Table 8
Comparison of selection of top test concentration for chemicals with different molecular weight in 2 mM or 1 mg/mL, whichever is higher.

Molecular weight	Selection of 2 mM or 1 mg/mL (whichever is higher)		
100	2 mM (0.2 mg/mL)	<	1 mg/mL (10 mM)
300	2 mM (0.6 mg/mL)	<	1 mg/mL (3.3 mM)
500	2 mM (1 mg/mL)	=	1 mg/mL (2 mM)
800	2 mM (1.6 mg/mL)	>	1 mg/mL (1.3 mM)
1000	2 mM (2 mg/mL)	>	1 mg/mL (1 mM)

Underlines show concentration to be selected.

carcinogenicity information, so we determined the biologic relevancy of *in vitro* CA induction based on the weight of evidence approach. Results from *in vitro* CA test with CHL cells only might lead to biased conclusions. However, the strength of our study is the high reliability of the test results due to the fact that all data were generated according to national or international test guideline under GLP conditions. Therefore, our analysis would be helpful to discuss on top concentration issues. In this analysis, many "irrelevant" positives by extreme culture conditions (low pH, high toxicity, and precipitation) were also identified. Note that CHL cells are often described as among the most sensitive cells, *i.e.*, effects observed at lower concentrations as compared to the other cell lines. The recently suggested improvements in testing are important to reduce irrelevant positives, in addition to defining the top concentration. Data from *in vitro* mammalian genotoxicity tests, using the criteria defined by this paper, should be helpful in genotoxic hazard identification.

Conflict of interest

There are no conflicts of interest.

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REVIEW

Micronucleus assays in rodent tissues other than bone marrow

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This report updates previous reviews that were conducted as part of the third and fourth International Workshops on Genetic Toxicology Testing of micronucleus (MN) assays in rodent tissues other than bone marrow. Tissues discussed here are liver, lung, skin, colon, spleen, testes and foetal/neonatal tissues with transplacental exposure. Previous reviews have been updated to include literature published after 2000. In addition to the previously described tissues, MN assays in bladder, buccal mucosal cells, stomach and vagina are also included. MN assays using tissues other than bone marrow are critical for risk assessments, for *in situ* evaluation and for studies of systemic genotoxic effects and modes of action. Protocols for the majority of assays in tissues other than bone marrow have not yet been well standardised and validated for regulatory application, and further development is needed to support regulatory studies.

Introduction

Evaluation of genetic toxicity is an important component of the safety assessment of chemicals, including pharmaceuticals, agricultural chemicals, food additives and industrial chemicals. Up to the present time, genotoxicity has been regulated mainly on the basis of qualitative outcomes of hazard identification assays, i.e. decisions are often based on classification as positive or negative for genotoxic potential. Recently, the field is moving towards quantitative risk assessments and with more reliance on weight of evidence (WOE) approaches (1–3). *In vivo* assays are critical components of both WOE analysis and of quantitative risk assessments. The *in vivo* micronucleus (MN) assay using rodent haematopoietic cells from bone marrow or peripheral blood is widely used for the assessment of clastogenicity/aneugenicity of chemicals, both as a hazard screening assay and as a component of exposure-based risk assessments. The assay is usually incorporated into the standard genotoxicity test battery as a representative *in vivo* assay together with an *in vitro* gene mutation assay using bacteria and an *in vitro* chromosomal aberration assay using cultured mammalian cells (4). The MN assay was originally established using rodent erythrocytes as a simple method of assessing cytogenetic damage, taking advantage of two important characteristics of the haematopoietic system: (i) the ease of scoring MN in the newly formed anucleate reticulocytes derived from bone marrow and (ii) the ability to identify the newly formed erythrocytes that had completed their last division during or shortly after exposure to the test agent as those staining positive for RNA content (5,6). These important advantages initially led to an almost exclusive focus on the haematopoietic cells as the target. However, in principle, MN can be scored in any dividing cell population, and the assay was soon extended to tissues other than bone marrow.

One of the early extensions of the assay was to male germ cells, which are a key cell population for genetic studies and which undergo continuous active division (7,8). The assay was also extended to liver by stimulating regeneration and hence cell replication, by partial hepatectomy (9–12) or pretreatment with hepatotoxicants (13,14). Later, colon, skin and young rodent liver were studied extensively and shown to be suitable for MN analysis. Studies using human tissues or cell preparations include corneal cells and exfoliated cells from urinary bladder, oesophagus, cells of the nasal cavity and oral buccal mucosa (15,16). Rodent embryonic cells have also been used to evaluate effects of transplacental application of test chemicals. In addition, human lymphocytes have been used to assess cytogenetic damage in epidemiology studies of environmentally or occupationally exposed individuals, as summarised previously. The MN assay using peripheral lymphocytes or other human tissues is reviewed separately in this special issue.

In regulatory testing, the rodent erythrocyte MN assay is the most common first choice among *in vivo* assays used for subsequent testing when *in vitro* genotoxicity test(s) are found to be positive. The unscheduled DNA synthesis assay using rat liver has also been used in this situation, but its sensitivity has been questioned (17). The single-cell gel electrophoresis (comet) assay and gene mutation assays using transgenic animals are frequently considered for use as follow-up assays when *in vitro* gene mutation assay(s) are positive. It is important to evaluate cytogenetic damage in those cell populations or tissues that are the relevant sites of distribution and metabolism *in vivo*, and so bone marrow haematopoietic cells alone cannot satisfy this need.

Scientific considerations as well as the animal welfare movement, which emphasises the reduction, replacement and/or refinement of animal use (the '3Rs'), have contributed to the implementation of protocols that allow the integration of genotoxicity end points into general toxicological tests. Assays that have been shown to be appropriate for integration with repeat-dose general toxicology studies include the bone marrow and peripheral blood erythrocyte MN assays, the peripheral blood lymphocyte assay and a new MN assay method using rat liver after an extended period of repeated dosing (18). The liver is a key site of xenobiotic metabolism

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and accumulation *in vivo*, and therefore the repeat-dose method, which appears to be applicable in adult rats, promises to be an important assay that could be incorporated into general toxicological studies.

MN assays using tissues other than bone marrow have previously been reviewed in conjunction with two International Workshops on Genotoxicity Testing (IWGT) (19,20) and have been discussed by Kirkland *et al.* (17,21). Here, we update the previous publications of the IWGT groups and revise the previous table of assay results to include studies published after 2000.

Literature search

The literature search was conducted by using PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and search terms that included 'micronucleus', 'rat', 'mouse' and specific tissue names with limitations of year and English language. The time limit was from 2000 to present, except for colon, liver and skin, in which cases the limit was set to 2005 or later because these tissues were evaluated in the third and fourth IWGT reviews (19,20). All abstracts identified in the searches were reviewed and then suitable articles were selected for further review and inclusion. Certain literature, for example publications of *in vitro* or *in vivo* studies of irradiation (e.g. X-ray, magnetic field), some MN studies on mouse models of human genetic disease or dietary deficiency and human studies were excluded. The tissues identified in which the rodent MN assays had been reported were alveolar, bladder, buccal mucosa, colon, liver, skin, spleen, stomach, vagina and foetal or neonatal tissues. No additional literature related to sperm or spermatid MN assays after 2000 was found. The identified assay results published within the above time periods are listed in Table I.

MN assays using tissues other than bone marrow

Liver

Liver is often a site of carcinogenic response, but liver cells replicate relatively slowly in adult animals. Therefore, in the early development stage of the liver MN assay, cell division was stimulated by partial hepatectomy (9–12) or treatment with hepatotoxicants (13,14). Early protocols were improved by subsequent investigators (25,59) who recognised that in rodents there is active cell division in liver soon after birth, i.e. up to 5–6 weeks in rat. Based on this characteristic, young rats (22,23,26,27,29) or mice (28) can be used for MN frequency determination without any additional physical or chemical treatment.

As noted above, recently a new protocol using adult rat liver in combination with repeated treatments is now under development (Narumi *et al.*, submitted for publication). This method should be easily integrated with general toxicological studies. As an example, adult rats that received daily *N*-nitrosodiethylamine (DEN) or 2,4-diaminotoluene (2,4-DAT) for 5, 14 and 28 days orally without any additional treatment had elevated frequencies of micronucleated hepatocytes after repeated treatments with DEN for $\geq$5 days or with 2,4-DAT for $\geq$14 days. The observed frequencies increased with the number of treatments, as expected. To verify the reliability of this method, cell proliferation in liver has been investigated with bromodeoxyuridine intake. Atrazine also induced MN in rat liver treated for 21 days (24). This method promises to allow monitoring of genetic damage in this important tissue during routine repeated-dose toxicology assays.

Colon and intestinal epithelium

The colon and digestive tract are important target sites of carcinogens (e.g. hydrazines). In addition, colon is an important tissue for metabolism by the intestinal microflora and excretion by feces (30). Therefore, the MN assay in colon and/or intestine is appropriate for hazard identification and risk evaluation of environmental chemicals that enter the human body through the food chain, such as pesticides and food ingredients, as well as for orally administrated pharmaceuticals (33). The colon MN assay with the mouse was developed by Goldberg *et al.* (60), and it has been improved by Vanhauwaert *et al.* (61) and Ohyama *et al.* (62). The colon MN assay has been used for the confirmation of the presence or absence of genotoxic activity in colon (30–33). Because cells in the colon originate from stem cells at the base of the crypts that are vascularised, it is also possible that MN in colon cells may be induced *via* blood-borne genotoxicants.

Skin

Skin is the major site of exposure to many chemicals, including industrial chemicals, pesticides, consumer products, cosmetics and pharmaceuticals. Skin is also an important tissue for protection from environmental agents, including light and chemicals. Although the use of *in vitro* photo-genotoxicity assays is diminishing because of oversensitivity and low specificity, the skin MN assay provides a more relevant method of assessing the risk of *in vivo* photo-genotoxicity associated with exposure to chemical and physical agents (35,36). The skin MN assay in the mouse was introduced around 1990 (63,64), and it was then developed in the rat and hairless mouse by Nishikawa *et al.* (34,65,66). In this assay, acridine orange fluorescent staining is playing an important role not only to identify MN but also to identify the appropriate cell population to be analyzed (34,35). Although many studies have been conducted using skin application with or without ultraviolet irradiation, detection of systemic effects in skin following oral administration is also important (67).

Spleen

The spleen MN assay with mouse splenocytes has been developed by Shindo *et al.* (68), using splenocytes as the target cell population. Evaluation of genotoxic effects in splenocytes during subchronic exposures provides another relevant target tissue in addition to haematopoietic cells in bone marrow or peripheral blood (37–40). *In vitro* and/or *in vivo* methods have been used in many studies (38,40–42).

Lung

Lung is one of the major target sites of those carcinogens for which inhalation is the major route of exposure. Therefore, lung cells are an important target site for detection of local genotoxic effects of environmental or industrial chemicals following or during inhalation exposures (43,45–47). Intratracheal instillation is also often used as a means of delivery to the lung in animal experiments (43). Studies of long-term exposure (several weeks or months) by inhalation are also possible (44,47). A lung MN assay was developed as an *in vivo*/*in vitro* assay in early 1990s (69,70), and in more recent

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Table I. MN assays in multiple organs in rodents published after the search in 4th IWGT[a]

Specific tissue/organ, chemical	Species tested	Administration		Sampling time after the final treatment	MN in the specific organ	
		Route	Times per duration		Result	Refs.
Liver						
2-Acetylaminofluorene	Rat[c]	po	Twice	3, 4, 5 days	+	(22)
Allyl alchohol	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Atrazine	Rat	po	7, 14, 21 days (once daily)	Not described	+	(24)
Bromobenzene	Rat[c]	po	Once	3, 4, 5 days	E[d]	(23)
Carbendazim	Mouse[e]	po	Once	4 days	+	(25)
4-Chloro-*o*-phenylenediamine	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
Chlorpromazine hydrochloride	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Clofibrate	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Colchicine	Mouse[e]	iv	Once	4 days	+	(25)
Cyclophsphamide	Rat[c]	po	Once	3, 4, 5 days	+	(22)
Cytochalasin B	Mouse[e]	iv	Once	4 days	+	(25)
2,4-Diaminotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
Diazepam	Mouse[e]	iv	Once	4 days	+	(25)
Di(2-ethylhexyl)phthalate	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
Diethylnitrosamine	Rat[c]	po	Once, twice	5 days	+	(22,23,26,27)
p-Dimethylaminoazobenzene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26,27)
1,2-Dimethylhydrazine dihydrochloride	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
Dimethylnitrosamine	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26)
2,4-Dinitrotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
2,6-Dinitrotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(23,27)
1,4-Dioxane	Mouse[c]	po	5 days (once daily)	24 h	+	(28)
Ethyl methanesulfonate	Rat[c]	ip, po	Twice	3, 4, 5 days	−	(22)
5-Fluorouracil	Rat[c]	ip	Once	3, 4, 5 days	−	(22)
Isoniazid	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Kojic acid	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
4,4'-Methylenedianiline	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
Methyl methanesulfonate	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
Mitomycin C	Rat[c]	ip	Once, twice	3, 4, 5 days	+	(22,27)
Alpha-naphthyl isothiocyanate	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Noscapin	Mouse[e]	iv	Once	4 days	+	(25)
Paclitaxel	Mouse[e]	iv	Once	4 days	+	(25)
Phenacetin	Rat[c]	po	Once	3, 4, 5 days	−	(23)
Quinoline	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26)
Thioacetamide	Rat[c]	po	Once	3, 4, 5 days	−	(23)
o-Toluidine	Rat[c]	ip or po	Once	3, 4, 5 days	−	(26)
Trichlorfon	Mouse[e]	iv	Once	4 days	+	(25)
Vinblastine sulfate	Rat[f]	ip	Once	24 h	+[g]	(29)
Vinblastine sulfate	Mouse[c]	ip	5 days (once daily)	24 h	+	(28)
Vitamin E	Rat	po	7, 14, 21 days (once daily)	Not described	−	(24)
Colon and intestinal epithelium						
Amaranth	Mouse	po	Twice	24 h	−	(30)
Colchicine	Mouse	po	Once	24, 36, 48 h	+	(31)
Cyclohexane chlorination products	Mouse	po	4 days (once daily)	24 h	+	(32)
Lambda-cyhalothrin	Rat	po	13 days (once per 48 h)	30 h	+	(33)
Dimethylaminoazobenzene	Mouse	po	Twice	24 h	−	(30)
1,2-Dimethylhydrazine	Mouse	po	Once	24 h	+	(30,31)
Mitomycin C	Rat	ip	Once	30 h	+	(33)
Okadaic acid	Mouse	po	Once	24, 36, 48 h	Inc[h]	(31)
Sunset yellow	Mouse	po	Twice	24 h	−	(30)
Tartrazine	Mouse	po	Twice	24 h	−	(30)
Skin						
Anthracene	Mouse	sa	3 days (daily)	24 h	−	(34)
Benz[*a*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Benzo[*a*]pyrene	Mouse	sa	Once	72, 96 h	+[i]	(35)
Benzo[*a*]pyrene	Mouse	sa	3 days (daily)	24 h	+	(34)
Benzo[*e*]pyrene	Mouse	sa	3 days (daily)	24 h	−	(34)
Chrysene	Mouse	sa	3 days (daily)	24 h	−	(34)
Dibenz[*a*,*c*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Dibenz[*a*,*h*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
7,12-Dimethylbenz[*a*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Kojic acid	Mouse	sa	Twice	48 h	−[i]	(36)
8-Methoxypsoralen	Mouse	sa	Twice, once	48 h, 24–168 h	+[i]	(35,36)
3-Methylcholanthrene	Mouse	sa	3 days (daily)	24 h	+	(34)
Mitomycin C	Mouse	sa	3 days (daily)	24 h	+	(34)
Pyrene	Mouse	sa	3 days (daily)	24 h	−	(34)

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Table I. *Continued*

Specific tissue/organ, chemical	Species tested	Administration		Sampling time after the final treatment	MN in the specific organ	
		Route	Times per duration		Result	Refs.
Spleen						
Aflatoxin B1	Rat	po (diet)	90 days	End of feeding	+	(37)
Benzo[*a*]pyrene	Mouse	po	4 weeks (twice weekly)	End of exposure	+[j]	(38)
Cyclophosphamide	Mouse	ip	Once	24 h	+	(39)
Ethylene oxide	Rat	inh	4 weeks (6 h/day, 5 days/week)	5 days	–[j]	(40)
Ethylene oxide	Rat	ip	Once	24 h	+	(40)
Fumonisin B1	Rat	po (diet)	90 days	Not described	+	(37)
Furan	Mouse	po	4 weeks (5 days/week)	24 h	+[j]	(41)
Furan	Mouse	po	Once	3 h	–[j]	(41)
Hydroquinone	Mouse	ip	6 days (daily)	24 h	+[j]	(42)
Methyl methanesulfonate	Mouse	ip	Once	3, 24 h	+	(41)
N-Methylmorphine chloride	Mouse	ip	Once	24 h	+	(39)
Metyrapone	Mouse	ip	Once	24 h	–	(39)
Morphine sulfate	Mouse	ip	Once	24 h	+	(39)
Lung						
Bleomycin	Rat	it	Once	72 h	+	(43)
Dioxazid	Rat	inh	3 months	Not described	–	(44)
Environmental cigarette smoke	Mouse	inh	15 days (6 h/day)	End of exposure	+	(45)
Ethylene oxide	Mouse	inh	4 h	72 h	+	(46)
Formaldehyde	Rat	inh	4 weeks (6 h/day, 5 days/week)	End of exposure	–	(47)
WC-Co dust[b]	Rat	it	Once	72 h	+	(43)
Stomach						
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	Mouse	po	Once	3, 4 days	+	(48)
N-nitroso-*N*-methylurea	Mouse	po	Once	3, 4 days	+	(48)
Bladder						
N-acetylcysteine	Mouse	ip	Once	10 days	–	(49)
Cyclohexane chlorination products	Mouse	po	4 days (daily)	24 h	+	(32)
Cyclophosphamide	Mouse	ip	Once	10 days	+	(49)
Dioxazid	Rat	inh	3 months	Not described	–	(44)
o-Phenylphenol	Rat	po (diet)	14–15 days	End of feeding	+	(50,51)
Sodium chloride	Rat	po (diet)	14 days	End of feeding	+	(51)
Sodium *o*-phenylphenol	Rat	po (diet)	15 days	End of feeding	+	(50)
Vitamin C	Mouse	ip	Once	10 days	–	(49)
Buccal mucosa						
Cadmium chloride	Mouse	po	6 weeks	Not described	+	(52)
Royal jelly	Mouse	po	6 weeks	Not described	–	(52)
Vagina						
Colchicine	Rat	ip	Once	Until three cycles of oestrus	+	(53)
Cyclophosphamide	Rat	ip	Once	Until three cycles of oestrus	+	(53)
5-Fluorouracil	Rat	va	5 days (daily)	Until three cycles of oestrus	+	(54)
20-Methylcholanthrene	Mouse	va	30 days (daily)	Not described	+	(55,56)
Metronidazole	Rat	va	5 days (daily)	Until three cycles of oestrus	+	(54)
Quercetin	Mouse	po (diet)	30 days	Not described	–	(56)
Vitamin E (alpha-tocopherol)	Mouse	po (water)	30 days	Not described	–	(56)
Foetal/neonatal tissues						
Fluconazole	Mouse[k]	tp by ip	3 days (PGDs 12–14, daily)	6–18 h after delivery	+	(57)
Cyclophosphamide	Mouse[k]	tp by ip	3 days (PGDs 12–14, daily)	6–18 h after delivery	–	(57)
Sodium dichromate dihydrate	Mouse[l]	tp by po (water)	18 days (PGDs 1–18)	End of feeding	–	(58)
Sodium dichromate dihydrate	Mouse[l]	tp by ip	Once (PGD 17)	1 day	+	(58)
Potassium dichromate	Mouse[l]	tp by po (water)	18 days (PGDs 1–18)	End of feeding	–	(58)
Potassium dichromate	Mouse[l]	tp by ip	Once (PGD 17)	1 day	+	(58)

inh, inhalation; ip, intraperitoneal injection; it, intratracheal instillation; po, per os; va, vaginal application; sa, skin application; tp, transplacental; PGD, pregnancy day; Inc, inconclusive; E, equivocal.
[a]For colon and intestinal epithelium, liver and skin, published data from 2005 to present. For lung, bladder, buccal mucosa, spleen, stomach, vagina and foetal/neonatal tissues, published data from 2000 to present.
[b]Consisted of 6.3% cobalt, 84% tangsten and 5.4% carbon.
[c]4-Week-old.
[d]Positive at toxic dose.
[e]Partial hepatectomy.
[f]5-Day-old.
[g]Erythrocytes from liver.
[h]Both positive and negative results.
[i]With light irradiation.
[j]*In vivo*/*in vitro*.
[k]Peripheral blood from neonatal 6–18 h after delivery.
[l]Peripheral blood and liver from foetus on PGD 18.

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studies, several types of lung cells have been used, including bronchoalveolar lavage cells, alveolar type II cells, Clara cells or pulmonary alveolar macrophages (46,47).

Stomach

Stomach is important because it is the first site of direct contact of many chemicals. Several *in vivo* genotoxicity assays using stomach have been developed, including the unscheduled DNA synthesis assay, comet assay and transgenic mutation assay. Recently, a stomach MN assay has been established by Okada *et al.* (48). Though data obtained are at present limited to two known stomach carcinogens (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and *N*-nitroso-*N*-methylurea), the assay appears promising for further development.

Bladder

Urinary bladder is a tumour target site of certain chemical classes such as naphthylamines, benzidines or aminobiphenyls. Because it is the tissue that collects urine excreted by kidneys, it will be exposed to water-soluble chemicals excreted *via* the urine. A bladder MN assay has been developed as a tool for studies of cancer risk, including intervention studies, in humans (71). Exfoliated or epithelial cells from urinary bladder have also been used in animal studies in which acute or subchronic exposures by several routes of administration were employed (32,44,49–51). The bladder MN assay is a good biomonitoring tool for humans exposed to carcinogenic or genotoxic chemicals, and analogous studies with animals can be used for identification and/or confirmation of genotoxic action in the bladder.

Buccal mucosa

Buccal mucosal cells have been used for biomonitoring of DNA damage potentially leading to oral cancer in humans (15,72). Although many studies have been conducted in humans, animal data are scant (52) or limited to animal models of human disease such as Alzheimer's disease (73). This may indicate that analysis of MN in buccal mucosal cells in rodents may not be suitable at present for detection of genotoxic effects and that technical development will be needed for general use of the detection of local genotoxic effects at this site in animals.

Vagina

Carcinoma of the uterine cervix is important in humans and has been shown to be inducible by chemical exposure in the mouse (55). A vaginal MN assay could be a useful tool for screening new chemopreventive agents in the rodent. Chakrabarti and Dutta (74) described a vaginal MN assay that could detect known MN inducers, but there is not yet sufficient data to evaluate the utility of this model (53–56).

Foetal/neonatal tissues

Assays using MN induction to evaluate transplacental genotoxicity were developed around 1980 (75–77) and many studies were published before 2000. Those studies have been summarised in previous IWGT reports (19,20). In the late 1980s, MacGregor *et al.* (78,79) showed that additional kinetic information could be obtained in the foetal blood MN assay by categorising newly formed red blood cells into age groups based on RNA aggregation state. Additional data from studies using peripheral blood from neonates or peripheral blood and liver from foetuses, found in our search of the literature published after 2005 are summarised in Table I (57,58). Foetal/neonatal MN assays using transplacental exposure will be useful for evaluation of agents associated with embryotoxicity, congenital malformations, tumours, heritable mutations in germ cells or other adverse developmental effects.

Spermatogonia/spermatids

A spermatogonial MN assay was proposed in the early 1980s (7,8), and early results of this assay were summarised in the previous IWGT reports (19,20). No new data were identified in our PubMed search. Initially, it was difficult to identify the specific developmental stage of spermatogonial cells and to restrict the cell population to be analysed. A fluorescent staining method using acridine orange and 4′,6-diamino-2-phenylindole concomitantly has now been introduced and it facilitates identification of the spermatogonial cells to be analysed (80). This staining method has also been applied to other tissues successfully because the cytoplasm and nucleus are clearly stained and provide increased accuracy of scoring micronucleated cells.

The evaluation of MN in reproductive tissue is obviously important in the evaluation of adverse effects to subsequent generations. The United Nations (UN) 'Globally Harmonised System of Classification and Labelling of Chemicals (GHS)' requests hazard classification of chemicals based on germ cell mutagenicity (81,82) and identifies the spermatid MN assay as an example of a mutagenicity test applicable to germ cells.

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Discussion

Many tissues other than bone marrow or peripheral blood have been used for evaluation of MN frequencies *in vivo* in rodents. In our literature search, liver, colon epithelium, skin, spleen, lung, stomach, bladder, buccal mucosa, vagina and foetal/neonatal tissues were identified as target tissues used for such assays. No new data on MN incidence in spermatogonia or spermatids were identified after the previous IWGT reviews (19,20). These MN assays have been used to detect local or systemic genotoxic effect in important tissues, including germ cells and progeny.

These assays are important tools for the prediction and evaluation of chemical carcinogenicity and also for the identification and study of agents with the potential to induce transmissible germ line mutations. Currently, there is emphasis on extending hazard identification techniques to evaluate genotoxic risk more quantitatively (1,2,83). One important objective of genotoxicity evaluation is the assessment of the mechanism of carcinogenicity, and whether genotoxic mechanisms are involved or not. Another is to assess adverse heritable effects of chemicals that may appear in subsequent generations. For the former purpose, assays are needed that permit assessment in the appropriate target tissues and allow development of mode of action (MOA) and a WOE of information in conjunction with information about exposure and metabolic transformation (84). The tissues with most active cell division are bone marrow, gastrointestinal tract, skin and reproductive tissues in mammals. These tissues have been used as experimental materials for the analysis of chromosomal damage, including evaluation of induction of chromosomal aberrations, and the ability to monitor MN frequencies in these same tissues is therefore important.

Given the recent emphasis on genotoxic risk characterisation, MOA determination and WOE approaches (84), the ability to study a wide range of tissues will become increasingly important. However, validated standard protocols

(e.g. Organization for Economic Co-operation and Development test guidelines) have not yet been established for most of these tissues, and methods remain primarily research oriented. Even liver, colon, skin and spermatogonia/spermatid MN assays, which have been used relatively frequently for research purposes, are still infrequently used for regulatory purposes. The target cell populations, cell preparation, suitable sampling time(s), cytotoxicity evaluation (dose selection) and/or historical background data are less well established in other tissues, such as lung, bladder, buccal mucosa, nasal epithelium and vagina. Investigation of cell division is a critical issue when studying MN in any tissue. Suitable sampling time(s) after the final treatment varies in different tissues or study designs including the parameters of treatment duration or route of exposure. Further development, especially to establish standard protocols and validation of these MN assays, is highly desirable and will facilitate their increasing application to support more meaningful and relevant assessments of clastogenic/aneugenic risks associated with chemical and physical exposures (1,2,17,21,83).

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Original Article

Lack of chronic toxicity and carcinogenicity of dietary administrated catechin mixture in Wistar Hannover GALAS rats

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ABSTRACT — Chronic toxicity and carcinogenicity of catechin mixture were examined in Wistar Hannover GALAS rats. Administration was in the diet at concentrations of 0, 0.02, 0.3, 1 or 3%. Slight increases in relative liver weight and centrilobular hypertrophy of hepatocytes associated with induction of CYP3A2 were found at the 3% in males of both studies. However, because there were no signs indicative of hepatotoxicity on serum biochemical and histopathological examinations, the changes observed in the liver were regarded as adaptation, and not adverse effects. The slight depressions of body weights at the 3% in females of the chronic toxicity study and in both sexes of the carcinogenicity study were observed. These decreases were because the diet at the highest concentration was frangible and nominal food consumption may not have reflected the actual food consumption resulting in decrease in caloric intake, rather than toxic effects. Thus it was concluded that catechin mixture had no toxicity. In addition, tumor incidences and types were comparable between treated and control groups. Based on the results, the no observed adverse effect levels estimated from the chronic toxicity study were 3% in both sexes equal to 1922.9 in males and 2525.7 mg/kg/day in females. Catechin mixture has no carcinogenic potential in male and female rats.

Key words: Catechin mixutre, Chronic toxicity, Carcinogenicity, Rat

INTRODUCTION

Green tea is consumed as a popular beverage in Japan and throughout the world. During the past decade, epidemiological studies have shown the tea catechin intake is associated with lower risk of cardiovascular disease (Hertog *et al.*, 1993). *In vitro* biochemical studies prevent oxidation of low-density lipoprotein (LDL), which is recognized as an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (de Whally *et al.*, 1990; Steinberg *et al.*, 1989). In addition, catechin has been known to have protective effects against some common types of cancer (Adhami *et al.*, 2003; Doss *et al.*, 2005; Stuart *et al.*, 2006; Kumar *et al.*, 2007). Epidemiological studies have indicated that Asian populations that consume tea on a regular basis have lowest incidence of many types of cancer including prostate cancer, than their western counterparts (Jian *et al.*, 2004; Sim and Cheng, 2005; Siddiqui *et al.*, 2006). The main catechin mixutre found in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3 gallate (ECG), and EGCG. Among these, EGCG is the most abundant and has attracted the most attention with respect of anticarcinogenic activity (Doss *et al.*, 2005). There is vast amount of scientific literature which suggests that EGCG is responsible for the majority of the potential health benefits attributed to green tea consumption (Khan *et al.*, 2008). Regarding chemopreventive mechanisms, inhibition of activation of specific receptor tyrosine kinases (RTKs) (Shimizu *et al.*, 2008) and related signaling pathways (Verschoyle *et al.*, 2007) or apoptosis induction (Khan *et al.*, 2006, 2010; Yang *et al.*, 2002) have been focuses of attention.

As agents considered to be beneficial and may be expected to be ingested over prolonged period, chronic exposure information is needed. However, the safety information available for catechin is limited. In Ames tests, catechins showed positive effects with or with-

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out cytotoxic levels (Takabe, 1997, Unpublished data). On the other hand, other genotoxicity studies concluded that catechin had no potential of genotoxicities to organisms in Ames, micronucleus, and chromosomal abbreviation tests (Sofuni, 1997 Unpublished data; Kurita, 1997 Unpublished data). In a subchronic study, data suggesting slight effects of catechin mixture on the liver were obtained without any morphological changes at dietary concentration of 5% (Takami *et al.*, 2008). However, information of chronic toxicity or carcinogenicity in rodents is lacking. Although many experimental and epidemiological data supports beneficial effects on human health described above, the controversial effects for genotoxicity are concern. Therefore, the present study was conducted to clarify the chronic toxicity and/or carcinogenicity of catechin mixture with oral administration of Wistar Hannover GALAS rats for 12 and 24 months. In addition, components of catechin mixture in the prepared diet were analyzed.

MATERIALS AND METHODS

Animals

Male and female specific-pathogen-free Wistar Hannover GALAS rat (BrlHan:WIST@Jcl Clea, Tokyo, Japan) at 5 weeks of age were used after 1-week acclimatization period. This strain rat has been recently distributed for international standardization of preclinical and toxicological research. The animals had free access to basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water during the acclimatization period. They were housed three per plastic cage with sterilized soft paper chips as bedding in a barrier-maintained animal room at 24 ± 1°C and 55 ± 5% and showing no abnormalities were used at 6 weeks of age. The protocol of the experiment was reviewed and approved by the Ethical Committee of Sasaki Institute (Tokyo, Japan).

Chemicals, dose selection, preparation of diet, and analysis of chemicals.

Catechin was supplied by TAIYO Kagaku Co. Ltd. (Mie, Japan) as catechin mixture produced by 3 companies (TAIYO KAGAKU, CO., LTD., MITSUI NORIN, CO.,LTD. Tokyo, Japan and ITOEN, LTD., Tokyo, Japan). The concentration of each component in the catechin mixture was analyzed at the beginning of study (Table 1). Treated doses in the present study were based on the results of 90-day dietary subchronic study in rats (Takami *et al.*, 2008). In this study, the over 10% depression of body weights in males and effect on the liver were observed in both sexes at 5%, the highest dose. Although no related histopathological abnormalities were detected, the study indicated that the concentration of 5% might be greater than the maximum tolerance dose (MTD) in males. Therefore, we chose dietary concentrations of 0, 0.02, 0.3, 1 or 3% in the present study. The lowest dose, 0.02% was considered to be an equivalent to the exposure levels of people consuming in a bottle of green

Table 1. The concentration of components in catechin mixture, and total amounts of catechin mixture, caffeine and polyhenol in the prepared diet

Components of catechin (%)			Catechin mixture (%)	Caffeine (%)	Total polyphenol (%)
The concentration of components in catechin mixture					
GC		1.058	76.444	0.199	89.0
EGC		11.738			
C		1.793			
EGCG		43.604			
EC		2.933			
GCG		1.692			
ECG		13.424			
CG		0.202			
Total amounts of catechin mixture, caffeine and polyhenol in the prepared diet					
Dose group	Catechin 0%		0.1762 ± 0.0944	0.00011 ± 0.0010	0.4884 ± 0.0845
	Catechin 0.02%		0.1900 ± 0.0908	0.0010 ± 0.0004	0.5006 ± 0.0485
	Catechin 0.3%		0.3143 ± 0.0975	0.0010 ± 0.0007	0.6528 ± 0.0820
	Catechin 1%		0.7448 ± 0.1187	0.0023 ± 0.0003	0.8847 ± 0.1114
	Catechin 3%		1.8960 ± 0.3016	0.0055 ± 0.0022	1.5110 ± 0.5879

GC, gallocatechin; C, catechin; EGCG, (-)-epigallocatechin-3-gallate; EC, (-)-epicatechin; GCG, gallocatechin; EGC, (-)-epigallocatechin; ECG, (-)-epicatechin-3 gallate; CG, catechin gallate.

tea in Japan.

Powder basal diet (CRF-1, Oriental Yeast) mixed with catechin mixture at designated concentrations was pelleted and was given rats *ad libitum*. Analysis of dietary concentration of each catechin component in prepared diet was performed using each lot. The control animals received basal diet (CRF-1) pelleted in the same way. Stability of dietary catechin mixture and each component up to 2 months after preparation was confirmed. The analyzed concentrations and stabilities of test materials in the diets were found to be generally acceptable (data not shown). The average concentrations of catechin mixture, caffeine and total polyphenols in the control and all treated groups were analyzed at diet preparation (Table 1).

Combined chronic/carcinogenicity study

The present study was designed as a combined one-year toxicity/carcinogenicity study in accordance with guidelines for designation of food additives and for revision of standards for use of food additives, Ministry of Health Labour and Welfare, Japan (MHLW, 1996). Details of the study protocol were as follows:

Chronic toxicity study

Rats were randomly allocated to 5 groups, each consisting of 10 males and 10 females, and given the pellet diet described above for 12 months. Throughout the study, rats had free access to tap water.

The animals were observed daily for clinical signs and mortality. Body weights and food consumption were recorded every week until week 12 and every 4 weeks thereafter. After 12 months treatment, urinalysis was conducted for all animals using test paper (Pretest, Wako Pure Chemical, Ltd., Osaka, Japan), and all rats were euthanized under deep anesthesia after on overnight fast. Blood samples were collected from the abdominal aorta for hematology and serum biochemistry. Hematological examination was performed using automatic hematology analyzer, M-2000 (Toa-Medical Electronics, Hyogo, Japan), and parameters for serum biochemistry were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation (3,000 rpm for 10 min.) After complete macroscopic examination, the brain, pituitary, heart, spleen, liver, adrenals, kidneys, testes or ovaries and prostate or uterus were weighed. In addition, the nasal cavity, eyes, Harderian glands, spinal cords (cervical, thoracic, and lumbar portions together with corresponding vertebral bones), salivary glands, stomach, small and large intestines, pancreas, urinary bladder, skin, mammary gland, mesenteric lymph nodes, trachea, lungs, esophagus, thyroid glands, tongue, thigh muscle, sciatic nerve, epididymis, seminal vesicles, prostate, uterus, ovaries, and vagina were also removed. All organs/ tissues were fixed in 10% neutral buffered formalin except for testes fixed in Bouin's solution overnight. Bone such as the nasal cavity, spinal column with spinal cords, sternum, and femur were decalcificated. After routine processing for paraffin embedding, sectioning and staining with hematoxylin and eosin, histological assessment was performed for all tissues of all animals.

To clarify immunohistochemical distribution of cytochrome P450s in the hypertrophied hapatocytes, serial liver sections in the 0 and 3% group of both sexes were incubated with anti-rat CYP1A1, 1A2 and 3A2 antibodies (Daiichi Chemicals, Tokyo, Japan), diluted 1:100 in Tris-buffered solution (Takara Bio, Shiga, Japan) with 1% skim milk at 37°C for 1 hr. After the incubation, the sections were exposed to secondary antibodies and linked with streptavidin peroxidase using a DAKO LSAB+ kit (DAKO cytomation, California, CA, USA). The binding was visualized by 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals).

Carcinogenicity study

Rats were randomly allocated to 5 groups, the same doses as the chronic study, each consisting of 50 males and 50 females. The animals were housed three or four per plastic cage, and were given the prepared pellets at each dose for 24 months. Clinical observation and measurements of body weights and food consumption were conducted in the same manner as for the chronic study. After 24 month treatment, all surviving animals were euthanized under deep anesthesia, and the liver were resected and weighed. All organs and/or tissues listed above, as well as macroscopic masses and any abnormalities were examined macroscopically and microscopically as in the chronic study. Moribund or dead animals were also completely necropsied and pathologically examined to determine their cause of death.

Statistical analysis

In multiple comparisons continuous data were analyzed with Bartlett test. If the variance was homogeneous, the data were assessed by the Dunnett's multiple comparison test. If not, the Steel's multiple comparison test was employed. The histopathology data were employed with Fisher's exact or chi-square test. The level of significance was set at $p < 0.05$ and 0.01.

RESULTS

General condition and mortality

No treatment-related clinical signs or deaths were found throughout the feeding period in either chronic or carcinogenicity studies (Fig. 1). The mortalities at termination of the carcinogenicity study were 36, 38, 32, 26 and 22% in males, and 28, 38, 28, 30 and 30% in females of the 0, 0.02, 0.3, 1.0 or 3% group, respectively.

Body weights in the chronic and carcinogenicity studies

In the chronic toxicity study, changes in body weights were comparable among the treated and control animals in males (Fig. 2). In females, suppression or a tendency for suppression of body weight gain by approximately 15% was observed several times at 3% only after Week 25 (Fig. 2). In the carcinogenicity study, suppressions of body weights within 10% was detected in both of males and females receiving the 3% dose in the latter half of the study (Fig. 3). No changes in body weights were observed in other treated groups.

Food consumption and chemical intakes in the chronic and carcinogenicity studies

The concentrations of catechin mixture in the prepared diet showed a dose dependent increase in the 0.3% group and higher (Table 1). Their concentrations were comparable between the control and the lowest dose groups. Data for food consumption are summarized in Figs. 4 (chronic study) and 5 (carcinogenicity study) and intakes of catechin mixture is Table 2. In both studies, increases or tendencies for increased food consumption were observed in males and females at 3%, especially at beginning of the studies. Since the pellets in the 3% group were more frangible than them in other treated or control groups, there were more pellet fragments in the cages of both sexes at 3% compared to other groups. The chemical intakes were shown in Table 2.

Table 2. Food consumption and intake of catechin in the 12-month chronic or 24-month carcinogenicity study, and total amounts of catechin mixture, caffeine and polyhenol in the prepared diet

Time/Sex	Dose (%)	No. of animals examined	Food consumption (g/rat/day)	Intake of catechins Daily (mg/kg bw/day)
Food consumption and intake of catechin				
12-month chronic				
Males	Catechin 0%	10	18.8 ± 0.8	0
	Catechin 0.02%	10	19.0 ± 0.5	9.7
	Catechin 0.3%	10	19.6 ± 1.1	146.0
	Catechin 1%	10	20.6 ± 1.1	517.7
	Catechin 3%	10	23.5 ± 2.7	1922.9
Females	Catechin 0%	10	18.6 ± 2.4	0
	Catechin 0.02%	10	17.8 ± 1.7	14.2
	Catechin 0.3%	10	18.1 ± 1.2	215.7
	Catechin 1%	10	18.8 ± 1.5	765.7
	Catechin 3%	10	19.5 ± 3.6	2525.7
24-month carcinogenic				
Males	Catechin 0%	50	19.3 ± 0.7	0
	Catechin 0.02%	50	19.1 ± 0.9	8.4
	Catechin 0.3%	50	19.0 ± 0.7	122.7
	Catechin 1%	50	19.3 ± 1.5	416.4
	Catechin 3%	50	22.0 ± 1.1	1265.8
Females	Catechin 0%	50	14.7 ± 1.2	0
	Catechin 0.02%	50	14.7 ± 0.8	10.1
	Catechin 0.3%	50	14.7 ± 0.4	148.4
	Catechin 1%	50	14.8 ± 0.8	497.2
	Catechin 3%	50	15.5 ± 0.9	1539.8

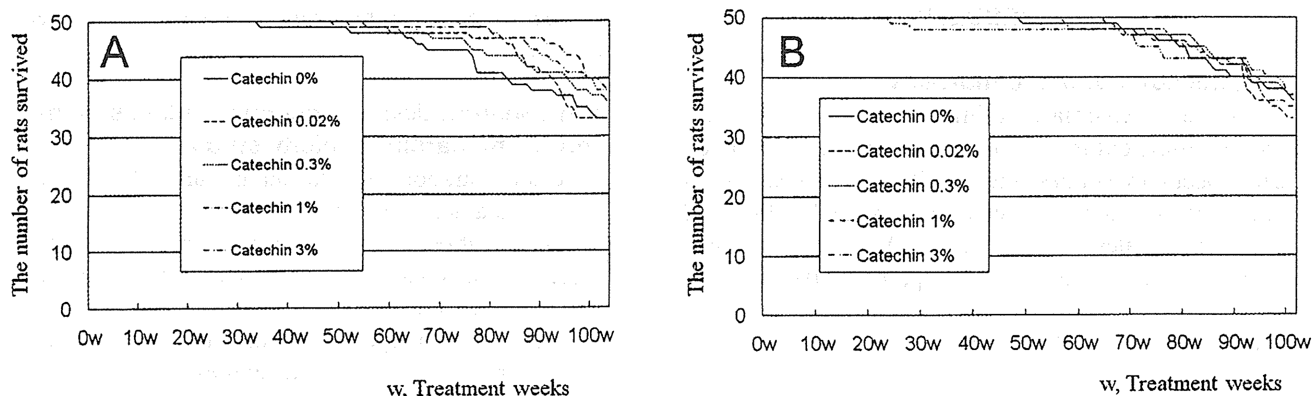

Fig. 1. Mortality of carcinogenicity study. A, males; B, females.

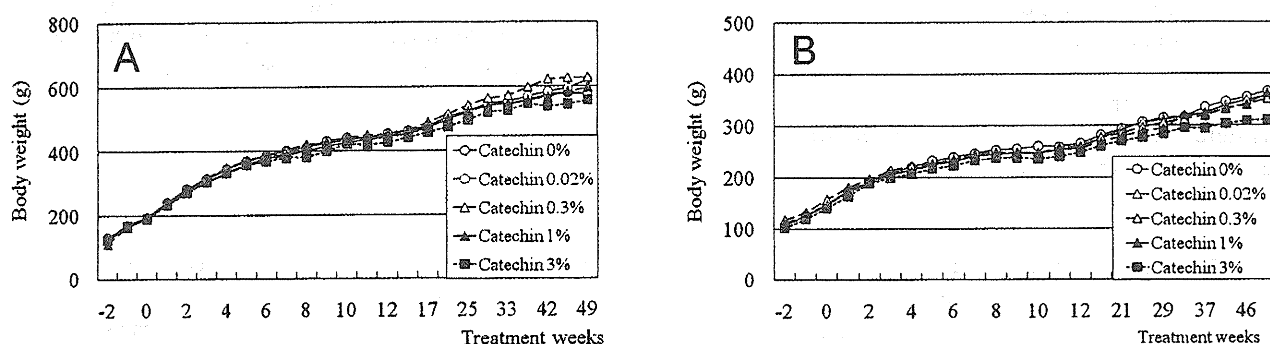

Fig. 2. Growth curve of chronic toxicity study. A, male; B, females.

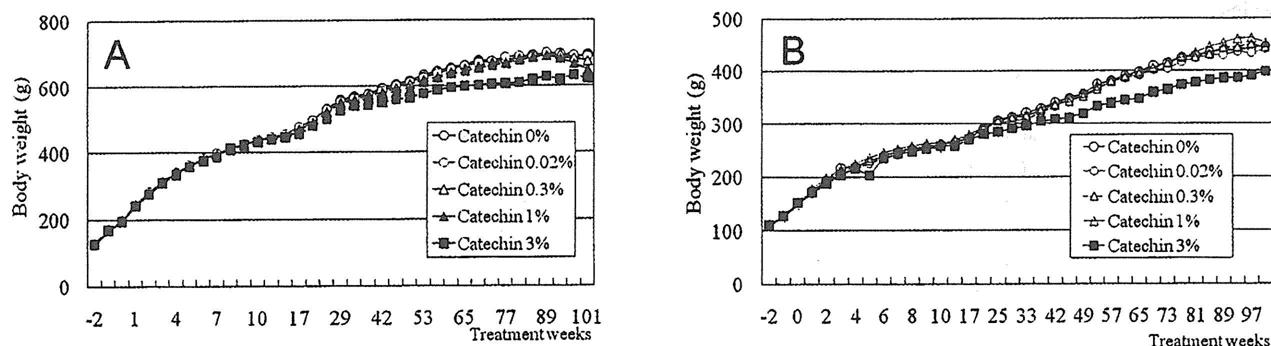

Fig. 3. Growth curve of carcinogenicity study. A, males; B, females.

Hematology and serum biochemistry

Although some significant changes were detected in hematology and blood biochemistry, no obvious dose-relation was found for any parameters among the treated groups (Tables 3 and 4).

Organ weights

In the chronic toxicity study, no significant changes were noted in the absolute and/or relative organ weights except an increase in the relative liver weight in males of the 3% group (Table 5). In carcinogenicity study, the liver weights in all treated groups were comparable to those of the control group in both sexes (Table 7).

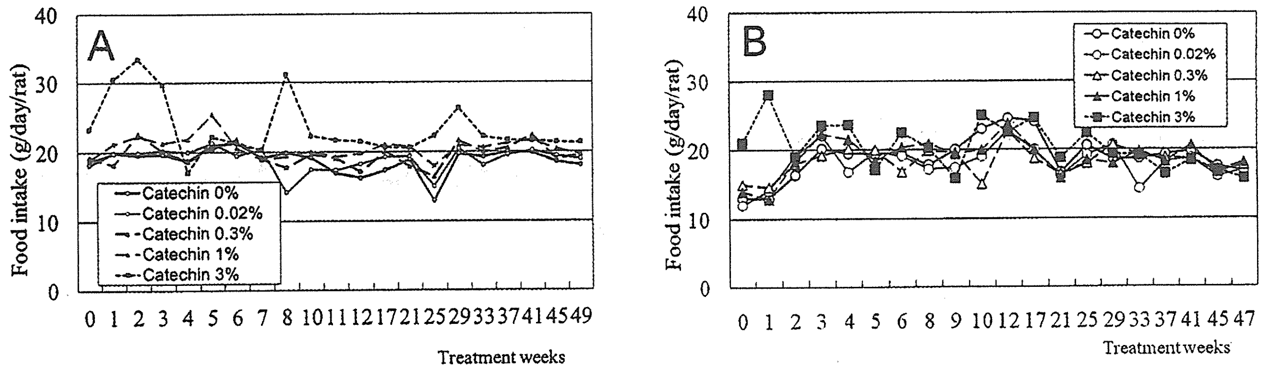

Fig. 4. Food intakes of chronic toxicity study. A, male; B, females.

Table 3A. Hematology in chronic toxicity study – Males –

Group		RBC × 10000/mm³	WBC /µl	Hb g/dl	HT %	MCV µ3	MCH pg	MCHC %
Catechin 0%	Mean	817.4	2662.5	15.1	49.4	60.5	18.4	30.5
(8)	SD	27.95	1140.1	0.38	1.27	1.77	0.52	0.53
Catechin 0.02%	Mean	820.8	3500.0	15.1	48.8	60.2	18.6	31.0
(10)	SD	36.93	1030.6	0.42	2.11	1.32	0.52	0.67
Catechin 0.3%	Mean	811.0	3500.0	15.1	48.8	60.2	18.6	31.0
(10)	SD	36.93	1030.6	0.42	2.11	1.32	0.52	0.67
Catechin 1%	Mean	820.6	3350.0	15.0	48.9	59.6	18.2	30.7
(10)	SD	32.62	861.9	0.40	1.41	2.01	0.79	0.48
Catechin 3%	Mean	793.3	3520.0	14.8	48.6	61.3	18.6	30.4
(10)	SD	25.46	853.5	0.45	1.38	1.89	0.70	1.07

( ): No. of rats examined.

Table 3B. Hematology in chronic toxicity study – Females –

Group		RBC × 10000/ mm³	WBC /µl	Hb g/dl	HT %	MCV µ3	MCH pg	MCHC %
Catechin 0%	Mean	709.2	1980.0	14.3	46.2	65.1	20.2	30.9
(10)	SD	24.04	533.90	0.50	2.66	2.59	0.60	0.97
Catechin 0.02%	Mean	693.1	1810.0	13.9	44.9	64.8	20.0	30.8
(10)	SD	22.24	438.30	0.46	1.60	1.48	0.67	0.63
Catechin 0.3%	Mean	711.0	2222.2	14.4	46.6	65.4	20.3	30.9
(9)[a]	SD	24.40	742.93	0.57	1.64	0.88	0.50	0.78
Catechin 1%	Mean	699.0	1700.0	14.2	45.7	65.4	20.2	31.0
(9)	SD	21.64	327.87	0.52	1.89	1.94	0.44	0.71
Catechin 3%	Mean	691.0	2033.3	14.1	45.9	66.4	20.4	30.7
(9)[a]	SD	19.47	259.81	0.40	1.27	1.88	0.53	0.71

( ): No. of rats examined. [a]: Female was the blood sample of one female rat could not available for analysis due to blood coagulation.

Necropsy and histopathology

In the chronic toxicity and carcinogenicity studies, no treated related macroscopic changes were detected. Microscopically, a number of non-neoplastic lesions were observed in both studies. In the chronic study, slight centrilobular hypertrophy of hepatocytes was significantly increased in males at 3% (Table 6, Fig. 6). However, this change was not observed in females at 3% or in the other treated groups. In immunohistochemistry, CYP3A2 expression was found in the hypertrophied

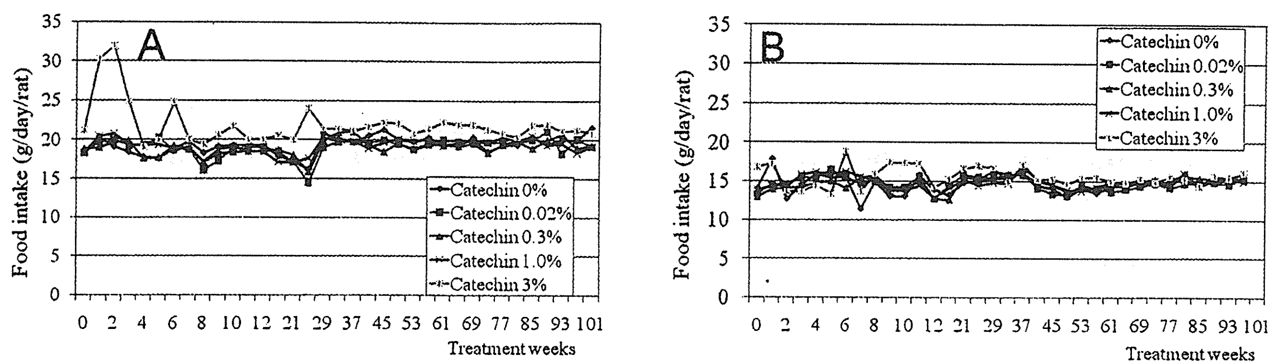

Fig. 5. Food intakes of in carcinogenicity study. A, male; B, females.

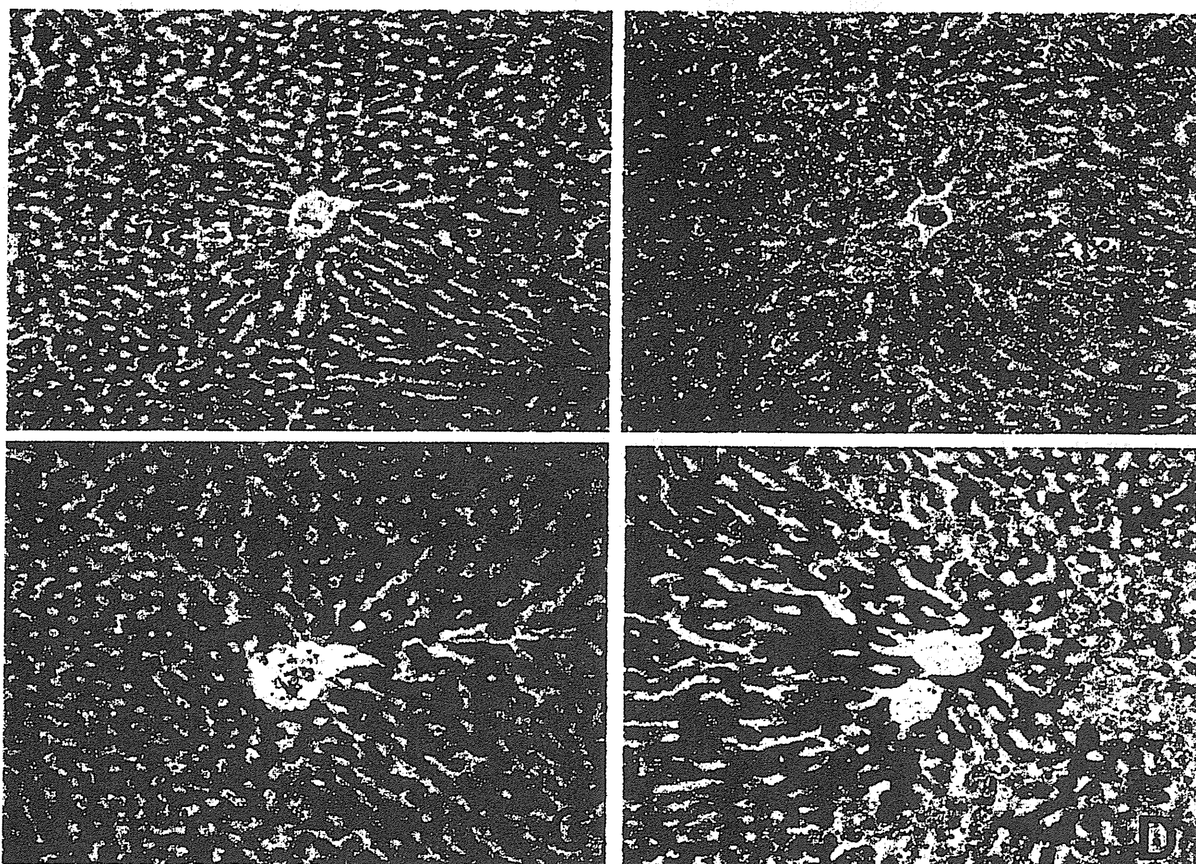

Fig. 6. Hisotpathological changes in the liver in the chronic toxicity study. A: A male in the control group (catechin 0%). No abnormalities detected. H-E stain. x 100. B: A male in the catechin 3% treated group. Slight centrilobular hypertrophy of hepatocytes. No degenerative, necrotic or inflammatory changes were detected. H-E stain. x 100. C: A male in the control group (catechin 0%). A slight positive area is apparent in the centrolobular area of hepatocytes. Immunohistochemical staining with CYP3A2 antibody. Visualized by DAB. x 100. D: A male in the catechin 3% treated group. Increased expression is apparent in the centrolobular area of hypertrophied hepatocytes, compared to that of C. Immunohistochemical staining with CYP3A2 antibodies. Visualized with DAB. x 100.

Table 4A. Serum biochemistry in chronic toxicity study – Males –

Group		TP g/dl	A/G	ALB g/dl	BIL mg/dl	TG mg/dl	TCHO mg/dl	CRE mg/dl	Na mEQ/l	K mEQ/l	Ca mg/dl	IP mg/dl	AST IU/l	ALT IU/l	ALP IU/l	γ-GTP IU/l
Catechin 0%	Mean	7.0	2.0	4.7	0.1	159.0	124.0	0.4	144.9	4.6	10.6	5.0	155.1	40.1	285.1	2 >
(8)	SD	0.17	0.34	0.29	0.02	52.80	26.49	0.04	0.64	0.31	0.23	0.76	36.05	8.84	51.75	
Catechin0.02%	Mean	7.2	1.8	4.6	0.1	189.6	149.6	0.4	144.7	4.6	10.8	5.2	148.4	42.1	267.0	2 >
(10)	SD	0.25	0.32	0.28	0.02	87.58	52.26	0.06	1.16	0.36	0.13	0.29	34.49	11.79	70.51	
Catechin 0.3%	Mean	7.3	1.8	4.6	0.1	224.0	124.3	0.3*	145.3	4.4	10.7	5.0	133.7	43.1	268.4	2 >
(10)	SD	0.31	0.31	0.18	0.03	96.76	19.65	0.03	1.49	0.32	0.19	0.42	42.75	30.90	94.59	
Catechin 1%	Mean	7.0	2.0	4.7	0.1	164.7	104.9	0.4	145.1	4.5	10.5	5.3	155.7	45.9	308.7	2 >
(10)	SD	0.28	0.19	0.21	0.02	56.19	13.40	0.04	0.88	0.28	0.28	0.48	27.13	9.99	47.66	
Catechin 3%	Mean	6.8	2.1	4.6	0.1	158.6	103.8	0.3**	145.4	4.5	10.7	5.6	137.2	54.5	347.6	2 >
(10)	SD	0.25	0.23	0.19	0.02	61.80	20.63	0.03	2.12	0.26	0.40	0.49	36.23	28.09	100.97	

( ): No. of rats examined. *, **: Significantly different from 0% ($p < 0.05$, $p < 0.01$).

Table 4B. Serum biochemistry in chronic toxicity study – Females –

Group		TP g/dl	A/G	ALB g/dl	BIL g/dl	TG mg/dl	TCHO mg/dl	CRE mg/dl	Na mEQ/l	K mEQ/l	Ca mg/dl	IP mg/dl	AST IU/l	ALT IU/l	ALP IU/l	γ-GTP IU/l
Catechin 0%	Mean	8.2	2.3	5.6	0.1	234.9	110.4	0.4	140.7	4.0	10.5	3.8	140.3	34.1	79.3	2 >
(10)	SD	0.79	0.44	0.29	0.05	164.08	30.45	0.04	2.67	0.37	0.37	0.61	31.66	5.34	25.66	
Catechin 0.02%	Mean	7.7	2.8	5.7	0.1	216.0	108.1	0.4	140.5	4.1	10.5	3.6	133.9	36.3	73.5	2 >
(10)	SD	0.53	0.46	0.44	0.05	104.58	16.87	0.04	2.68	0.37	0.32	0.41	35.26	10.86	20.97	
Catechin 0.3%	Mean	7.9	2.4	5.5	0.1	270.9	107.9	0.4	141.1	4.3	10.4	3.7	134.5	35.4	86.3	2 >
(10)	SD	0.54	0.51	0.27	0.03	148.59	28.07	0.04	3.07	0.39	0.35	0.91	30.89	9.36	41.10	
Catechin 1%	Mean	7.6	2.6	5.5	0.1	190.7	103.9	0.4	141.7	4.0	10.5	4.2	133.1	38.1	85.9	2 >
(9)	SD	0.39	0.40	0.26	0.03	63.08	32.25	0.05	3.12	0.44	0.42	0.47	27.67	13.53	37.53	
Catechin 3%	Mean	7.7	3.0**	5.7	0.1	153.3	98.5	0.3	142.5	4.3	10.4	3.6	137.9	42.7	92.4	2 >
(10)	SD	0.42	0.53	0.35	0.03	83.66	17.36	0.03	3.47	0.25	0.37	0.35	31.88	14.31	11.14	

( ): No. of rats examined. **: Significantly different from 0% ($p < 0.01$).

Table 5A. Organ weights in chronic toxicity study – Males –

Absolute weight

Group		Final B.W. (g)	Brain (mg)	Pituitary (mg)	Heart (mg)	Spleen (mg)	Liver (mg)	Adrenals (mg)	Kidneys (mg)	Testis (mg)	Prostate (mg)
Catechin 0%	Mean	579.9	2070.6	13.8	1358.0	834.3	12971.8	60.1	2791.9	3730.6	676.8
(8)	SD	61.01	70.47	2.25	85.60	93.23	1236.93	6.85	383.37	235.53	184.03
Catechin0.02%	Mean	605.0	2148.6	11.8	1366.0	928.5	14798.1	65.2	2808.8	3938.0	653.0
(10)	SD	46.53	87.45	1.62	172.66	103.21	2667.65	13.02	417.57	313.14	141.96
Catechin 0.3%	Mean	571.0	2085.4	12.0	1393.3	1019.3	14856.8	64.8	2945.8	3460.1	668.8
(10)	SD	71.27	220.38	2.16	118.92	154.07	2504.39	7.44	316.11	574.62	138.77
Catechin 1%	Mean	586.0	2144.6	12.4	1387.7	937.9	13387.3	69.6	2921.3	4027.6	639.5
(10)	SD	52.90	82.21	1.84	164.06	153.27	1508.17	8.32	474.54	410.94	137.47
Catechin 3%	Mean	560.0	2177.5	12.1	1308.0	977.7	14289	60.0	3014.8	3884.1	611.5
(10)	SD	52.90	82.21	1.84	164.06	153.27	1508.17	8.32	474.54	410.94	137.47

Relative weight (mg/BWg × 100)

Group		Brain	Pituitary	Heart	Spleen	Liver	Adrenals	Kidneys	Testis	Prostate
Catechin 0%	Mean	360.7	2.4	235.8	145.5	2249.1	10.4	487.1	650.9	117.3
	SD	41.29	0.35	22.32	25.22	229.89	1.31	90.28	89.67	31.92
Catechin 0.02%	Mean	356.9	2.0	225.2*	153.4	2435.0	10.8*	463.0	654.5	107.5
	SD	28.72	0.29	15.04	12.19	291.61	1.92	45.34	71.21	21.07
Catechin 0.3%	Mean	343.4	2.2	227.0	168.4	2399.2	10.7	478.9	576.4	109.9
	SD	63.00	0.20	19.78	41.07	192.96	2.06	36.32	142.60	26.70
Catechin 1%	Mean	368.4	2.1	236.5	159.8	2283.6	11.9	496.4	689.4	109.1
	SD	32.35	0.31	13.92	20.49	131.24	1.28	47.07	66.34	20.67
Catechin 3%	Mean	399.1	2.2	236.6	176.4	2566.7*	10.9	541.8	707.8	110.6
	SD	64.71	0.38	29.45	27.48	269.34	2.05	42.90	108.35	21.64

( ): No. of rats examined *: Significantly different from 0% ($p < 0.05$).

Table 5B. Organ weights in chronic toxicity study – Female –

Absolute weight

Group		Final B.W. (g)	Brain (mg)	Pituitary (mg)	Heart (mg)	Spleen (mg)	Liver (mg)	Adrenals (mg)	Kidneys (mg)	Ovaries (mg)	Uterus (mg)
Catechin 0%	Mean	366.4	2011.5	25.0	978.6	673.9	7474.4	86.9	1969.5	76.9	1320.2
(10)	SD	40.12	59.24	7.07	73.76	114.77	1130.63	20.51	178.91	33.31	302.82
Catechin 0.02%	Mean	353.1	1978.7	24.2	902.6	610.8	7403.1	79.4	1954.7	87.3	1037.5
(10)	SD	56.39	107.17	7.08	97.84	98.26	915.07	16.60	171.13	27.64	469.12
Catechin 0.3%	Mean	350.0	1899.3	22.3	935.9	644.1	7468.9	85.2	1981	73.7	1059.2
(10)	SD	40.34	299.20	3.86	58.98	63.09	715.38	12.79	167.66	18.79	273.15
Catechin 1%	Mean	349.8	2024.1	20.3	905.8	664.3	7596.2	80.7	1998.8	93.9	1047.8
(9)	SD	54.47	67.51	3.32	102.43	88.48	1185.61	15.41	381.89	29.73	420.52
Catechin 3%	Mean	305.8	1970.9	18.9	876.0	667.3	6746.8	76.3	1862.2	90.3	1136.1
(10)	SD	21.58	66.46	3.28	78.92	82.44	487.23	5.44	169.20	50.44	445.64

Relative weight (mg/BWg × 100)

Group		Brain	Pituitary	Heart	Spleen	Liver	Adrenals	Kidneys	Ovaries	Uterus
Catechin 0%	Mean	554.8	6.5	269.3	185.7	2044.5	24.1	541.7	21.3	368.7
	SD	61.74	1.00	28.85	37.74	247.63	6.89	60.99	9.20	119.78
Catechin 0.02%	Mean	575.9	7.0	259.0	174.1	2114.1	22.9	565.4	24.7	301.4
	SD	114.88	2.23	29.83	20.95	178.17	5.52	94.88	6.24	139.59
Catechin 0.3%	Mean	550.2	6.5	269.7	185.7	2148.1	24.5	570.0	21.5	302.9
	SD	111.73	1.36	25.87	23.01	210.31	3.91	53.26	6.79	72.07
Catechin 1%	Mean	589.9	5.9	261.9	194.0	2176.7	23.6	573.4	26.6	314.2
	SD	83.01	1.29	32.96	42.41	155.71	6.54	78.23	8.78	161.91
Catechin 3%	Mean	646.7	6.2	286.4	219.9	2210.3	25.1	609.1	29.6	371.2
	SD	39.26	1.07	14.72	36.76	148.15	2.57	36.55	16.48	140.34

( ): No. of rats examined, *, **: Significantly different from 0% ($p < 0.05$, $p < 0.01$).