susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by a carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). Such gender-related variation is also reported in humans, mostly for drugs, such as more severe adverse effects with greater improvement in response to antipsychotic drugs such as chlorpromazine and fluspirilene in women (Fletcher et al., 1994; Harris et al., 1995). The various causes of these gender differences are indicated mainly for toxicokinetic determinants. It is well-known that hepatic metabolism differs between the sexes, with males generally having higher activity than females in rats (Gad, 2006). Furthermore, gender differences in membrane transport in various organs of the body including the kidneys, liver, intestine, and brain have emerged relatively recently (Morris et al., 2003). In the case of HDBB, it is difficult to discuss the cause of the gender differences because no other data are available on toxicity, including the toxicokinetics. However, because male rats showed higher susceptibility to various effects of HDBB (on the liver, heart, blood, etc.) consistently, such differences in metabolism or transports between the sexes might increase the blood concentration of causative substances (HDBB or its metabolites) in males.

For gender differences, it goes without saying that sexual hormones play an important role. In fact, Wang et al. (2001) reported that orchidectomy completely abolished the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine. Because testosterone decreased cholinesterase inhibition in gonadectomized males and females, it is apparent that testosterone interferes with the effects of rivastigmine. On the other hand, estrogen has been shown to act as a dopamine antagonist (Fletcher et al., 1994; Harris et al., 1995), which is considered to contribute at least in part to sex differences in response to antipsychotic drugs. It would be interesting to investigate the role of sex steroids in the mediation of sex differences in toxic susceptibility to HDBB, too. For the metabolic enzyme cytochrome P450, involved in the metabolism of many substances, gonadal hormones are known to play an important role in regulating the expression; however, gonadal hormones do not act directly on the liver to confer the sex-dependent pattern, but rather, indirectly via the hypothalamus, which regulates the pituitary and its secretion of the polypeptide hormone, growth hormone (Waxman and Chang, 2005).

Based on the findings of this study, the NOAEL for females was concluded to be 2.5 mg kg^{-1} day^{-1} based on the induction of hypertrophy and increased mitosis of hepatocytes and degeneration and hypertrophy of the myocardium at 12.5 mg/kg. On the other hand, the NOAEL for males could not be determined because hypertrophy and decreased incidence of fatty change of hepatocytes and bile duct proliferation were noted at the lowest dose of 0.5 mg/kg. Considering the toxic effects observed at a relatively low dose and the incomplete recovery, more severe damage by the longer exposure is a concern; therefore, we

are currently performing a 52-week repeated-dose toxicity study to clarify the potential toxic effects of this chemical.

CONCLUSION

The current results showed that the oral administration of HDBB for 28 days principally affected the liver and heart, and male rats were more susceptible to the toxic effects of this chemical than female rats. The NOAEL for repeated-dose toxicity was concluded to be less than 0.5 mg kg^{-1} day^{-1} in male rats and 2.5 mg kg^{-1} day^{-1} in female rats.

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DNA Repair

Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells

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ABSTRACT

DNA double strand breaks (DSBs) are usually repaired through either non-homologous end-joining (NHEJ) or homologous recombination (HR). While HR is basically error-free repair, NHEJ is a mutagenic pathway that leads to deletion. NHEJ must be precisely regulated to maintain genomic integrity. To clarify the role of NHEJ, we investigated the genetic consequences of NHEJ repair of DSBs in human cells. Human lymphoblastoid cell lines TSCE5 and TSCE105 have, respectively, single and double I-SceI endonuclease sites in the endogenous thymidine kinase gene (*TK*) located on chromosome 17q. I-SceI expression generated DSBs at the TK gene. We used the novel transfection system (Amaxa Nucleofector) to introduce an I-SceI expression vector into the cells and randomly isolated clones. We found mutations involved in the DSBs in the TK gene in 3% of TSCE5 cells and 30% of TSCE105 cell clones. Most of the mutations in TSCE5 were small (1–30 bp) deletions with a 0–4 bp microhomology at the junction. The others consisted of large (>60) bp deletions, an insertion, and a rearrangement. Mutants resulting from interallelic HR also occurred, but infrequently. Most of the mutations in TSCE105, on the other hand, were deletions that encompassed the two I-SceI sites generated by NHEJ at DSBs. The sequence joint was similar to that found in TSCE5 mutants. Interestingly, some mutants formed a new I-SceI site by perfectly joining the two original I-SceI sites without deletion of the broken-ends. These results support the idea that NHEJ for repairing I-SceI-induced DSBs mainly results in small or no deletions. Thus, NHEJ must help maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations.

1. Introduction

DNA double strand breaks (DSBs) are the most dangerous form of DNA damage. They can be caused by ionizing radiation (IR) or radiometric chemicals, and they can occur spontaneously during DNA replication. Other DNA damage, such as single strand breaks, easily convert to DSBs when a replication fork encounters them [1,2]. The non- or misrepair of DSBs can cause cell death or neoplastic transformation [3,4], so the accurate repair of DSBs is important for maintaining genomic integrity [5]. DSBs are generally repaired through non-homologous end-joining (NHEJ) or homologous recombination (HR) [6,7]. NHEJ joins sequences at the broken ends, which have little or no homology, in a non-conservative manner, and some genetic information is lost. HR, on the other hand, requires extensive tracts of sequence homology and is

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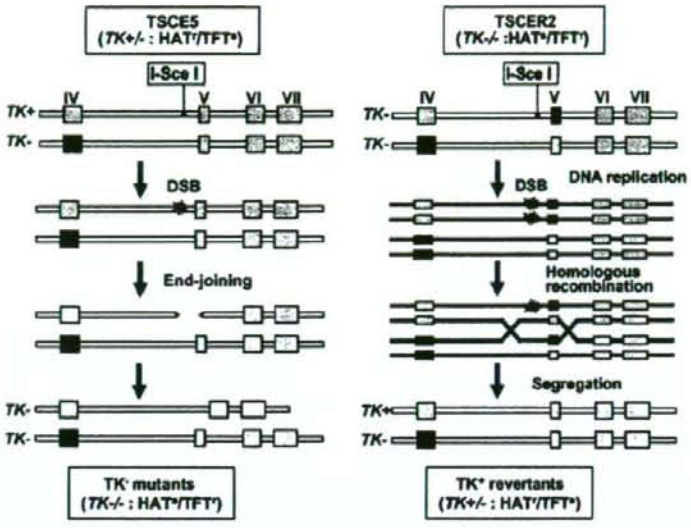

Fig. 1 – Schematic representation of the experimental system. Shadowed and closed rectangles represent the wild type and mutant exons of the TK gene, respectively. In TSCE5 cells, when a DSB at the I-SceI site is repaired by NHEJ and causes an exon 5 deletion, TK-deficient mutants are selected in TFT medium. In TSCER2 cells, when a DSB at the I-SceI site is repaired by HR, TK-proficient revertants are selected in HAT medium.

basically error-free [8]. HR is the primary DSB repair pathway in yeast and prokaryotes, but NHEJ is believed to be the primary pathway in mammalian cells [9]. HR is preferable to NHEJ because it is error-free, but NHEJ may have a different way to maintain genomic integrity.

We previously developed a human cell system to trace the fate of a DSB occurring in an endogenous single copy gene (Fig. 1) [10]. The human lymphoblastoid cell line, TSCE5, is heterozygous (+/–) and TSCER2 is compound heterozygous (–/–) for the thymidine kinase gene (*TK*), and both have an I-SceI endonuclease site in intron 4. DSBs can be generated at the I-SceI site by the introduction of an I-SceI enzyme expression vector. When DSBs occur at the TK locus, NHEJ in TSCE5 cells produces TK-deficient mutants, while HR between the alleles produces TK-proficient revertants in TSCER2 cells. Positive–negative drug selection for the TK phenotypes permits the distinction between NHEJ and HR repair mechanisms. Using the same system, we previously found that almost all I-SceI-induced DSBs in human cells are repaired by NHEJ and result in mainly 100–4000 bp deletions [10]. Drug selection, however, does not recover cells with genetic changes that are too small to influence TK function, and the resulting spectrum of mutations and reversions may be biased quantitatively as well as qualitatively.

To better understand the fate of DSBs in human cells, we randomly isolated non-selected clones after introducing DSBs and directly analyzed their DNA. A novel transfection system (Amaxa Nucleofector™) can introduce the I-SceI expression vector into most of cell population [11] and efficiently produces DSBs at the *TK* gene. With this improved method, we were able to detect cells with deletions at DSBs without drug selection and to trace the fate of DSBs without bias. We also developed a new cell line that has two I-SceI sites in the *TK* gene and can be used as a model for clustering DSBs. DNA sequence analysis of the mutants in this strain revealed that both single and double DSBs were repaired predominantly by NHEJ, producing only small genetic changes, or none. We discuss how NHEJ maintains genomic integrity.

2. Materials and methods

2.1. *Human cell lines for detecting NHEJ and HR induced by a single DSB*

Human lymphoblastoid cell lines TSCE5 and TSCER2 were previously created from TK6 cells [10], which are heterozygous for a point mutation in exon 4 of the *TK* gene (*TK*+/–) (Fig. 1). TSCE5 has a 31 bp DNA flagment containing the 18 bp I-SceI site inserted 75 bp upstream of exon 4 of the *TK*+ allele and retains TK function. TSCER2 is a TK-deficient mutant spontaneously arising from TSCE5. It has a point mutation (G:A transition) at 23 bp of exon 5 of the *TK*+ allele of TSCE5. TSCER2 is compound heterozygote (*TK*–/–) for the *TK* gene. NHEJ for a DSB occurring at the I-SceI site results in TK-deficient mutants in TSCE5 cells, while HR between the alleles produces TK-proficient revertants in TSCER2 cells.

2.2. *I-SceI expression and isolation of mutant clones*

We introduced the I-SceI expression vector (pCBASce) by suspending 5×10^6 cells in 0.1 ml Nucleofector solution V (Amaxa

Biosystem, Koeln, Germany) with 50 μg of uncut pCBASce vector (or without the vector as a control), following the manufacturer's recommendations. We then plated the cells into 96-microwell plates at 1 cell/well. Two weeks later, we randomly isolated single colonies and independently expanded them for DNA analysis.

We maintained the cell culture for 3 days and then seeded them into 96-microwell plates in the presence of 2.0 μg/ml trifluorothymidine (TFT) for isolating TK-deficient mutants or HAT (200 μM hypoxanthine, 0.1 μM aminopterin, 17.5 μM thymidine) for isolating TK-proficient revertants. We counted the drug-resistant colonies 2 or 3 weeks later [12] and calculated the mutation and revertant frequencies according to the Poisson distribution [13].

2.3. *Creating a cell line containing two I-SceI sites*

The targeting vector, pTK10, which we had used to make TSCE5 cells, consists of about 6 kb of the original TK gene encompassing exons 5, 6, and 7 and an I-SceI site in intron 4 [10]. We constructed pTK13 by inserting an additional 21 bp DNA fragment containing the 18 bp I-SceI sequence into pTK10 at the Nco I site in intron 5 (152 bp down stream of exon 5) using site-directed mutagenesis (GeneTailer, Invitrogen) (Fig. 4a). To obtain TK-revertant clones with two I-SceI sites in the TK gene, we transfected TSCER2 cells (5×10^6) with 20 μg of linearized pTK13 vector using the Nucleofector system. After 72 h, we seeded the cells into 96-microwell plates containing HAT. We identified one revertant clone, TSCE105, as correctly targeted and confirmed its molecular structure by DNA sequencing.

2.4. *DNA analysis*

To analyze mutations in the isolated TSCE5 and TSCE105 clones, we amplified the part of the TK gene containing the I-SceI sites by PCR, labeling forward primers with a fluorescent dye. We used the following primers for the I-SceI site in intron 4: forward (166F), 5′-TGG GAG AAT TAA GAG TTA CTC C-3′; reverse (196R), 5′-AGC TTC CAC CCC AGC AGC AGC T-3′. We used the following for the I-SceI site in intron 5: forward (251F), 5′-GGA TGG GCA CAG AGA CAC CA-3′; reverse (241R), 5′-CTG ATT CAC AAG CAC TGA AG-3′. For TSCE105 clones, we used 166F and 241R to amplify the regions containing both I-SceI sites. Amplification was performed by denaturation at 96 °C for 5 min, followed by 25 cycles of 96 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and extension at 72 °C for 10 min. We analyzed the PCR products using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and sequenced them with an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA).

3. Results and discussions

3.1. *Efficiency of the system for detecting NHEJ and HR repair of chromosomal DSBs using Amaxa nucleofection*

The lymphoblastoid cell lines, TSCE5 and TSCER2, which we previously developed, can trace the genetic consequences of chromosomal DSBs in the human genome. NHEJ for a DSB occurring at the I-SceI site results in TK-deficient mutants in TSCE5 cells, while HR between the alleles produces TK-proficient revertants in TSCER2 cells (Fig. 1) [10]. To introduce the I-SceI expression vector into the cells, we now used the Amaxa nucleofection system. The Amaxa Nucleofector™ can directly transfer DNA into the nucleus of the cells at high efficiency. It was designed for primary cells and hard-to-transfected cell lines such as the human B-cell lymphoblastoid [11,14]. Twenty-four hours after the nucleofection, approximately 65% of the transfected TSCE5 cells expressed the I-SceI enzyme, suggesting that DSBs were efficiently introduced into the cells (data not shown; Takashima et al., under submission).

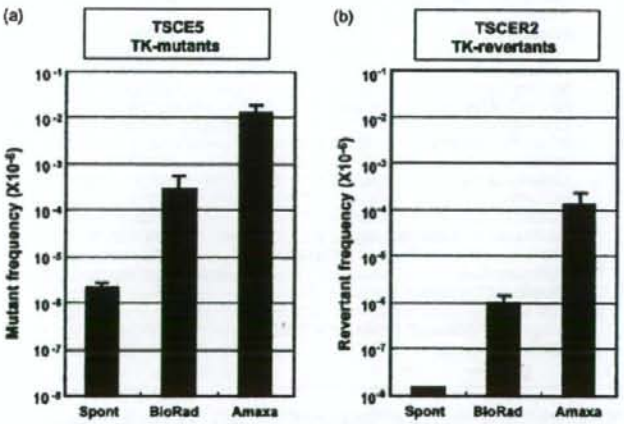

Fig. 2 – Detection of NHEJ and HR repaired DSBs using Amaxa nucleofection or BioRad electroporation. (a) Transfection of TSCE5 with the I-SceI expression vector using Amaxa nucleofection increased the TK-deficient mutant frequency more than 40-fold compared with BioRad electroporation. (b) Transfection of TSCER2 with the I-SceI expression vector using Amaxa nucleofection increased the TK-proficient revertant frequency more than 100-fold compared with BioRad electroporation.

Following Amaxa nucleofection, the mean TK mutant frequency in TSCE5 cells was 1.21%, which was more than 40-fold higher than the frequency we observed with the transfection system we had used previously (BioRad electroporation) (Fig. 2a), and the mean TK-proficient revertant frequency in TSCER2 cells was 1.22×10^{-4}, which was more than 100-fold higher than we observed previously (Fig. 2b). These results demonstrate that the Amaxa nucleofection system effciently introduced the expression vector and generated DSBs with high efficiency in the TSCE5 and TSCER2 cell lines. The relative contribution of NHEJ and HR for reparing the DSBs was 100:1. The value may be biased, however, because the drug selection assay recovers certain classes of NHEJ and HR.

3.2. *Genetic consequences of a chromosomal DSB in non-selected clones*

Because the I-SceI site is inserted into intron 4 of the functional TK allele 75 bp upstream of exon 5, any small deletions caused by NHEJ that do not affect TK function will not be recovered as TFT-resistant mutants in the TSCE5 assay. Similarly, in the TSCER2 assay, short tract gene conversion events that do not extend to exon 5 will not be recovered as TK revertants. Thus, recovery of TK mutants and revertants by drug selection may be biased. Because nucleofection can efficiently generate DSBs at the I-SceI site, however, the system enables detection of deletions and recombination in the TK gene without drug selection. We randomly isolated 926 transfected clones without TFT selection and directly analyzed DNAs from them. We observed that 29 (3.13%) of them had an I-SceI mutation; these were usually small (<60 bp) deletions, insertions, or rearrangements (Table 1). Fig. 3 shows the DNA sequences of 21 mutants with small genetic changes. Three of them (1659, 1841, and 1893) contained a 1 bp deletion at a CCC tract within the I-SceI site. Others had mostly 0–4 bp microhomologies at the junction, suggesting that the NHEJ machinery was involved. The mutant that had a 1 bp insertion at a TT tract within the I-SceI site (2018) might have been generated by misalignment of the cohesive ends. The mutant that exhibited a complicated DNA rearrangement involving a 50 bp deletion combined with a 9 bp inverted sequence that was a part of deleted sequence (1614) was probably the result of sister chromatid fusion and breakage after DNA replication, as described previously [10]. Five of the mutants showed large deletions (17.2%). This fraction may correspond to the TK mutants in the drug selection assay. The large deletions which were commonly detected in the drug selection assay ranged from 1070 to 4030 bp, and had 4–7 bp microhomology at their junctions (data not shown) [10].

One mutant was the product of gene conversion between homologous alleles. It had lost the I-SceI site and retained

Table 1 – Analysis of non-selected TSCE5 clones after I-SceI expression

Total clones	Mutant clones	Mutants (%)
926	29 (Total)	3.13
	23 (Small deletion, insertion, rearrangement; <60 bp)	2.48 (79.3)
	5 (Large deletion; >60 bp)	0.54 (17.2)
	1 (Gene conversion)	0.11 (3.4)

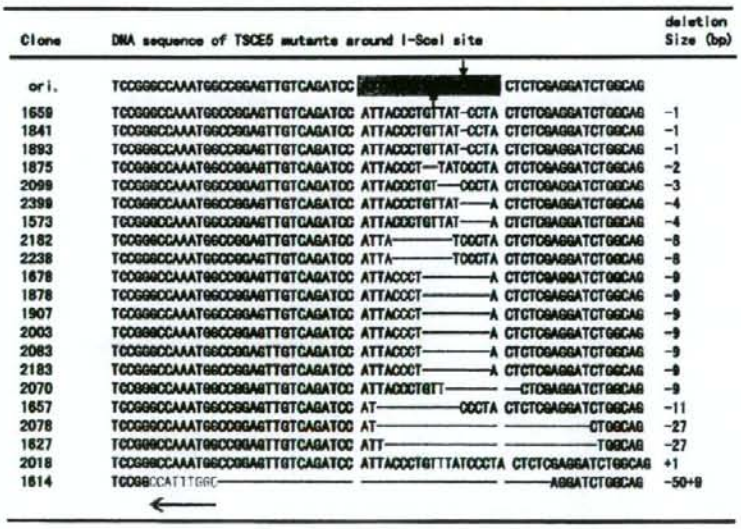

Fig. 3 – DNA sequences at the repair junction of 21 of the 26 non-selected I-SceI mutants with small (<60 bp) genetic changes in TSCE5 cells ("ori." is original sequence). The I-SceI recognition site is highlighted in orange. Arrows indicate I-SceI cleavage sites. The 1 bp deletion in the CCC tract is shown in blue and the 1 bp insertion in the TT tract is shown in green. Microhomologous sequences at junctions are shown in red. The sequence in yellow with a left arrow indicates an inverted sequence from part of a deleted sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

intron 4 of the TK gene that had been originally connected to the I-SceI site. The appearance of HR mutants was infrequent in the non-biased assay, too, suggesting that I-SceI-induced DSBs are mainly repaired by NHEJ, resulting in small deletions [10,15–17]. This does not mean that HR rarely works for DSBs, however, because our I-SceI system does not cover all HR events.

Most I-SceI systems have been developed using artificial reporter substrates based on exogenous drug-resistance or fluorescence genes and are biased in favor of detecting certain classes of deletions and recombination events [18–20]. In the present system, however, we conducted a survey of DSBs occurring in the endogenous single-copy gene, and investigated the consequences of the DSB without selection bias. We first demonstrated the mutational spectrum induced by I-SceI endonuclease in the human genome. However, it does not necessarily reflect the fate of DSBs occurring spontaneously or induced by irradiation, because our I-SceI system does not monitor sister chromatid HR, which must be the major HR pathway in mammalian cells. Other I-SceI systems setting up two tandem copies of the selective gene on the same chromosome can not also evaluate sister chromatid HR quantitatively, because both chromatids are theoretically cleaved during S/G2 phase. We may underestimate the contribution of HR in the I-SceI system.

Although the I-SceI expression vector was introduced into about 65% of the cells, the frequency of mutants at the I-SceI site in the non-selection assay was still only 3.1%. Three possibilities could explain this: (1) only a small proportion of TSCE5 cells expressing the I-SceI vector may undergo a DSB, (2) most cells with DSBs may undergo apoptosis, and (3) some DSBs may go back to their original sequence by perfect joining. The last possibility would be important to the maintenance of genomic integrity following DSB repair, but its demonstration would be difficult because it is impossible to distinguish between non-cleaved and perfectly repaired I-SceI sites.

3.3. *Genetic consequences of two closely separated DSBs*

To efficiently generate DSBs in the genome, we developed a cell line containing two I-SceI sites in the TK gene. We constructed a targeting vector, pTK13, consisting of 6kb of original TK gene including exon 5, 6, 7 and two I-SceI sites flank-

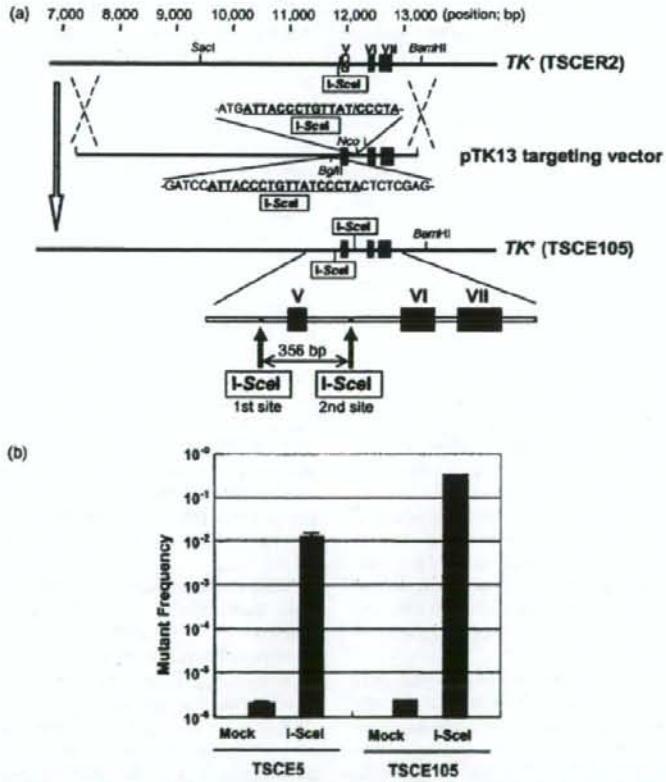

Fig. 4 – (a) Creating the TSCE105 cell line with two I-SceI sites. The functional TK allele in the TSCE105 cell line has two I-SceI recognition sites flanking exon 5, 356 bp apart. (b) The TK-deficient mutant frequency in the TSCE105 cells after introduction of DSBs by Amaxa nucleofection. The mutant frequency was 30-fold higher in TSCE105 than in TSCE5.

Table 2 - Analysis of TSCE105 mutants after I-SceI expression

Total Mutants	Type of Mutation	356bp (1st 2nd)	Number of Mutants(%)
125	Only 1st I-SceI		1 (0.8)
	Only 2nd I-SceI		47 (38)
	Both, independent		6 (4.8)
	Both, combined		70 (56)
	Perfect joining		4
	Joining with small deletion (<60bp)		31
	Joining with large deletion (>60bp)		29
	Joining with rearrangement		6
	Recombination		1 (0.8)

ing exon 5, and transfected it to TSCER2 cells (Fig. 4a). One HAT-resistant recombinant, TSCE105, had another I-SceI site at intron 5 of the TK gene in addtion to the original I-SceI site in TSCE5. The two I-SceI sites are 356 bp apart, flanking exon 5 (Fig. 4a). TSCE105 was also a TK heterozygote and was TFT-sensitive. When we nucleofected the I-SceI expressing vector into TSCE105, the TK-deficient mutant frequency by the TFT selection assay, surprisingly, was extremely high (31.3%) (Fig. 4b). We also examined non-selectecd clones after nucleofection. Among 283 non-selected clones, 83 (29.3%) of them had a deletion mutation involving one or both I-SceI sites. This mutation frequency was about the same as the TK-deficient mutation frequency in the TFT selection assay, suggesting that most mutations in TSCE105 were deletions involving coding sequence of the TK gene.

To investigate the genetic changes induced by the two DSBs, we analyzed 125 mutants (42 TFT-selected and 83 non-selected) and classified them into 4 types depending on whether they occurred (1) only at the first I-SceI site, (2) only at the second I-SceI site, (3) independenly at both I-SceI sites, or (4) at the combined first and second I-SceI sites (Table 2). The majority (56%) were the last type. Interestingly, four of them joined the two I-SceI sites perfectly, creating a new I-SceI site. Fig. 5 shows the DNA sequences around the joint sites of 26 of the 31 mutants that had small deletions. Almost all of them had a 0–4 bp microhomology at the junction, and the sequences were similar to those found around single DSB repair sites (Fig. 3).

While a single DSB in TSCE5 cells caused predominantly small deletions, two closely occuring DSBs in TSCE105 cells were not repaired independently and caused large deletions involving the two I-SceI sites, indicating that multiple DSBs enhance genetic changes qualitatively as well as quantitatively. Mammalian cells may have difficulty retaining small DNA fragments generated by multiple DSBs. High doses of ionizing irradiation, too, not only increase mutation frequency but also change the mutation type to predominantly large deletions [21,22]. The genomic changes observed in TSCE5 and TSCE105 may reflect a dosage-effect, bringing about different numbers of DSBs. In both cases, however, NHEJ is involved and injury is minimized.

The mutants with perfect joining were generated by NHEJ without exonuclease processing in which the cleaved two flanking I-SceI ends simply join. Most of I-SceI-induced DSBs in TSCE5 and TSCE105 cells may be perfectly joined and create a new I-SceI site. Because the I-SceI enzyme is continuously expressed for at least 48 h after nucleofection (Takashima et al., under submission), the new I-SceI sites generated by perfect joining are cleaved again and again. When the DSBs are occasionally joined after exonuclease processing, they accumulate as deletional mutations and are not cleaved any more (Fig. 6). Thus, the perfect joining by NHEJ is important for repairing DSBs, at least endonuclease-induced DSBs. The perfect joining by NHEJ was also reported in other I-SceI-induced DSB systems [23,24]. Van Heemst et al. demonstrated that a blunt DSB induced by the E. coli transposon Tn5 were repaired without loss of nucleotides in Chinese hamster cell lines, suggesting that compatible ends precisely join without deletions [25]. The efficiency or accuracy of precise NHEJ was reduced in Ku80, DNA-PK, XRCC4, or p53 deficient cells [23–26].

NHEJ in mammalian cells involves seven components—Ku70, Ku80, DNA-PKcs, Artemis, XRCC4, Cernunnos/XLF, and Ligase IV [4,7,27–29]. Although the exact role of these proteins remains unknown, three steps have been suggested: (1) end-binding, (2) terminal processing, and (3) ligation [9]. Karanjawala et al demonstrated that defects in Artemis and DNA-PKcs, which are key components in step 2 and possess substantial nucleolytic activity, do not cause severe phenotypes or genomic instability [30]. On the other hand, deficiency of Ku (step 1) or Ligase IV (step 3) confers severe radiosensitivity or lethality [30]. Thus, the second step may not be essential in NHEJ of DSBs, especially of endonuclease-induced DSBs, because the cleaved DNA ends are ligatable

Clone	DNA sequence of TSCE105 mutants around junction site		Deletion Size (bp)	
		1st site 2nd site		
perfect	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	GGTCTGTGCAGACTGC		
2412	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356	(0)
2429	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356	(0)
2445	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356	(0)
2465	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356	(0)
2703	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTAT-CCTA GGTCTGTGCAAACTGC	-357	(-1)
2650	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGT——CCCTA GGTCTGTGCAAACTGC	-359	(-3)
2393	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTG——CCCTA GGTCTGTGCAAACTGC	-360	(-4)
2453	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTA————TCCCTA GGTCTGTGCAAACTGC	-364	(-8)
2434	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	AT————ATCCCTA GGTCTGTGCAAACTGC	-365	(-9)
2345	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTCTGCAAACGGC	-365	(-9)
2689	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT ····· A GGTCTCTGCAAACGGC	365	(9)
2714	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTCTGCAAACGGC	-365	(-9)
2764	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTCTGCAAACGGC	-365	(-9)
2444	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTCTGCAAACGGC	-365	(-9)
2446	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTGTGCAAACTGC	-365	(-9)
2424	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCT————A GGTCTGTGCAAACTGC	-365	(-9)
2304	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	AT————CCCTA GGTCTGTGCAAACTGC	-367	(-11)
2442	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGT———— ————GCAAACTGC	-372	(-16)
2443	TCCGGGCCAAATGTCCGGAGTTGTCAGATCC	————TA GGTCTGTGCAAACTGC	-372	(-16)
2402	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTAT———— ————AACTGC	-372	(-16)
2425	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTTATC—— ————TGC	-374	(-18)
2435	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	ATTACCCTGTT———— ————GC	-378	(-22)
2713	TCCGGGCCAAATGGCCGGAGTTGTC————	———— ——TGTGCAAACTGC	-384	(-28)
2735	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC	———— ————CTGC	-378	(-22)
2405	TCCGGGCCAAATGGCCG————	———— —TCTGTGCAAACGGC	-382	(-36)
2437	TCCGGCC————	———— ————AAACTGC	-409	(-53)

Fig. 5 – DNA sequences at the NHEJ repair junction around the I-SceI junction site in TSCE5 cells. "Perfect" is the DNA sequence when two I-SceI sites join perfectly and create a new I-SceI site (highlighted in orange). Sequences in black are upstream of the first I-SceI site and those in blue are downstream of the second I-SceI site. A total of 26 TSCE105 mutants with deletions combining two I-SceI sites are shown. Underlining indicates a new I-SceI recognition sequence produced by error-free NHEJ. Red indicates microhomologous sequences at junctions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and do not require terminal processing. Perfect joining by NHEJ probably skips the second step. Naturally occurring DSBs produced by oxidative stress, ionizing radiation, and DNA-damaging agents, however, do not have directly ligatable DNA ends and need some form of nucleolytic processing [7,9]. Their repair by NHEJ results in deletions, even if it works properly. In the present study, the size of the deletions caused by NHEJ, however, were relatively small. No recovered TSCE5 or TSCE105 mutants exhibited large deletions or translocations similar to those frequently observed

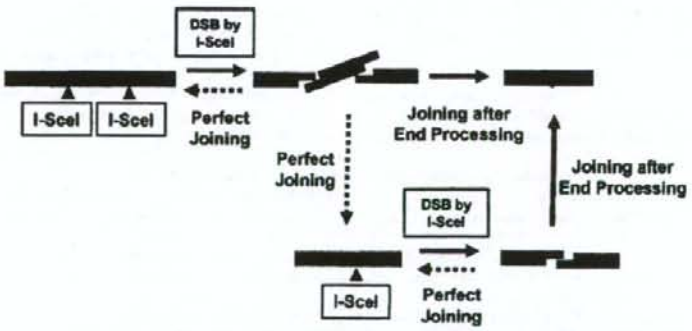

Fig. 6 – A model for NHEJ generating deletions in TSCE105 cells. When a DSB is repaired by perfect joining, an I-SceI site newly generates and is cleaved again. The rare DSB that is joined after exonuclease processing converts to a deletional mutation and accumulates in the cell population.

at the chromosome level in cancer cells [4]. This suggests that NHEJ helps maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations.

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Original Article

Evaluation of statistical tools used in short-term repeated dose administration toxicity studies with rodents

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ABSTRACT — In order to know the different statistical tools used to analyze the data obtained from twenty-eight-day repeated dose oral toxicity studies with rodents and the impact of these statistical tools on interpretation of data obtained from the studies, study reports of 122 numbers of twenty-eight-day repeated dose oral toxicity studies conducted in rats were examined. It was found that both complex and easy routes of decision trees were followed for the analysis of the quantitative data. These tools include Scheffe's test, non-parametric type Dunnett's and Scheffe's tests with very low power. Few studies used the non-parametric Dunnett type test and Mann-Whitney's *U* test. Though Chi-square and Fisher's tests are widely used for analysis of qualitative data, their sensitivity to detect a treatment-related effect is questionable. Mann-Whitney's *U* test has better sensitivity to analyze qualitative data than the chi-square and Fisher's tests. We propose Dunnett's test for analysis of quantitative data obtained from twenty-eight-day repeated dose oral toxicity tests and for qualitative data, Mann-Whitney's *U* test. For both tests, one-sided test with p=0.05 may be applied.

Key words: Statistics; 28-day repeated toxicity study; Rodents; Dunnett's test; Mann-Whitney's *U* test

INTRODUCTION

Short-term repeated oral toxicity study conducted for 14 or 28 days is aimed to (1) predict appropriate doses of test substance for future subchronic or chronic toxicity studies, (2) determine NOELs for some toxicology endpoints and (3) to allow future studies in rodents to be designed with special emphasis on identified target organs (USFDA, 2000). This study also provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time (USEPA, 2000; OECD, 1995). Though these guidelines provide all the information required for the conduct of the study, no information is provided on the appropriate statistical tools to be used to analyze the data obtained from the study. Use of right statistical tool to analyze the data obtained from theses studies is very crucial as the interpretation of the data is mostly based on the results of the statistical analysis.

The statistical tools used to analyze the data obtained from 122 numbers of twenty-eight-day repeated dose oral toxicity tests in rats were examined in the present study. The objective of the study was to know the different statistical tools that are used in these studies and the possible impact of these statistical tools on interpretation of the data. A brief discussion on the use and the property of the different statistical tools used in the studies are also given. The purpose of this article wished for the standardization of statistics and the analysis methods. Finally, the authors made an attempt to suggest statistical techniques that may best suit twenty-eight-day repeated dose oral toxicity studies in rodents.

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MATERIALS AND METHODS

Studies examined

A total number of 122 studies conducted in various test facilities in Japan were examined (MHLW, 2006). The chemical of these examinations was executed with existing chemical substances by the guideline of the Chemical Substance Control Law (1986). The number of studies conducted in each test facility is given in parenthesis: Food and Drug Safety Center, Kanagawa (22), An-Pyo Center, Shizuoka (22), Mitsubishi Chemical Safety Institute Ltd., Ibaraki (18), Safety Research Institute for Chemical Compounds Co., LTD, Hokkaido (15), Bozo Research Center Inc., Shizuoka (12), Research Institute for Animal Science in Biochemistry & Toxicology, Kanagawa (11), Panapharm Laboratories, Kumamoto (10), Nihon Bioresearch Inc., Gifu (9) and National Institutes of Health, Tokyo (3).

Quantitative and qualitative items

Several quantitative and qualitative items are evaluated in twenty-eight-day repeated dose oral toxicity tests in rats, as per the regulatory guidelines. The quantitative items that require statistical analysis are body weight, food consumption, water consumption, leucocytes, erythrocytes, hemoglobin, hematocrit, platelets, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential leucocyte counts, prothrombin time, activated partial thromboplastin time, total protein, albumin, albumin/globulin ratio, total bilirubin, alanine aminotransferase, aspartate aminotransferase, γ-glutamic transaminase, alkaline phosphatase, acetylcholinesterase, total cholesterol, tryglycerides, phospholipids, glucose, blood urea nitrogen, creatinine, inorganic phosphorous, calcium, sodium, potassium, chlorides, urine volume, specific gravity of urine, absolute organ weights and relative organ weights. Qualitative items that require statistical analysis are mortality, functional observation battery, clinical signs, urinalysis (color, pH, protein, glucose, ketone bodies, bilirubin, occult blood, urobilinogen, epithelial cells, erythrocytes, leucocytes, casts and crystals) and pathological findings (macroscopic and microscopic). But the regulatory guidelines do not indicate the specific statistical techniques to be used to analyze these data.

Which test to be used - One-sided or two-sided?

When the *t*-test and Dunnett's multiple comparison test (Dunnett's test) are used, the significant difference detection rate of a two-sided test is about 85% as compared with a one-sided test (Kobayashi, 1997a). In toxicological studies, usually a dosed group is compared with the control group. For this comparison, one-sided test is ideal, hence Yoshimura and Ohashi (1992) recommend the one-sided test for comparing a dosed group with the control group.

Is analysis of variance (ANOVA) necessary?

It is a common practice to subject the data, if they are from more than two groups, ANOVA. If ANOVA shows a significant difference among the groups, multiple comparison tests are used to find the significant difference between any two groups. In recent years, several authors suggested that the error of the second kind can be prevented by carrying out direct multiple comparison tests, without subjecting the data to ANOVA (Hamada *et al.*, 1998; Kobayashi *et al.*, 2000a; Sakaki *et al.*, 2000). It may be worth mention in this context that Dunnett (1964) did not recommend ANOVA prior to multiple comparison tests.

Is Bartlett's homogeneity test necessary?

Generally Bartlett's test is used to examine the homogeneity of variance if the number of animals in a group is 10 or more. Therefore, this test is not used in the toxicity studies with dogs, where the number of animals in the group is less. According to Kobayashi *et al.* (1998), Bartlett's test is not required to examine the homogeneity of variance, when the number of animals in a group is less.

Non-parametric type Dunnett's test

The non-parametric Dunnett's multiple comparison test has two techniques - 'joint type' and 'separate type' or Steel's test. When the Steel's test shows the highest dosage correlation, the number of animals required in the dosage groups to detect a significant difference in the low dosage group is four (Inaba, 1994; Kobayashi *et al.*, 1995). On the contrary, 'joint type' needs 15 animals in each group.

Transformation of data

If the data show heterogeneity of variance as per Bartlett's test, sometimes the data are transformed, for example to logarithmic values and then they are subjected to non-parametric tests. According to Finney (1995), "when a scientist measures a quantity such as concentration of a chemical compound in body fluid, his interest usually lies in the scale, perhaps mg/ml, that he has used; he is less likely to be interested in a summary of results relating to a transformed quantity such as the logarithm of blood concentration. If he analyzes in terms of logarithms, encouraged perhaps by an elementary but uncritical statistical textbook or by a convenient software package, he may find significant differences but to express his conclusions in meaningful numbers may be impossible. I do not assert

that a scientist should never transform data before analysis; I urge that data should be transformed only after careful consideration of all consequence". Therefore, transformation should be done cautiously.

Power of Scheffe's test

Use of Scheffé's test is discouraged in recent years because this test may not show a significant difference in the dosage groups even if the dosage groups show a difference of 60-53% compared to control group (Kobayashi *et al*., 1997b).

Power of non-parametric tests using ranked data

In four groups setting with the highest dosage correlation, the minimum numbers of animals required in the low-dose group to detect a significant difference, compared to control, using the statistical tools of Scheffé's type, Dunn's test, Tukey type, Dunnett type, Williams-Wilcoxon test, Steel test and Mann-Whitney's *U* test are 22, 19, 18, 15, 8, 4 and 3, respectively. Therefore, in the twenty-eight-day repeated dose oral toxicity tests in rats, where the number of animals is 5/sex/group, except Steel and Mann-Whitney's *U* tests, other tests are not used. Inaba (1994) made a similar observation on the power of the above tests.

Power of Chi-square and Fisher's tests

When a finding in the animals of a control group is 0, in order to find a significant difference of the finding between the control group (n=5) and dosage group (n=5) by chi-square test, all the 5 animals in the dosage group (n=5) should show the finding, whereas by Fisher's test 4 animals should show the finding. When 1 animal in the control group shows a finding, even if the finding is seen in all the animals in the dosage group, a significant difference is not detected by chi-square test, but it is detected by Fisher's test. In the light of the above it may be stated that power of one-sided Fisher's test is better than the Chi-square test.

Dunnett's test is the expanded version of *t*-tests

Dunnett's test becomes *t*-test when two groups are analyzed (Kobayashi *et al*., 1997c). Therefore, when comparing the recovery groups in the twenty-eight-day repeated dose oral toxicity tests in rats, where number of the groups is 2, it does not make any difference, whether the analysis is carried out by Dunnett test or *t*-test.

Power of Mann-Whitney's *U* test

This test is generally used for the analysis of pathology data (Kobayashi *et al*., 1997d). A significant difference by a one-sided test is detected if the calculated *U* value is four or less. Since one-side is expected in studies like twenty-eight-day repeated dose oral toxicity tests in rats, a one-sided Mann-Whitney's *U* test is used to analyze pathology data obtained from these studies.

RESULTS

Quantitative data

Out of 122 studies examined, 79 studies used statistical tools that follow a complicated course (tool numbers; 2, 3, 4, 5, 8, 9, 10, 12, 15, 16 and17) and 43 studies used statistical tools that follow simple course (tool numbers; 1, 6, 7, 11, 13 and14) (Table 1; Fig. 1). The statistical tools describing the method of analyzes, in the case of three or more groups and two groups were mentioned in 6 studies, whereas this description was not found in 11 studies. Only eight studies used trend test (Jonckheere, 1954). In the tool number 10, the significance level of ANOVA and Kruskal-Walis's *H* test were set at p=0.10. For comparing with the control, this tool set the significance level of p=0.05. Tool numbers 13 and 14 did not perform Bartlett's test for testing the homogeneity of variance. Use of one-sided or two-sided test is not indicated in 87 studies. Only one study indicated use of non-parametric test.

Qualitative data

Since urinalysis data were classified into many grades, chi-square test was used to analyze these data in most of the studies. For macro- and microscopic pathological findings, Mann-Whitney's *U* test, Fisher's test and Chi-square test were used. Most of the studies did not indicate the alpha. Only the pathological findings of 3 studies were examined for dose-relationship (Table 2).

Use of a one-sided test was more common than a two-sided test in the case of analysis of both quantitative and qualitative data (Table 3).

DISCUSSION

National Toxicology Program, USA published technical reports of long-term carcinogenicity studies and short-term toxicity tests carried out with more than 500 substances in rat and mouse (NIH, 2006). Most of these studies used the statistical tools almost similar to the ones currently used to analyze the data obtained from the toxicity tests of agricultural chemicals and medical drugs (Kobayashi *et al*., 2000b).

On examination of 122 studies, it was found that complex and easy courses of analytical techniques were used for the analysis of the quantitative data. These tools may be classified into 4 different categories. Five tools (tool

numbers; 4, 5, 8, 16 and17) are the advanced type of the algorithm, similar to the one developed by Yamazaki *et al.* (1981). These tools include Scheffé's test, non-parametric type Dunnett's and Scheffé's tests with very low power. Six tools (tool numbers; 3, 7, 9, 10, 12 and 15) are again advanced type of algorithm developed by Sano and Okayama (1990), which can be used even if the number of animals in the groups are different. Use of the non-parametric Dunnett type test with low power is also seen in few studies. Mann-Whitney's *U* test was also used (tool number; 9) in 14 studies in order to retain the power. Three tools (tool numbers; 2, 6 and 11) are an improved version of non-parametric type Dunnett's test ('joint type') and Steel's test ('separate type'). Dunnett's or Scheffé's tests is independently used for 3 tools (tool numbers; 1, 13 and 14). Though use of Scheffé's test has the advantage of comparison of groups in various combinations, for example, control+mid dose *vs.*high dose, low dose+mid dose *vs.* high dose, etc., it has extremely low detection power. Hence, this test is not widely used in recent years.

Yoshimura (1987) used Bartlett's test to analyze the difference in distribution of variance among the groups, where number of animals in the group is more than 10. The power of Bartlett's test decreases when the number of animals in the group is less.

Dunnett's test is the expanded version of *t*-tests, hence, it becomes *t*-test when two groups are analyzed by Dunnett's test. Therefore, for the comparison of two groups either Dunnett test or *t*-test can be used.

The most important purpose of applying statistical analysis in toxicity studies is to know whether the items estimated in the experimental group has increased or decreased compared to the control. Therefore, a one-sided test is used. Detection rate of two-sided test is half of the one-sided test, hence it is important to mention in the study report whether a one-sided or two-sided test is used. It may

Table 1. Classification of number of studies based on the statistical tools used for the analysis of quantitative data.

Tool. No.	Description of statistical tools	Number of studies
1	Dunnett's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	5
2	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, Steel's test	7
3	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	9
4	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, Non-para type Dunnett's test, non-parametric type Scheffe's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	10
5	Bartlett's test, NOVA, Dunnett's test, Duncan's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test	9
6	Bartlett's test, Dunnett's test, Steel's test	20
7	Bartlett's test, Dunnett's test, non-parametric type Dunnett's test	10
8	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Scheffé's test	23
9	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, Mann-Whitney's *U* test	14
10	Bartlett's test, ANOVA (p=0.10), Dunnett's test, Kruskal-Walis's *H* test (p=0.10), Mann-Whitney's *U* test, When compared with control setting (p=0.05)	1
11	Bartlett's test, Dunnett's test, Steel's test	3
12	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test: Three groups or more; Student's *t*-test or Mann-Whitney's *U* test: Two groups	1
13	Dunnett's test: Three groups or more; *t*-test or Mann-Whitney's *U* test: Two groups	4
14	Dunnett's or Scheffé's tests: Three groups or more; *t*-test or Mann-Whitney's *U* test: Two groups	1
15	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test	3
16	Bartlett's test, ANOVA, Dunnett's test, Jaffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Jaffé's test	1
17	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Scheffé's test: Three groups or more; Student's *t*-test: Two groups	1
	Jonckheere's trend test (Not included in the number of tools)	8
	Total	122

Statistical tools used in short-term toxicity studies.

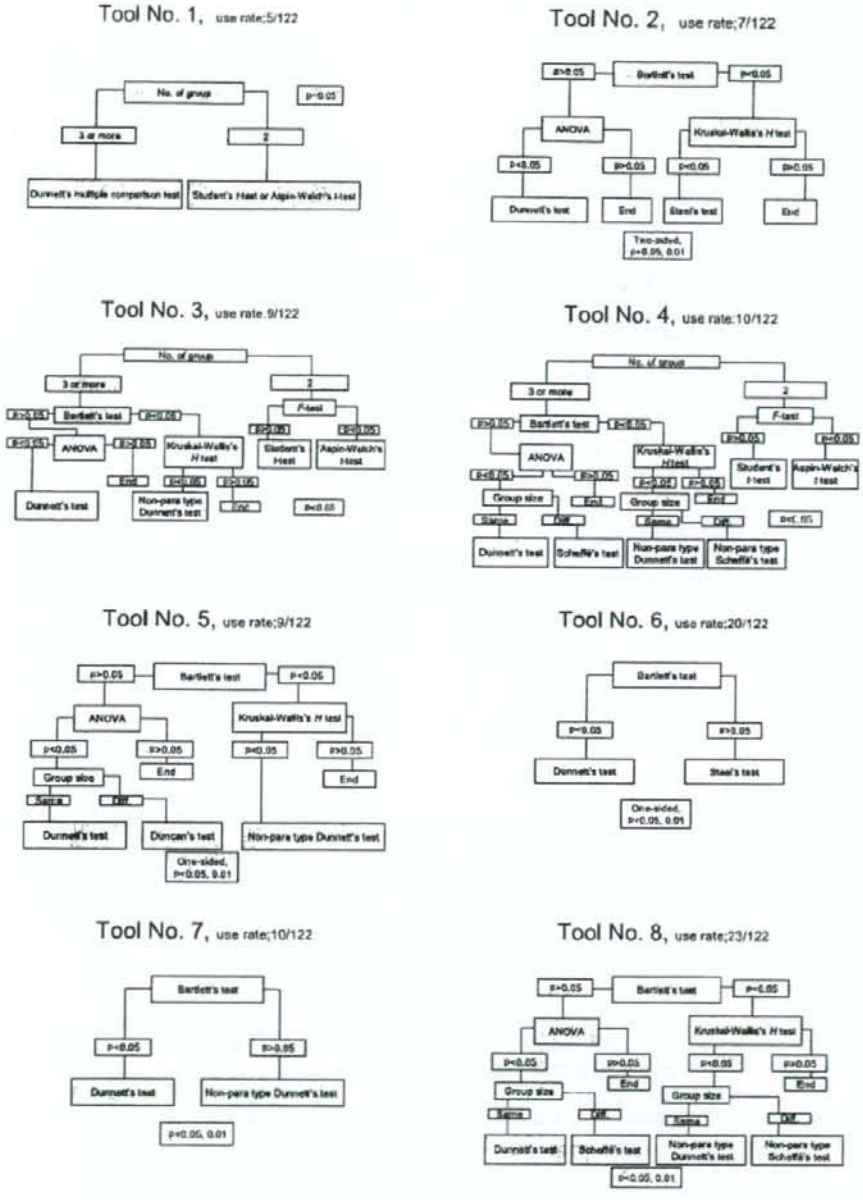

Fig. 1. Classification of number of studies based on the statistical tools used for the analysis of quantitative data.

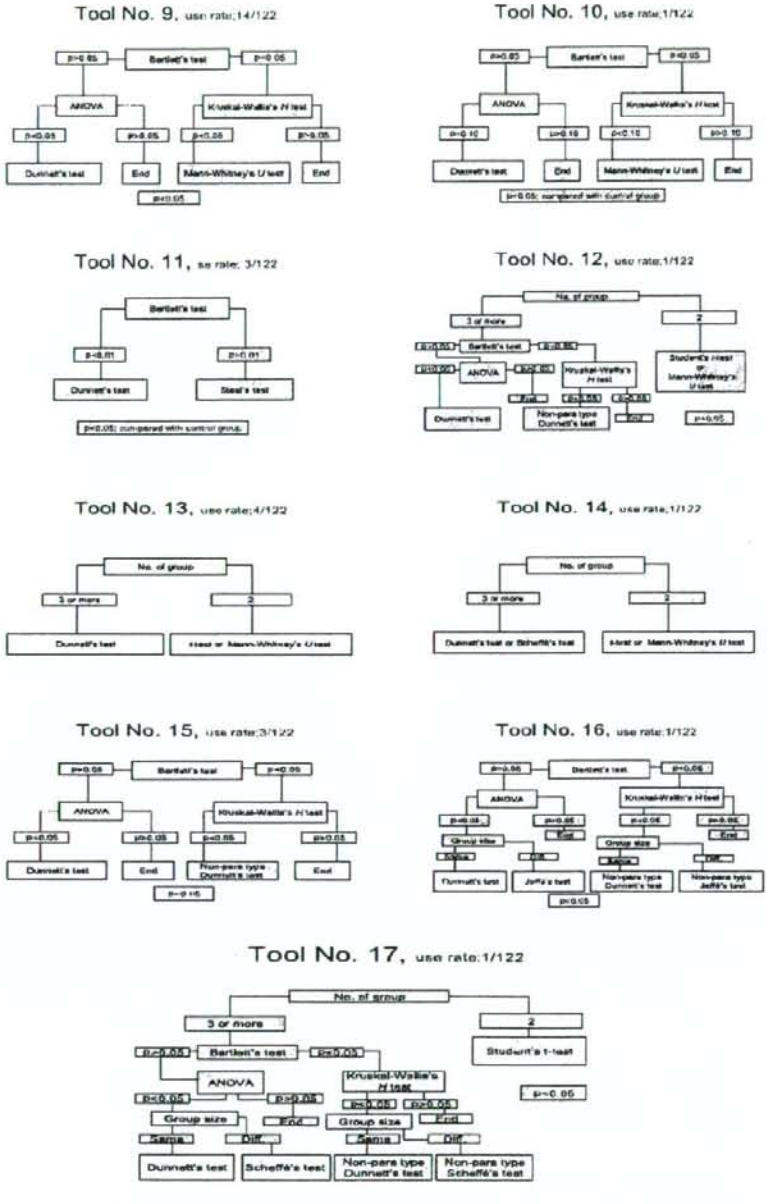

Fig. 1. Continued.

be noted that use of ANOVA causes the error of the second kind. Because of this, some of the recent studies skipped ANOVA in the decision tree and straight away used the statistical tools for *post hoc* comparison (Sumida *et al.*, 2006; Nagano *et al.*, 2006).

For the analysis of qualitative data, chi-square and Fisher's tests do not seem to be appropriate, though Fisher's test is slightly more sensitive than the chi-square test. These two tests do not detect a significant difference between a finding in the dosage group and control group, when all the animals (5/5) show the finding in the dosage group and 2 animals in the control group (2/5). On the other hand, Mann-Whitney's *U* test, which converts the scores into numerical values, detects a significant difference, when the finding in the dosage group is 5/5 and control group is 2/5. Therefore, Mann-Whitney's *U* test has better sensitivity to analyze qualitative data than the chi-square and Fisher's tests. Trend test like Jonckheere test can be used to determine no observed adverse effect level/ no observed effect level (NOAEL/NOAL) in the twenty-eight-day repeated dose oral toxicity tests. The statistical tools used, especially in the case of non-parametric tests, to determine the NOAEL/NOAL may be clearly elaborated in the study report.

We propose Dunnett's test for the analysis of quantitative data obtained from twenty-eight-day repeated dose oral toxicity tests in rodents and for qualitative data, Mann-Whitney's *U* test. For both tests, one-sided test with p=0.05 may be applied.

Table 2. A classification of number of studies based on the statistical tools used for the analysis of qualitative data.

Tool. No.	Description of statistical tools		Number of studies
	Scored data	Frequency data	
1	Mann-Whitney's *U* test (two-sided, p<0.05)	Fisher's test (one-sided, p<0.05)	6
2	Urinalysis	Pathological findings	7
	Cumulated Chi-square test (two-sided, p<0.05, p<0.01)	Mann-Whitney's *U* test (two-sided, p<0.05, p<0.01)	
3	Urinalysis	Pathological findings	13
	Cumulated Chi-square test (p<0.05)	Mann-Whitney's *U* test (two-sided, p<0.05)Fisher's test (one-sided test, p<0.05)	
4	Pathological findings		26
	Fisher's test (one-sided test, p<0.05)		
5	Pathological findings		19
	Chi-square test (p<0.05)		
6	FOB, urinalysis and differential leucocytes		15
	Kruskal-Wallis's *H* test, Mann-Whitney's *U* test (p<0.05)		
7	Urinalysis and pathological findings		9
	Mann-Whitney's *U* test (two-sided, p<0.05, p<0.01)		
8	Pathological findings		1
	Fisher's test		
9	FOB, sense function test and macroscopic and microscopic findings of pathology		1
	Wilcoxon rank-sum test, Fisher's test and Mann-Whitney's *U* test (p<0.05, p<0.01)		
10	Pathological findings		4
	Nonparametric type Dunnett's test or non-parametric type Scheffe's test, and Cochran-Armitage's trend test		
11	FOB, sense function test and macroscopic and microscopic findings of pathology		21
	No statistical tool mentioned		
Total			122

Table 3. Use of one-sided or two-sided test for short-term repeated dose administration toxicity studies with rats.

Data	One-sided	Two-sided	No mentioned	Total
Quantitative	22	13	87	122
Qualitative	34	22	70	126

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Relevance and follow-up of positive results in *in vitro* genetic toxicity assays: An ILSI-HESI initiative☆

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Abstract

In vitro genotoxicity assays are often used to screen and predict whether chemicals might represent mutagenic and carcinogenic risks for humans. Recent discussions have focused on the high rate of positive results in *in vitro* tests, especially in those assays performed in mammalian cells that are not confirmed *in vivo*. Currently, there is no general consensus in the scientific community on the interpretation of the significance of positive results from the *in vitro* genotoxicity assays. To address this issue, the Health and Environmental Sciences Institute (HESI), held an international workshop in June 2006 to discuss the relevance and follow-up of positive results in *in vitro* genetic toxicity assays. The goals of the meeting were to examine ways to advance the scientific basis for the interpretation of positive findings in *in vitro* assays, to facilitate the development of follow-up testing strategies and to

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