Original Article

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Acute oral toxicity test of chemical compounds in silkworms

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Summary **This study performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines as standard substances for a cytotoxicity test, and median lethal dose (LD_{50}) werecalculated for each compound. Acute oral LD_{50} values in mammals are listed in OECD guidelines and acute oral LD_{50} values in silkworms were determined in this study. R^2 for the correlation between LD_{50} values in mammals and LD_{50} values in silkworms was 0.66. In addition, the acute oral toxicity test in silkworms was performed by two different facilities, and test results from the facilities were highly reproducible. These findings suggest that an acute oral toxicity test in silkworms is a useful way to evaluate the toxicity of compounds in mammals.**

Keywords: Acute oral toxicity test, animal model, silkworm

1. Introduction

Acute toxicity of chemical compounds can be evaluated by calculating LD_{50} values, *i.e.*, the dose that will kill 50% of animals in a test group. From the viewpoint of animal welfare, sacrificing a large number of mammals in an experiment raises ethical issues. At the end of 2002, the Organization for Economic Cooperation and Development (OECD) deleted TG 401, which is the method for evaluating the acute oral toxicity of compounds by calculating LD_{50} values in mammals (*1*). Given the issue of animal welfare, the fixed dose procedure (FDP; OECD 420), the acute toxic class method (ATC; OECD 423), and the up and down procedure (UDP; OECD 425) have been developed as alternatives to animal testing in TG 401. However, mammals must be sacrificed even when using these alternate methods. Cytotoxic tests of mammalian cells could predict the acute toxicity of compounds in mammal by measuring IC_{50} values (*2*). However, predicting the toxicity of compounds in mammals with those tests would be difficult since the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body are not taken into account. Techniques to predict the acute toxicity of compounds in mammals without sacrificing those animals must be devised to overcome these problems.

Silkworms have been bred over the long history of sericulture, and subjecting silkworms to a toxicity test poses fewer ethical problems. Moreover, techniques to rear a large number of genetically uniform individuals have been established. The current authors also recently reported that metabolic pathways in silkworms are similar to those in mammals and that the toxicity of some compounds to silkworms accords with their toxicity to mammals once weight is taken into account (*3*). Moreover, silkworms are large enough to inject a precise amount of a sample solution into either the hemolymph or the midgut (*4*). Hemolymph is akin to blood in humans and injection into the midgut is akin to oral administration in humans. In light of these facts, an acute oral toxicity test in silkworms might be alternative to testing mammals. Thus, the current study was conducted.

This study performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines (*5*), and LD_{50} values

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were calculated for each compound. LD_{50} values in silkworms were calculated in this study and LD_{50} values in mammals are listed in OECD guidelines. LD_{50} values in silkworms and LD_{50} values in mammals were subjected to linear regression analysis. The acute oral toxicity test in silkworms was conducted two different facilities, and this study evaluated the between-laboratory reproducibility of their results.

2. Materials and Methods

2.1. *Acute oral toxicity tests in silkworms*

The toxicity of 59 compounds was evaluated in silkworms using an acute oral toxicity test. These compounds (Table 1) are listed as standard substances in OECD Guidelines. *Bombyx mori* eggs (Hu Yo × Tsukuba Ne or Kinshu × Showa) were purchased from Ehime Sansyu (Ehime, Japan) or Kougensha (Nagano, Japan) and raised as previously described (*6,7*). A two-fold dilution series containing each compound (50 μL) was injected into the midgut of fifth instar silkworm larvae with a disposable plastic syringe (Terumo, Tokyo, Japan). Silkworms were fed with Silkmate 2S (Nosan Co., Yokohama, Japan) every day and reared at 27°C. After 2 or 7 days, survival rates were measured and LD_{50} values were calculated based on the survival curve ($n = 7$).

Acute oral LD_{50} values in silkworms were determined at 2 days and acute oral LD_{50} values in mammals are listed in OECD guidelines. Linear regression analysis of the common logarithm of LD_{50} values in silkworms and LD_{50} values in mammals was performed to determine the correlation between these 2 values.

2.2. *Reproducibility of an acute oral toxicity test in silkworms conducted by two different facilities*

Two different facilities (Laboratory 1: Genome Pharmaceuticals Institute Co., Ltd., Tokyo, Japan, Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory, Kanagawa, Japan) performed an acute oral toxicity test in silkworms, and LD_{50} values for 59 compounds were calculated after 2 days. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was determined in order to examine the between-laboratory reproducibility of the toxicity test results.

3. Results

3.1. *Comparison of acute oral toxicity test results in silkworms and in mammals*

LD_{50} values in mammals and IC_{50} values in 3T3 cells and NHK cells are listed in OECD guidelines. Linear regression analysis of the LD_{50} values was performed, and the regression line and the determination coefficient were defined as below.

$\log (LD_{50}$ in mammals$) = 0.499 \times \log (IC_{50}$ 3T3 cells$) + 1.65 \quad (R^2 = 0.33)$

$\log (LD_{50}$ in mammals$) = 0.445 \times \log (IC_{50}$ NHK cells$) + 1.81 \quad (R^2 = 0.37)$

LD_{50} values for 59 compounds were calculated based on an acute oral toxicity test in silkworms (Table. 1). LD_{50} values in silkworms were calculated in this study and LD_{50} values in mammals are listed in OECD guidelines. Linear regression analysis of the 2 values was performed, and the regression line and the determination coefficient were defined as below.

$\log (LD_{50}$ in mammals$) = 0.860 \times \log (LD_{50}$ in silkworms$) + 0.168 \quad (R^2 = 0.66)$

Some of the 59 compounds produced LD_{50} values in silkworms that differed from LD_{50} values in mammals listed in OECD guidelines (Table 1). The LD_{50} values for physostigmine, nicotine, lindane, carbamazepine, propylparaben, and sodium hypochlorite were 10 or more times lower in silkworms than in mammals. The LD_{50} values for cycloheximide, sodium selenate, and phenylthiourea were 10 or more times higher in silkworms than in mammals.

3.2. *Reproducibility of an acute oral toxicity test in silkworms performed by two different facilities*

LD_{50} values in silkworms that are calculated by different facilities must be highly reproducible if an acute oral toxicity test in silkworms is to serve as an alternative to acute oral toxicity testing in mammals. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88

4. Discussion

Cytotoxicity tests of compounds in mammalian cells or cell lines have been proposed as a way to predict the acute oral toxicity of compounds in mammals (*2*). IC_{50} values in 3T3 cells or NHK cells and LD_{50} values for 59 compounds in mammals are listed in OECD guidelines (*5*). Linear regression analysis of those IC_{50} values yielded an R^2 of 0.33 and linear regression analysis of LD_{50} values in mammals yielded an R^2 of 0.37. LD_{50} values that were calculated in the acute oral toxicity test in silkworms and LD_{50} values listed in OECD guidelines for mammals were subjected to linear regression analysis, which yielded an R^2 of 0.66 (Figure 1). These results suggest that, in contrast to a toxicity test in mammalian cells, an acute oral toxicity

Table 1. Acute oral LD_{50} values in silkworms calculated in this study

No.	Name	*Ref.* acute oral LD_{50} (*5*) (mg/Kg)	Laboratory 1		Laboratory 2	
			LD_{50} (Day 2) (mg/Kg)	LD_{50} (Day 7) (mg/Kg)	LD_{50} (Day 2) (mg/Kg)	LD_{50} (Day 7) (mg/Kg)
1	1,1,1-Trichloroethane	12,078	6,200	5,200	N.D.	1,700
2	2-Propanol	5,105	22,000	22,000	14,000	11,000
3	5-Aminosalicylic acid	3,429	460	160	N.D.	N.D.
4	Acetaminophen	2,163	1,700	870	2,300	1,800
5	Acetonitrile	3,598	43,000	36,000	25,000	20,000
6	Acetylsalicylic acid	1,506	1,400	1,400	1,900	1,500
7	Arsenic III trioxide	25	100	100	16	16
8	Atropine sulfate	819	3,400	1,100	N.D.	1,300
9	Boric acid	3,426	3,100	1,600	1,300	830
10	Busulfan	12	42	42	N.D.	N.D.
11	Cadmium II chloride	135	57	51	140	87
12	Caffeine	310	560	290	800	300
13	Carbamazepine	2,805	280	120	N.D.	210
14	Carbon tetrachloride	3,783	3,400	2,400	1,200	1,200
15	Chloral hydrate	638	1,700	990	1,600	1,580
16	Citric acid	5,929	10,000	9,500	6,500	6,500
17	Colchicine	15	23	1.8	N.D.	5.4
18	Cupric sulfate $5H_2O$	474	650	460	940	610
19	Cycloheximide	2	1,875	1,875	2,300	2,100
20	Dibutyl phthalate	8,892	45,000	30,000	N.D.	18,000
21	Dichlorvos	59	310	15	0.42	0.42
22	Diethyl phthalate	9,311	3,800	3,500	4,400	4,000
23	Digoxin	28	96	6.1	24	17
24	Dimethylformamide	5,309	20,000	18,000	25,000	16,000
25	Ethanol	11,324	56,000	34,000	28,000	25,000
26	Ethylene glycol	7,161	33,000	41,000	46,000	46,000
27	Gibberellic acid	6,040	7,100	4.4	N.D.	N.D.
28	Glycerol	19,770	35,000	35,000	54,000	54,000
29	Haloperidol	330	1,100	69	N.D.	N.D.
30	Hexachlorophene	82	20	12	67	59
31	Lactic acid	3,639	36,000	30,000	5,100	4,500
32	Lithium I carbonate	590	220	5.4	N.D.	N.D.
33	Mercury II chloride	40	120	50	58	40
34	Methanol	8,710	6,900	3,900	8,300	8,000
35	Nicotine	70	15	3.3	3.6	3.7
36	Phenol	548	860	790	1,000	940
37	Phenylthiourea	3	320	170	240	240
38	Potassium Cyanide	7	25	25	74	82
39	Potassium I chloride	2,799	6,800	5,800	10,000	7,900
40	Procainamide	1,950	7,000	4,100	N.D.	5,900
41	Propranolol HCL	466	570	540	540	490
42	Propylparaben	6,332 (mouse)	670	370	640	580
43	Sodium chloride	4,046	6,500	5,000	6,800	4,900
44	Sodium Dichromate Dihydrate	51	170	170	610	530
45	Sodium hypochlorite	10,328	1,500	980	830	650
46	Sodium I fluoride	127	110	70	110	96
47	Sodium oxalate	633	1,100	1,100	1,300	1,300
48	Sodium selenate	3	50	39	150	110
49	Thallium I sulfate	25	92	10	1,100	1,100
50	Trichloroacetic acid	5,229	5,200	3,500	1,500	1,200
51	Valproic acid	995	3,500	3,500	7,900	6,100
52	Verapamil HCL	111	490	370	1,200	1,100
53	Xylene	4,667	2,200	2,200	2,100	1,100
54	Amitriptyline HCL	348	390	210	340	290
55	Chloramphenicol	3,491	1,100	980	350	320
56	Epinephrine Bitartrate	4 (mouse)	14	14	N.D.	N.D.
57	Lindane	100	27	12	2.3	2.3
58	Physostigmine	5	0.76	0.46	0.75	0.65
59	Strychnine	6	44	4.1	N.D.	N.D.

"*Ref.* acute oral LD_{50}" means LD_{50} values for adult laboratory rats according to the literature (*5*) unless otherwise specified.

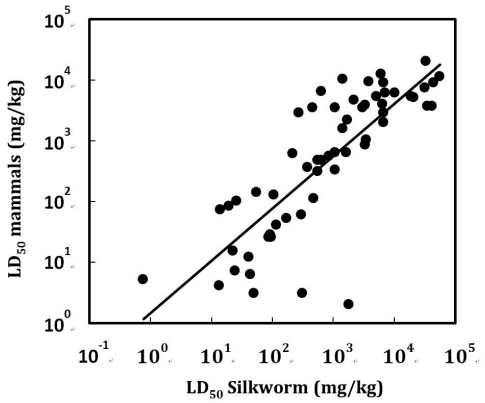

Figure 1. Linear regression analysis of acute oral LD_{50} values for 59 compounds (listed in OECD guidelines) in silkworms and in mammals. Acute oral LD_{50} values for 59 compounds were calculated using the data on silkworm survival after 2 days. Acute oral LD_{50} values in mammals were obtained from the literature. The common logarithm of LD_{50} values in silkworms and in mammals was determined and regression analysis of these 2 values was performed. The regression line was defined as: log (LD_{50} in mammals) = 0.860 × log (LD_{50} in silkworms) + 0.168 (R^2 = 0.66)

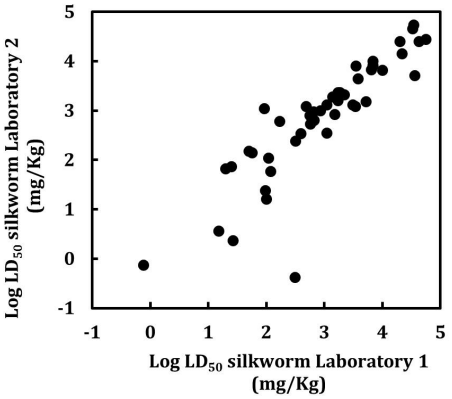

Figure 2. Comparison of acute oral LD_{50} values in silkworms calculated by two different facilities. An acute oral toxicity test in silkworms was performed by two different facilities (Laboratories 1 and 2). Acute oral LD_{50} values for 59 compounds (LD_{50} in silkworms: mg/Kg) after 2 days were calculated by each facility. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88. Laboratory 1: Genome Pharmaceutical Institute Co., Ltd. Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory.

test in silkworms yields results that are more closely in line with the results of an acute oral toxicity test in mammals. A previous study by the current authors reported that some compounds are metabolized in silkworms in the same manner as in mice (*3*). An *in vitro* cytotoxicity test cannot reflect the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body, and this leads to disparities in the oral acute toxicity and cytotoxicity of some compounds. These problems might be overcome through the use of silkworms and more consistent results with mammalian test could be obtained rather than cytotoxicity test.

In this study, the LD_{50} values for some compounds were 10 or more times lower in silkworms than in mammals or 10 or more times higher in silkworms than in mammals. Compounds with LD_{50} values that were 10 or more times lower in silkworms were physostigmine, nicotine, lindane, carbamazepine, propyl paraben, and sodium hypochlorite. Compounds with LD_{50} values that were 10 or more times higher in silkworms were cycloheximide, sodium selenite, and phenyl thiourea. Physostigmine is a carbamate insecticide and dichlorvos has been used as an organic phosphorus-based insecticide; physostigmine and dichlorvos are known to inhibit acetylcholinesterase (*8,9*). Nicotine, an acetylcholine receptor agonist, has been used as an insecticide (*10*). Lindane is an insecticide that is known to be a GABA receptor antagonist (*11*). Therefore, silkworms may be more sensitive to these compounds than mammals. Cycloheximide was one compound with an LD_{50} value that was 10 or more times higher in silkworms than in mammals. Cycloheximide may be hydrolyzed in the midgut of silkworms because of the alkaline environment there (pH 9-11).

This study evaluated the reproducibility of an acute oral toxicity test in silkworms by having two different facilities perform the test. Each facility calculated LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88 (Figure 2). Therefore, the acute oral toxicity test in silkworms has a high level of between-laboratory reproducibility. The two facilities found that LD_{50} values for some compounds, such as thallium sulfate and dichlorvos, differed by 10 times or more in silkworms. In the acute oral toxicity test, each sample was directly injected into the midgut of silkworms. Therefore, this discrepancy in LD_{50} values may be due to how adeptly the sample was injected.

Results of this study indicated that, in contrast to a cytotoxicity test using mammalian cells, an acute oral toxicity in silkworms yields results that are more closely in line with the results of an acute oral toxicity test in mammals. Furthermore, the current results suggest that an acute oral toxicity test in silkworms has a high level of between-laboratory reproducibility. Therefore, an acute oral toxicity test in silkworms is a useful alternative to testing the acute oral toxicity of chemical compounds in mammals.

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