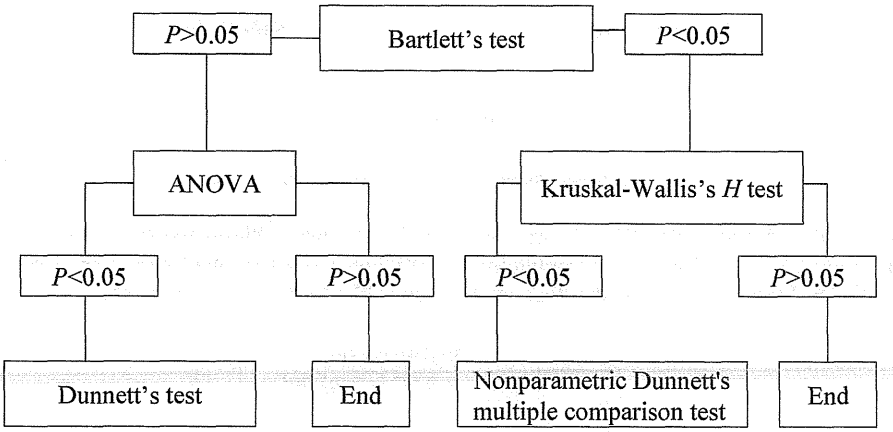

Fig. 3: The Traditional Decision Tree for the Analysis of Toxicological Data Used in Japan for Several Years.

2.1.5. In 1998

In the decision tree (Fig. 4) proposed by Hamada et al. [13], the individual values were transformed to logarithmic values for the data that do not show the homogeneity of variance. The decision tree traces scatter plots or box-plot, Bartlett's test, log-transformation, Bartlett's test for log data, checking outliers, absolute maximum value of Studentized