Toxicological Assessment of *Trans*-resveratrol

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Abstract: **Objective** To evaluate toxicity and safety of *trans*-resveratrol (*t*-RSV). **Methods** For assays of acute toxicity, genetic toxicity, and sub-chronic toxicity, Ames test, mice bone marrow erythrocyte micronucleus, and mice sperm abnormality were performed. **Results** In the acute oral toxicity tests, maximum tolerable dose (15 g/kg) in male and female Kunming mice showed no toxicological signs. For 90-d feeding of *t*-RSV at dosage range of 167–500 mg/(kg·d) in both male and female Sprague-Dawley rats, no noticeable toxicological effects were observed. **Conclusion** *T*-RSV has no acute toxicity and no genotoxicity, no harmful effects on the human body at the tested dosage range and thus resveratrol is safe for human consumption.

Key words: toxicity; toxicological assessment; *trans*-resveratrol
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Introduction

Trans-resveratrol (*trans*-3, 5, 4'-trihydroxystilbene, *t*-RSV), a compound found in grapes, mulberries, peanuts, and Chinese herb *Polygonum cuspidatum* Sieb. et Zucc., is a phytoalexin that plants produce in response to stress, fungal attack, or elicitor treatment to defend themselves (Soleas, Diamandis, and Goldberg *et al*, 1997). Scientists became interested in exploring potential health benefits of *t*-RSV after it was found in red wine in 1992, and speculated that *t*-RSV might help explain the "French Paradox" (Siemann and Creasey, 1992). Reports on the potential for *t*-RSV to inhibit the development of cancer (Jang *et al*, 1997) and extend lifespan (Baur *et al*, 2006) have continued to generate scientific interest. However, there is few literature on the safety of resveratrol (RSV) in spite of the long history of ingestion of RSV from various foods, such as peanuts, grapes, and red wine. A small scale of the safety study on oral *t*-RSV in ten healthy volunteers showed that a single dosage of up to 5 g of *t*-RSV caused no serious adverse effects (Boocock *et al*, 2007). No effects on male SD rats with ig administration *t*-RSV (20 mg/kg) daily for 28 d consecutively were observed. However, this single-dose study by using only six control and eight RSV-fed male rats would not produce appropriate parameters of toxicity (Juan Vinardell, and Planas, 2002). Scientists from the National Cancer Institute conducted a four-week study in rats to evaluate potential toxicity of *t*-RSV. Dosages of ig administration were 300, 1000, and 3000 mg/(kg·d), respectively, and the observed adverse effect level (NOAEL) showed on the dosage of 300 mg/kg. The 1000 mg/kg dosage caused slight weight loss in female rats and slight but significant elevation in white blood cell count (WBC) in the male rats. However, 3000 mg/kg dosage showed significant toxicity, such as increased incidence and severity of kidney damage, as well as reduced body weight and food consumption (Crowell *et al*, 2004). Therefore, a complete toxicological assessment of *t*-RSV, including test of genotoxicity, acute oral toxicity, and a 90-d sub-chronic toxicity should be conducted in order to study scientifically defensible safety data and NOAEL on *t*-RSV as well.

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Materials and methods

Preparation of RSV

T-RSV (lot# 080135, Restrol^{TM}) extracted from the root of *P. cuspidatum* was provided by World-Way Inc., Changsha, Hunan, China.

P. cupsidatum roots (Hunan, China) were selected and crushed, then fermented for 8 d. The fermented materials were extracted for three times with 80% ethanol. The aqueous alcohol extract were concentrated by vacuum, centrifugated at 1500 r/min and the supernatant was 50% crude extract. To dissolve the crude extract with ethanol and pass through an alumina column, concentrate the flowthrough liquid the coarse crystals were collected. The coarse crystals were decolored by charcoal-carbon and recrystallized, then vacuum drying, smashing, and sieving were carried out to produce the product.

The compound identity was confirmed by LC-MS and NMR, the purity was determined by HPLC to be (99.0 ± 0.5)%. The substance was stored at room temperature, ambient humidity, but was protected from light.

Instruments and chemical agents

Abbott CD3400 CELL-DYN Hematology Systems, Olympus AU400 Automatic Biochemistry Analyzer, and LD5-10B Centrifuge are the main instruments used. Albumin, alanine transaminase (ALT), asparate amino-transferase (AST), blood urea nitrogen (BUN), glucose (GLU), cholesterol (CHOL), and triglyceride (TG) were purchased from Shanghai Fuxi Changzeng Medical Science Co.; Creatinine was bought from Diasys Diagnostic Systems (Shanghai) Co., Ltd.

Animals and animal housing

Male and female Kunming mice and SD rats were purchased from Dongchuang Laboratory Animal Service Company in Changsha, China (laboratory animal reproduction license # SCXK [HN] 2006-0001). Animal foods were purchased from the same company. The studies were conducted in compliance with Good Laboratory Practices at Hunan Disease Control and Prevention (CDC) (laboratory animal use permit # SYXK [HN]2003-0002). Temperature was controlled at 22–24 ℃, humidity was controlled at 52%–58%, and light control was maintained as a 12-h cycle of dark-light throughout the test period. All animals were examined for their general physical conditions upon adoption and acclimatized for one week before any test.

Dosing formulations

Because of the low solubility of *t*-RSV in water, a vehicle solution of 10 g/L carboxymethylcellulose in ddH_2O was used for suspension. Each dosing suspension was prepared individually by mixing *t*-RSV with the vehicle solution in a homogenizer. Dosing formulations were stored at 4 ℃, homogenized daily prior to dosing for at least 2 min, allowed to warm up to room temperature before administration, and stirred continuously while the dosing procedure was ongoing.

Experiment designs and methods

Acute oral toxicity test Maximum tolerable dose (MTD) test was conducted since *t*-RSV is not known to be toxic or causes adverse effects in human beings from literature searching. Twenty of 7-8 week-old Kunming mice, ten male and ten female, were used. *T*-RSV (15.0 g) was mixed with 40 mL of vehicle solution and was ig administered twice at 4 h intervals, 20 mL/kg for each administration, the accumulated *t*-RSV dose was 15.0 g/kg. Mice were departed from feed for 16 h before the first administration. Toxicological signs and morbidity were observed daily for 2 weeks after ig administration.

Ames test Ames test was preformed with or without metabolic activation (S-9 obtained from Aroclor 1254-induced rat livers). Four certificated histidine-requiring strains of *Salmonella typhimurium* TA97, TA98, TA100, and TA102 were cultured overnight in a nutrient broth (pH 7.4, 5.0 g peptone, 2.5 g beef extract, 2.5 g NaCl, 1.3 g $K_2HPO_4{\cdot}3H_2O$, dissolved in 50 mL ddH_2O) at 37 ℃ under shaking. Tester strain (0.1 mL), testing solution (0.1 mL), and phosphate buffer solution (PBS, 0.5 mL) or S-9 mixture (when metabolic activator needed) were added to 2 mL of molten top agar (0.6% agar, 0.5% NaCl, and 10 mL of 0.5 mmol/L histidine-biotion solution) that was preheated to approximate 45 ℃. The mixture was poured immediately onto minimal agar (1.5% agar, 40% glucose, and PBS) Petri dish. After incubation at 37 ℃ for 48 h, the number of His^+ revertant clones was counted and compared to the number of spontaneous revertant clones of negative solvent control (ddH_2O) plates. All srains were tested using three plates per dose. The five testing doses of *t*-RSV were 5000, 1000, 200, 40, and 8 μg/dish. The maximum dose level of 5000 μg/dish followed the

recommendation of ICH Harmonized Tripartite Guideline S2 (R1). Negative solvent control and strain-specific positive controls were included in each test. The test substance will be considered to be mutagenic if the revertant clones are two or more folds the number of spontaneous revertant clones on negative solvent control plates. A dose response relationship was observed in at least 2 concentration. The test was repeated under the same conditions to confirm the results.

Mice bone marrow erythrocyte micronucleus assay Fifty of 7-8 week-old Kunming mice, 25 male and 25 female, were divided into five groups randomly. Cyclophosphamide (0.04 g/kg) was used as positive control; vehicle solution was used as negative control. The *t*-RSV dose levels of testing groups were 7.500, 3.750, and 1.875 g/kg, respectively. The testing samples were freshly prepared by mixing *t*-RSV (15.00, 7.50, and 3.75 g) with vehicle solution to a total volume of 40 mL, respectively. Cyclophosphamide (100 mg) dissolved in ddH_2O (50 mL) was used as positive control. Testing materials were ig administered twice at 0.20 mL/10 g at 24 h intervals. Execution *via* cervical vertebra dislocation was conducted 6 h after the last dose administration. Sternum bone was removed and the bone marrow cells were pulled out and mixed with fetal bovine serum immediately following the sacrifice. One drop of the mixture was smeared onto a clean slide and air-dried. The slides were briefly flamed, then fixed with immersion in 95% methanol for 10 min, and stained in ordinary staining jars with Giemsa Working Solution for 15 min. Stained slides were washed gently with ddH_2O, air-dried, and cover-slipped for microscope examination. All slides were coded to ensure that the evaluation was blinded. Micronucleus frequencies were determined for each animal by counting 1000 polychromatic erythrocytes (PCE) and the micronucleus occurrence rate per one thousand PCE was recorded. The proportion of immature erythrocytes (i.e. PCE) to total erythrocytes [PCE + NCE (normochromatic erythrocytes)] was determined for each animal by counting a total of 200 erythrocytes. Mean ± SD of micronucleus occurrence rate and PCE/NCE ratio of each group was compared using SPSS11.0 software.

Mice sperm abnormality test Twenty-five of 7-8 week-old male Kunming mice were randomly divided into five groups. The testing samples were prepared by mixing *t*-RSV (15.00, 7.50, and 3.75 g) with vehicle solution to a total volume 40 mL, respectively. Cyclophosphamide (100 mg) was dissolved in 50 mL ddH_2O and used as positive control. Negative control group was vehicle solution. *T*-RSV dose levels of testing groups were 7.500, 3.750, and 1.875 g/kg, respectively. Intubations occurred daily at 0.20 mL/10 g for 5 d. Execution was conducted *via* cervical vertebra dislocation 30 d after the last dose administration. Epididymis was isolated and placed on a flat dish containing 2 mL of 0.9% NaCl solution. The epididymis was cut by using ophthalmological scissors longitudinally once or twice, allowed to settle for 3–5 min, vibrated gently, and filtered with four layers of microscopy cleaning paper. One drop of the filtrate was smeared onto a clean slide and air-dried. Slides were then fixed with immersion into 95% methanol for 5 min, stained with 2% HE for 1 h, washed gently with ddH_2O, and air-dried. Each animal counted a total of 1000 sperm in an optical microscope at 1000 × augmentations in a bright field. The percentage of abnormalities was calculated, first as a total, and then further classified in relation to the specific location of each abnormality in the sperm.

90-d Feeding study Eighty SD rats, half male and half female, male body weight (78.2 ± 6.9) g, female body weight (77.0 ± 8.2) g, were randomly divided into four groups with equal amount of male and female, one control group and three treatment groups. Rats were caged individually in stainless steel open-mesh cages. Feeding should meet the nutritional requirements, and rats and water were provided *ad libitum* during the study period. Testing samples were prepared by mixing *t*-RSV (3.33, 6.65, and 9.98 g) with vehicle solution to a total volume of 200 mL. Rats were ig adminstrated once a day with 0 (vehicle only), 167, 333, or 500 mg/kg of *t*-RSV 10 mL/(kg·d) for 90 d consecutively. The volume administrated was based on the most recently measured body weight.

Routine cage-side observations were conducted on all animals once a day throughout the study for general behavior and toxicological signs. Feed was added 2–3 times per week, individual food consumption (food added–food left) was recorded. Body weight measurement, food consumption, as well as physical exams were

conducted weekly. In the middle of the study (day 45), animals were fasten for 16 h, blood samples were collected from the orbital sinus and put into anticoagulant and nonanticoagulant tubes for hematology and clinical chemistry measurements (anesthetic: CO_2-O_2 = 70%-30%). Anti-coagulant samples were analyzed using Abbott CD3400 Hematology Systems for hemoglobin (Hb), hematocrit (HCT), red blood cell count (RBC), WBC, platelet count, and sub-classification of WBC. Non-anticoagulant tubes were analyzed using Olympus AU400 Automatic Biochemistry Analyzer for total protein, albumin, creatinine (Cr), BUN, ALT, AST, CHOL, TG, and GLU. In the later period of the experiment (one day after the last dose on day 90), animals were fasten for 16–18 h and euthanized by CO_2 asphyxiation. Then blood samples were collected and the hematology and clinical chemistry analysis were conducted as described above. Necropsy after taking blood samples was a thorough and systematic examination by dissection of the viscera and carcass. All tissues and organs collected at necropsy were examined microscopically for the vehicle control and high-dose groups. If treatment related effects were found in certain tissues, then specific tissues from the next lower dose level would be examined. All tissue changes received a severity grade where: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. Mean group severity scores for each change were determined by dividing the sum of the severity scores by the number of tissues examined in that group.

Statistical analyses The SPSS Statistical System was used to analyze the data for variance homogeneity. Homogenous data were analyzed using a One-Way Analysis of Variance (ANOVA), heterogeneous data were analyzed using the Kruskal-Wallis test, and the significance of inter-group differences between the control and treatment groups was assessed using Dunnett *t*-test for pair-wise comparisons to the control group. All statistical tests were performed at the $P < 0.05$ and $P < 0.01$ levels of significance.

Results

Acute oral toxicity

For both male and female Kunming rice fed with 15.0 g/kg *t*-RSV by ig administration, no toxicological effects were observed in 14-d period, in terms of body weight, food consumption, organ weight, gross and histologic pathology. Therefore, the acute oral toxicity MTD of *t*-RSV in Kunming mice is over 15.0 g/kg.

Ames test

No evidence of cytotoxicity (reduced rate of spontaneously occurring clones and visible thinning of the bacterial lawn) was observed at all testing dosage levels of *t*-RSV. The mean number of revertants per plate of *t*-RSV treatment groups at all dose levels for four strains of TA97, TA98, TA100, and TA102 with or without S-9, were negative. None of the treated groups has two folds or more revertant counts than the concurrent control and no dose-relationship was observed. The mean number of revertant clones of the negative control was within the historical range of the laboratory. The positive control mutagens induced the increases in revertant clones, confirming the validity of the assay. The results of Ames test indicate that *t*-RSV has no genetic toxicity (Table 1).

Mice bone marrow erythrocyte micronucleus trial

The micronucleus of the *t*-RSV treatment groups at all dose levels as well as the negative control group were significantly lower than those in positive control group treated with cyclophosphamide ($P < 0.01$), confirming the validity of the trial. There were no significant differences of micronucleus between the negative control group and the *t*-RSV treatment groups ($P > 0.05$). The PCE/NCE ratio of each group was within normal range. The result indicating that *t*-RSV is not mutagenic *in vivo* at the tested dosage range (Table 2).

Mice sperm abnormality trial

The sperm abnormality of the *t*-RSV treatment groups at all dose levels had no significant differences compared with the negative control group ($P > 0.05$). The sperm abnormality ratios in all treatment groups as well as the negative control group was significantly lower than those in positive control group treated with cyclophosphamide at 40 mg/kg ($P < 0.01$) (Table 3).

90-d Subchronic oral toxicity study

No decease, abnormal behaviors or treatment related toxic signs were observed for the mice throughout the experimental period. Table 4 shows the body weight, body weight gain and food consumption data from the 90-d study.

Table 1 Effects of *t*-RSV on Ames test

Groups	Dose / (μg·dish^{-1})	TA97		TA98		TA100		TA102	
		+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9
t-RSV	5000	152 ± 11	151 ± 16	37 ± 5	34 ± 4	176 ± 14	170 ± 19	284 ± 17	294 ± 21
	1000	150 ± 16	149 ± 12	37 ± 3	39 ± 6	168 ± 20	172 ± 14	295 ± 16	292 ± 22
	200	156 ± 11	155 ± 15	37 ± 7	36 ± 4	175 ± 15	172 ± 12	291 ± 18	292 ± 21
	40	156 ± 20	149 ± 10	39 ± 4	35 ± 5	171 ± 19	173 ± 18	285 ± 26	287 ± 15
	8	156 ± 14	154 ± 13	34 ± 5	38 ± 4	174 ± 20	175 ± 15	286 ± 25	294 ± 21
Concurrent	–	156 ± 14	151 ± 18	39 ± 4	37 ± 5	174 ± 14	170 ± 20	287 ± 16	294 ± 21
Solvent	–	156 ± 19	157 ± 10	36 ± 5	37 ± 6	175 ± 16	173 ± 19	291 ± 20	289 ± 15
Positive	–	1400 ± 239	1206 ± 210	2489 ± 321	2242 ± 245	2232 ± 251	2300 ± 287	1001 ±116	2358 ± 238

Note: The result was mean ± SD of 3 plates. Positive control: TA97 + S9, TA98 + S9, TA100 + S9 used 2-AF at 10.0 μg/dish
TA97 – S9, TA98 – S9 used 9-Fluorenone at 0.2 μg/plate; TA100 – S9 used NaN_3 at 1.5 μg/plate
TA102 +S9 used 1,8-dihydroxyanthraquinone at 50.0 μg/plate; TA102 –S9 used MMC at 0.5 μg/plate

Table 2 Effects of *t*-RSV on mice bone marrow micronucleus and PCE/NCE ratio (n = 5)

Sex	RSV Dose / (mg·kg^{-1})	Micronucleus ratio			PCE/NCE		
		Total PCE counted	Total micronucleus contain PCE	Micronucleus ratio ($\bar{X} \pm s$) / %	Total PCE counted	Total NCE identified	PCE/NCE ($\bar{X} \pm s$)
Male	7500	5000	5	0.1 ± 0.1	1000	860	1.164 ± 0.048
	3750	5000	4	0.08 ± 0.08	1000	881	1.137 ± 0.057
	1875	5000	6	0.12 ± 0.08	1000	865	1.158 ± 0.050
	0.000	5000	5	0.1 ± 0.1	1000	870	1.150 ± 0.019
	40 (cp)	5000	152	3.04 ± 0.63**	1000	1086	0.923 ± 0.048
Female	7500	5000	3	0.06 ± 0.09	1000	877	1.142 ± 0.044
	3750	5000	4	0.08 ± 0.11	1000	877	1.142 ± 0.049
	1875	5000	5	0.0 ± 0.07	1000	865	1.159 ± 0.063
	0.000	5000	5	0.1 ± 0.07	1000	873	1.149 ± 0.073
	40(cp)	5000	126	2.52 ± 0.43**	1000	1043	0.960 ± 0.038

**P < 0.01 *vs* negative group

Table 3 Effects of *t*-RSV on sperm counts and sperm morphology in mice (n = 5)

Dose / (mg·kg^{-1})	Total sperms counted	Total abnormal sperms	Abnormal ratios ($\bar{X} \pm s$) / %	Percentage of abnormal morphology ($\bar{X} \pm s$) / %						
				Ghost-like	Banna shape	Large round head	Amorphous	Kinks tail	Two heads	Two tails
7500	5000	105	2.10 ± 0.21	24.7 ± 4.2	23.9 ± 3.0	21.9 ± 3.4	29.6 ± 4.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3750	5000	97	1.94 ± 0.11	21.6 ± 1.5	26.6 ± 5.6	22.7 ± 2.5	29.1 ± 5.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1875	5000	101	2.02 ± 0.15	22.6 ± 5.0	24.6 ± 6.1	22.8 ± 5.6	29.9 ± 6.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.000	5000	102	2.04 ± 0.42	20.1 ± 4.7	21.1 ± 4.2	26.1 ± 4.9	32.7 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
40(cp)	5000	549	10.98 ± 0.69**	24.4 ± 2.6**	21.0 ± 2.0	20.6 ± 4.0	32.2 ± 3.9	0.9 ± 0.6	0.7 ± 0.4	0.96 ± 0.038

**P < 0.01 *vs* control group

Male and female rats' body weight as well as body weight gain in the experiment period had no significant differences (P > 0.05) except the body weight of a male rat group at day 35 (P < 0.05). Further analysis on the day-35 male rat body weight using Dunnett *t*-test that compares each dose level group with the control group shows no significant difference with or without RSV treatment (P > 0.05).

Food consumption of female rat intermediate dose group at week 11, intermediate and high dose groups at week-13 were significantly lower than that in the control group (P < 0.05 or P < 0.01). Food consumption and food

Table 4 Body weight and food consumption of rats in 90-d study

Dose / ($mg \cdot kg^{-1} \cdot d^{-1}$)	Male rats				*P* value	Female rats				*P* value
	Control	Low dose	Intermediate dose	High dose		Control	Low dose	Intermediate dose	High dose	
Body weight and body weight gain: ($\bar{X} \pm s$) / g										
Baseline	78.6± 7.7	78.8± 7.9	79.2± 6.4	79.5 ± 6.8	0.992	72.6± 9.1	72.7± 9.0	72.2± 7.3	72.7± 8.8	0.999
Week 1	122.2±10.3	123.7±13.3	127.3± 9.2	123.7±15.2	0.811	108.5±10.9	112.9±16.9	110.6± 7.8	112.1±15.5	0.891
Week 2	160.5±12.3	163.5±12.0	161.9±13.5	166.4±10.2	0.728	133.1±11.9	135.8±21.9	134.5± 9.2	132.1±17.6	0.958
Week 3	196.0±14.7	191.6±19.8	195.5±20.1	191.7±17.8	0.917	155.1±14.4	158.2±18.4	163.6±17.9	156.7±19.3	0.720
Week 4	228.0±14.6	213.5±21.7	226.1±14.8	218.9±18.9	0.251	173.5±16.7	171.2±16.2	179.5±11.5	172.8±17.9	0.668
Week 5	260.7±19.7	240.5±17.8a	261.1±21.1b	245.9±18.5c	a:0.065 b:1.000 c:0.222	190.0±16.0	194.7±18.4	200.8±16.1	188.6±24.9	0.489
Week 6	289.3±24.6	260.8±20.8	290.4±36.0	276.4±25.1	0.069	207.0±18.6	215.8±21.0	226.4±23.8	212.9±23.3	0.262
Week 7	310.1±25.5	286.0±23.3	311.1±30.2	295.7±28.4	0.132	216.1±16.8	225.8±17.2	231.6±28.7	218.7±27.6	0.441
Week 8	335.6±25.2	312.7±34.2	337.1±35.2	330.5±33.6	0.322	229.9±16.0	237.2±15.9	239.4±32.2	231.6±24.6	0.771
Week 9	356.6±30.0	333.7±35.5	365.4±41.4	358.9±26.3	0.189	244.9±15.3	251.0±25.8	258.2±38.5	244.0±21.6	0.619
Week 10	376.7±33.7	351.2±44.6	382.8±52.4	375.8±32.1	0.349	258.2±15.0	260.3±30.0	269.4±36.7	252.9±28.0	0.489
Week 11	396.9±38.6	375.9±53.3	401.2±63.0	389.8±32.6	0.666	268.1±19.2	268.5±28.9	273.2±41.8	258.7±26.4	0.750
Week 12	416.2±35.8	400.0±50.5	423.6±66.0	406.4±37.6	0.716	276.1±20.9	277.9±28.3	276.2±43.8	265.4±26.5	0.779
Week 13	429.6±37.8	423.9±55.8	432.8±68.6	415.5±35.8	0.883	282.1±26.5	283.3±29.2	277.5±42.8	269.8±28.1	0.785
BW Gain	351.0±42.2	345.1±52.4	353.6±68.0	336.0±40.4	0.877	209.5±29.8	210.7±29.9	205.2±38.4	197.2±23.7	0.758
Weekly feed intake: ($\bar{X} \pm s$) / g										
Week 1	107.6±14.8	110.9±14.6	116.6±12.0	110.7±19.6	0.629	99.9±17.5	107.9±15.7	108.5±15.6	109.7±20.1	0.581
Week 2	113.2±14.5	119.2±15.4	111.7±15.7	121.0±15.7	0.425	100.1± 5.9	100.0±14.9	102.1± 7.5	94.3± 7.1	0.191
Week 3	131.7±16.1	126.1±25.7	130.5±17.5	120.1±14.1	0.519	118.3±13.3	116.0±13.9	126.6±14.5	126.6±18.3	0.276
Week 4	137.2±15.1	126.8±22.1	140.7±20.1	133.5±17.7	0.411	123.9± 9.4	118.5± 7.5	125.2±18.8	125.9±17.7	0.649
Week 5	146.2±21.9	142.6±16.3	151.4±24.0	142.3±22.5	0.760	133.2±12.7	139.4±21.7	140.2±14.7	136.4±12.0	0.746
Week 6	165.7±19.8	157.8±18.0	168.1±18.7	171.2±22.6	0.481	147.4± 6.5	155.0±17.2	161.0±14.8	155.0± 9.2	0.108
Week 7	167.9± 9.9	169.8±19.7	167.7±19.4	165.6±20.0	0.962	149.7± 9.0	158.8±14.8	146.6± 7.8	153.5± 8.5	0.071
Week 8	177.8±14.9	183.1±30.2	185.3±31.3	203.5±25.3	0.164	153.0±16.8	152.5±14.7	155.7±12.3	153.4±12.4	0.959
Week 9	192.1±21.7	197.1±30.4	205.2±22.6	210.0±28.7	0.429	159.0±15.5	157.3±12.4	159.8±17.4	150.1±11.1	0.426
Week 10	187.8±27.5	191.1±29.1	185.7±26.7	188.8±26.1	0.976	163.6±18.2	154.9±11.4	162.0± 9.2	151.4±10.3	0.126
Week 11	191.8±19.2	202.6±31.8	189.8±31.4	188.2±15.1	0.586	160.5±12.5	147.5±11.9a	142.0±14.2 b**	149.4±11.7c	a:0.069 b:0.006 c:0.139
Week 12	193.5±27.0	198.8±14.6	198.4±11.2	186.4±12.6	0.362	158.5±10.1	164.5±10.6	150.6±16.3	161.8±12.3	0.093
Week 13	200.8±25.2	213.3±23.3a	181.2±11.6b	180.2±10.4c	a:0.326 b:0.067 c:0.052	164.8±15.4	151.9±11.3a	147.2±9.7 b**	146.8±13.3 c**	a:0.072 b:0.010 c:0.008
Total feed intake	2113.1±57.9	2139.1±94.2	2132.1±137.0	2121.3±73.8	0.932	1831.8±34.3	1824.2±41.4	1828.4±50.3	1814.4±30.1	0.782

**$P < 0.01$ *vs* control group

Single *P* value indicated that *P* value of four groups comparison, otherwise were Dunnett *t*-test by treatment group *vs* control group

a: p value in low dose group; b: p value in intermediate dose group; c: p value in high dose group

utilization of the male rats had no significant differences compared with the female rats at all other dose levels at other testing weeks ($P > 0.05$). No significant differences in total food consumption and total food utilization between the treatment and control groups.

All hematological parameters had no statistically significant differences between the *t*-RSV treatment and control groups in the middle and end periods of the 90-d study (Tables 5 and 6). All clinical chemistry measurements had no significant differences between the treatment and control groups in the middle (Table 7) and end (Table 8) periods of the 90-d study. However, the Cr levels of the intermediate and high dose groups of male rats and BUN of female rat low and intermediate dose groups on day 45 were significantly lower than those in the control group ($P < 0.05$ or $P < 0.01$).

No statistically significant differences in absolute organ weight and organ-to-body weight ratios (Fig. 1) were observed, except for that the liver-to-body weight ratio of the high dose group of male rats was significantly

Table 5 Midway (day-45) hematological results of 90-d feeding test ($\bar{X} \pm s$)

Items	Unit	*t*-RSV of male rats				*t*-RSV of female rats			
		Control	Low	Intermediate	High	Control	Low	Intermediate	High
Erythrocyte count	$10^{12}\cdot L^{-1}$	7.68 ± 0.43	7.67 ± 0.32	7.70 ± 0.69	7.73 ± 0.35	7.43 ± 0.25	7.48 ± 0.39	7.54 ± 0.45	7.28 ± 0.53
Hemoglobin concentration	$g\cdot L^{-1}$	141 ± 10	138 ± 6	144 ± 10	142 ± 7	137 ± 5	139 ± 5	141 ± 6	132 ± 6
Hematocrit	$L\cdot L^{-1}$	0.625 ± 0.047	0.619 ± 0.027	0.638 ± 0.048	0.633 ± 0.034	0.612 ± 0.026	0.616 ± 0.017	0.633 ± 0.028	0.589 ± 0.029
Total leukocyte count	$10^{9}\cdot L^{-1}$	15.7 ± 7.4	16.7 ± 8.0	16.6 ± 4.7	12.8 ± 3.7	13.2 ± 5.0	11.6 ± 4.5	12.2 ± 5.3	14.2 ± 3.0
Lymphocytes	%	71.0 ± 8.9	71.5 ± 9.2	74.5 ± 7.2	68.9 ± 7.6	73.0 ± 8.3	71.7 ± 6.1	70.6 ± 9.0	74.8 ± 4.7
Neutrophils	%	20.6 ± 7.7	21.2 ± 8.0	17.6 ± 6.8	22.7 ± 7.1	19.0 ± 9.2	20.5 ± 5.7	21.4 ± 7.5	16.4 ± 4.4
Monocytes	%	5.36 ± 1.31	4.77 ± 2.50	5.52 ± 1.00	5.70 ± 1.11	5.86 ± 1.48	5.53 ± 1.88	5.11 ± 1.54	6.47 ± 2.25
Eosinophils	%	1.20 ± 0.70	0.90 ± 0.56	0.57 ± 0.16	0.89 ± 0.39	0.82 ± 0.57	0.93 ± 0.54	1.22 ± 0.97	0.65 ± 0.49
Basophils	%	1.82 ± 0.75	1.64 ± 1.04	1.78 ± 0.91	1.80 ± 0.78	1.36 ± 0.43	1.36 ± 0.34	1.64 ± 0.82	1.71 ± 0.93
Platelet count	$10^{9}\cdot L^{-1}$	726 ± 150	647 ± 161	751 ± 46	619 ± 170	743 ± 233	708 ± 154	749 ± 194	699 ± 158

Table 6 Termination (day-90) hematological results of 90-d feeding test ($\bar{X} \pm s$)

Items	Unit	*t*-RSV of male rats				*t*-RSV of female rats			
		Control	Low	Intermediate	High	Control	Low	Intermediate	High
Erythrocyte count	$10^{12}\cdot L^{-1}$	8.32±0.60	8.26±0.67	8.23±0.57	8.33±0.43	7.64±0.35	7.81±0.57	7.61±0.57	7.59±0.84
Hemoglobin concentration	$g\cdot L^{-1}$	148±11	145±9	146±7	150±8	143±7	145±5	139±12	138±12
Hematocrit	$L\cdot L^{-1}$	0.659±0.058	0.644±0.031	0.653±0.028	0.667±0.038	0.634±0.029	0.645±0.023	0.620±0.052	0.613±0.05
Total leukocyte count	$10^{9}\cdot L^{-1}$	13.4±5.5	12.3±5.9	12.1±7.3	12.1±4.1	9.6±1.5	9.8±2.4	10.1±3.0	10.4±3.3
Lymphocytes	%	69.6±10.5	67.4±10.3	69.4±8.2	67.9±4.8	73.3±5.7	73.2±5.6	67.3±9.3	70.0±6.8
Neutrophils	%	24.1±7.0	24.0±7.7	23.0±8.0	24.2±3.3	18.8±5.4	20.3±5.7	24.8±8.0	22.1±5.8
Monocytes	%	3.96±3.23	5.33±3.38	5.31±2.64	5.32±2.29	5.15±2.22	4.36±1.70	5.52±1.75	5.87±1.91
Eosinophils	%	1.18±0.84	1.63±1.11	1.05±0.58	0.92±0.43	1.55±1.13	1.26±0.71	0.94±0.97	0.71±0.33
Basophils	%	1.21±0.83	1.61±0.85	1.32±0.81	1.63±1.01	1.19±0.52	0.88±0.30	1.40±0.56	1.24±0.48
Platelet count	$10^{9}\cdot L^{-1}$	863±178	802±142	752±287	618±296	750±210	716±121	819±186	867±111

Table 7 Midway (day-45) clinical results of 90-d feeding test ($\bar{X} \pm s$)

Items	Unit	*t*-RSV of male rats				*t*-RSV of female rats			
		Control	Low	Intermediate	High	Control	Low	Intermediate	High
Alanine aminotransferase	U·L^{-1}	42.88 ± 6.22	47.13 ± 5.63	50.45 ± 7.80	48.47 ± 8.08	41.32 ± 7.83	36.87 ± 4.69	45.61 ± 12.35	42.97 ± 8.34
Aspartate aminotransferase	U·L^{-1}	181.48 ± 27.67	195.84 ± 28.43	200.88 ± 30.97	185.82 ± 26.66	176.00 ± 22.84	178.94 ± 21.73	180.82 ± 27.05	192.18 ± 27.05
Total protein	g·L^{-1}	71.61 ± 3.96	70.71 ± 3.58	73.76 ± 4.86	70.42 ± 1.89	73.77 ± 4.07	73.82 ± 2.44	74.37 ± 3.78	74.04 ± 5.22
Albumin	g·L^{-1}	35.90 ± 1.84	34.47 ± 2.34	37.43 ± 2.57	36.42 ± 1.44	36.01 ± 1.31	36.51 ± 1.86	37.91 ± 2.12	36.38 ± 2.44
Cholesterol	mmol·L^{-1}	1.70 ± 0.24	1.80 ± 0.25	1.89 ± 0.40	1.76 ± 0.33	1.99 ± 0.39	1.96 ± 0.46	2.05 ± 0.29	2.13 ± 0.43
Triglyceride	mmol·L^{-1}	0.93 ± 0.28	1.13 ± 0.43	1.04 ± 0.23	0.98 ± 0.39	1.09 ± 0.43	0.92 ± 0.24	0.81 ± 0.20	1.23 ± 0.50
Urea Nitrogen	mmol·L^{-1}	5.75 ± 1.52	5.39 ± 1.39	5.00 ± 0.73	4.75 ± 0.70	6.94 ± 1.06	5.79 ± 1.18*	5.86 ± 0.66*	6.35 ± 0.72
Creatinine	μmol·L^{-1}	45.42 ± 2.35	43.46 ± 2.69	42.01 ± 3.27*	41.68 ± 2.09**	47.63 ± 4.02	45.16 ± 2.95	47.80 ± 1.65	45.92 ± 3.29
Blood Glucose	mmol·L^{-1}	4.50 ± 0.68	4.37 ± 0.73	4.28 ± 0.49	4.56 ± 0.50	4.70 ± 0.43	4.39 ± 0.58	4.65 ± 0.55	4.67 ± 0.80

*$P < 0.05$; **$P < 0.01$ *vs* control group

Table 8 Termination (day-90) clinical results of 90-d feeding test ($\bar{X} \pm s$)

Items	Unit	*t*-RSV of male rats				*t*-RSV of female rats			
		Control	Low	Intermediate	High	Control	Low	Intermediate	High
Alanine aminotransferase	U·L^{-1}	45.31 ± 8.27	44.95 ± 8.06	55.58 ± 14.06	50.19 ± 10.85	38.64 ± 6.64	43.54 ± 12.10	36.61 ± 5.58	39.46 ± 7.78
Aspartate aminotransferase	U·L^{-1}	175.50 ± 34.66	152.04 ± 30.83	148.64 ± 33.88	144.88 ± 21.20	166.28 ± 30.97	174.69 ± 31.33	152.58 ± 31.77	143.84 ± 19.04
Total protein	g·L^{-1}	75.01 ± 4.62	74.16 ± 4.34	71.62 ± 5.38	72.82 ± 4.71	72.99 ± 2.03	73.49 ± 4.90	71.64 ± 5.53	73.96 ± 3.41
Albumin	g·L^{-1}	37.08 ± 2.46	35.14 ± 1.99	36.07 ± 2.51	35.83 ± 3.01	37.11 ± 2.31	38.85 ± 2.58	35.96 ± 4.89	38.19 ± 2.03
Cholesterol	mmol·L^{-1}	1.65 ± 0.33	1.70 ± 0.34	1.48 ± 0.28	1.53 ± 0.25	1.88 ± 0.52	2.01 ± 0.52	1.76 ± 0.54	1.93 ± 0.47
Triglyceride	mmol·L^{-1}	1.12 ± 0.38	1.38 ± 0.76	1.14 ± 0.21	1.14 ± 0.51	0.91 ± 0.22	0.98 ± 0.34	0.65 ± 0.18	0.96 ± 0.24
Urea Nitrogen	mmol·L^{-1}	6.23 ± 0.93	5.70 ± 0.74	6.14 ± 1.00	6.13 ± 0.84	7.12 ± 1.66	7.01 ± 1.48	8.30 ± 2.81	8.15 ± 1.47
Creatinine	μmol·L^{-1}	54.09 ± 4.06	50.78 ± 2.97	51.28 ± 5.54	49.58 ± 3.18	56.98 ± 3.73	53.92 ± 3.58	54.77 ± 7.38	55.62 ± 3.75
Blood Glucose	mmol·L^{-1}	4.54 ± 0.71	4.45 ± 0.35	4.68 ± 0.65	5.09 ± 0.81	4.86 ± 0.28	5.10 ± 0.61	4.65 ± 0.60	4.97 ± 0.59

higher than that of the control group ($P < 0.05$).

In the tests of gross anatomy, the size, color, and shape of heart, lung, liver, spleen, kidney, stomach, intestine, testis, and ovary of all the tested rats were normal, as evaluated by eye observation. The pathological examination was conducted by high dose group *vs* the control group only. No pathological changes of liver (Table 9), kidney (Table 10), spleen, intestine, stomach, testis, and ovary tissues were observed for the high dose group (data not shown), *vs* the control group. In summary, no toxic side effects were observed in the 90-d study.

Discussion

To obtain first hand information of the acute oral toxicity of *t*-RSV, we tested the MTD since *t*-RSV is not known to be toxic or causes adverse effects in human beings from literature search. For male and female Kunming mice at 15 g/kg, the absence of symptoms, the lack of negative effect on growth, and the

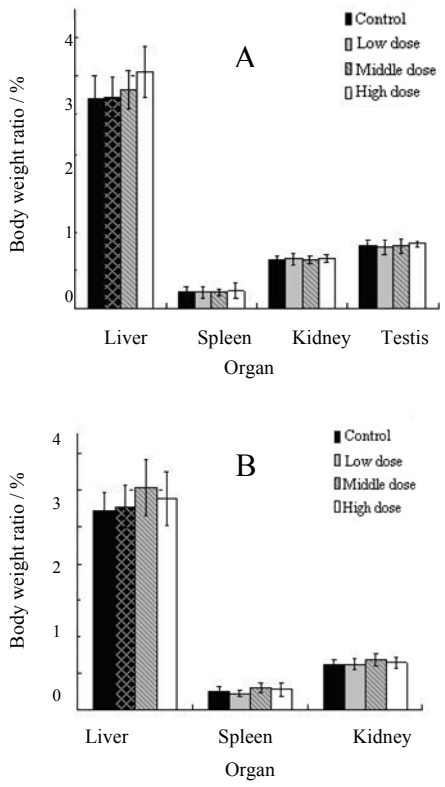

Fig. 1 Influence of RSV on body weight ratio

A: Males B: Females

normal appearance of live organs in the gross anatomy suggested that *t*-RSV is practically nontoxic under these acute oral toxicity assay conditions. The MTD of *t*-RSV in mice is over 15 g/kg. According to the *Toxicity Classes of Hodge and Sterner Scale*, the tested substance, *t*-RSV, is considered harmless and nontoxic grade material.

We choose ICH to optimize two battery approaches using Ames test and two *in vivo* assays for genotoxicity evaluation, to confirm or reject the early evidence of genotoxicity of RSV *in vitro* in mammalian cells (Schmitt *et al*, 2002; Matsuoka *et al*, 2001). Extensive reviews have shown that a positive result of Ames test likely indicates that a compound is a carcinogen for rodent animals. The *in vitro* mammalian tests not only increase sensitivity and broaden the spectra of genetic events detected, but also decrease the specificity of prediction. Our results demonstrate that *t*-RSV is neither cytotoxic nor mutagenic at 5000 mcg/plate-the ICH recommended maximum test dosage level, and other diluted dosage levels for *S. typhimurium* TA97, TA98, TA100, and TA102 in the absence and presence of a microsomal metabolizing system. The trials of the *in vivo* bone marrow erythrocyte micronucleus and sperm abnormality of Kunming mice at 7.5 g/kg *t*-RSV do not increase the numbers of micronucleated PCEs and abnormal sperms under the experiment conditions, nor affect the proportion of PCEs to total erythrocytes. RSV by ig administration is well absorbed (Crowell *et al*, 2004; Gescher and Steward, 2003) and daily ig administration of RSV to adult male rats was reported to enhance spermatogenesis (Juan *et al*, 2005). Therefore, there is no doubt that the oral dosed resveratrol has reached the target tissue. The two *in vivo* negative results in combination with the negative Ames test results suggest that *t*-RSV has no genotoxicity under the testing conditions.

In the sub-chronic toxicity study, the *t*-RSV dosage was made on the basis of a 28-d trial, in which RSV (0, 300, 1000, and 3000 mg/(kg·d) were administered to rats. The group administered RSV 3000 mg/(kg·d) resulted in nephrotoxicity, and the RSV 1000 mg/(kg·d)

Table 9 Liver pathological examination of 90-d feeding study

Pathological changes		Male rats		Female rates	
		High dose	Control	High dose	Control
Heptocyte	Fatty degeneration	0/10	0/10	0/10	0/10
	Necrosis	0/10	0/10	1/10	1/10
	Inflammatory cell infiltration	0/10	0/10	1/10	1/10
Interstitial tissue	Vascular dilatation	0/10	0/10	0/10	0/10
	Inflammatory cell infiltration	0/10	0/10	0/10	0/10
	Fibrosis	0/10	0/10	0/10	0/10

Note: Observed pathological changes shown by 0/10 means 0 out of 10
Found pathological changes shown by 1/10 indicated 1 out of 10, Table 10 is the same

Table 10 Kidneys pathological examination of 90-d feeding study

Pathological changes		Male rats		Female rats	
		High dose	Control	High dose	Control
Glomerulus	Degeneration	0/10	0/10	0/10	0/10
	Necrosis	0/10	0/10	0/10	0/10
	Inflammatory cell infiltration	0/10	0/10	0/10	0/10
Renal tubules	Swelling	0/10	0/10	0/10	0/10
	Necrosis	0/10	0/10	0/10	0/10
	Inflammatory cell infiltration	0/10	0/10	0/10	0/10
Interstitial tissue	Vascular dilatation	0/10	0/10	0/10	0/10
	Inflammatory cell infiltration	0/10	0/10	1/10	0/10
	Fibrosis	0/10	0/10	0/10	0/10

reduced body weight gain (females) and elevated WBC (males).

The dosage for NOAEL was reported to be 300 mg/(kg·d) (Crowell *et al*, 2004). In this study, three dosages, 167, 333, and 500 mg/kg, were given to rats daily for consecutive 90 d to evaluate the sub-chronic toxicity at lower dosage levels and a longer period of time.

Crowell demonstrated that the administration of RSV 3000 mg/(kg·d) for 28 d resulted in nephrotoxicity, which was shown with significantly elevated serum BUN and Cr levels, kidney weight increasing, gross and histopathological changes in the kidneys (Crowell *et al*, 2004). Our 90-d study showed that the body weight, food consumption, food utilization index, midway (day-45), and termination (day-90) hematological tests in both male and female rats of each testing dose level group had no statistically significant differences compared with the control group ($P > 0.05$). Most of blood chemistry parameters had no statistically significant differences in comparison with the control group. However, the blood Cr of the 333 and 500 mg/kg male rat groups and the blood urea nitrogen of the 167 and 333 mg/kg female rat groups were significantly lower than those of the control groups ($P < 0.05$ or $P < 0.01$) at midway of the experiment. The lower BUN or Cr of the treatment groups indicated the function of *t*-RSV on the renal protection rather than nephrotoxicity. Necropsy and pathological examination showed no observable kidney abnormalities, suggesting that *t*-RSV in the tested dosage range of 167–500 mg/kg for 90 d has no nephrotoxicity.

In a 4-week rat toxicity study Juan reported that administration of RSV (20 mg/kg) resulted in mild changes in serum liver enzyme AST levels (Juan, Vinardell, and Planas, 2002). In this study, a significant change of the liver-to-body weight ratio was observed in the high dose group of male rats, but no significant changes in serum liver enzyme levels (AST and ALT) and no liver histological changes were observed in all dose levels. Our findings are consistent with the results of Crowell, in which no liver toxicity at 15-fold higher dosage than used by Juan was observed (Crowell *et al*, 2004). Our study suggests that *t*-RSV administered at the tested dosage within the range of 167–500 mg/kg for 90 d has no hepotoxicity.

Food consumption of the female rat intermediate dose group at week-11 and the intermediate and high dose groups at week-13 was significantly lower than that of the control group ($P < 0.05$ or $P < 0.01$). Although there were no significant differences in total food consumption and total food utilization between the treatment and control groups, the final body weight and body weight gain of the high dose female rat group were about 4.4% and 5.9% lower than those of the control groups. The final body weight and body weight gain reduction was apparently not statistically significant, but in agreement with the results of Crowell, in which a mild reduction in final body weight of approximately 5% and in weight gain of 14% ($P < 0.05$)

in the females after treated with RSV (1000 mg/kg) for 28 d was observed (Crowell *et al*, 2004). Toxic effects of *t*-RSV in longer period or at a larger dosage might need to be examined.

The lipoprotein profile *in vivo* was not affected by the ig administration of *t*-RSV (167–500 mg/kg), and was consistent with the findings of Turrens in which a daily ip injection of *t*-RSV at two doses, 20 and 40 mg/kg for 21 d. Their treatments did not alter the proportion of cholesterol bound to high density lipoprotein (HDL) or low density lipoprotein (LDL) (Turrens, Lariccia, and Nair, 1997). Some of the health benefits of red wine have been attributed to the presence of *t*-RSV, such as a decrease in LDL, an increase in HDL, and reduced coagulation. However, none of these indeices were significantly affected by high doses of *t*-RSV in this *in vivo* study on the health rats.

In a review article, Gescher (Gescher and Stewad, 2003) attempted to determine an equivalent human dosage to reproduce the apparent cancer "chemo-preventive" benefits of RSV observed *in vitro* and *in vivo* animal studies. The authors found that most animal studies used dosage ranging from 1.0 to 100 mg/(kg·d), which would equate to a daily human dose of about 10–1000 mg/d for a 60 kg adult according to the animal to human dosage convert formula.

In summary, the lack of acute toxicity, no mutagenic effects and lack of harmful effects found in the hematology, clinical chemistry and histopatology in this safety assessment indicate that *t*-RSV has a large safety margin.

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