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Safety Assessment of Musk Substitute from Nutria of *Myocastor Coypus* in Mice

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ABSTRACT

Musk is an important crude drug in Traditional Chinese Medicine and is used in the manufacture of perfume. Traditionally, the gland was picked after killing the male deer. As a highly valued ingredient of Chinese medical remedies, the wide use of this substance has made this animal near the edge of extinction. Hence it is necessary to find a substitute for the same or similar use. The gland from nutria (*Myocastor coypus*) has been used as a musk substitute in some Taiwan areas. To assess the safety of nutria gland extract (NE), we carried out this study.

For the safety evaluation, we have completed the animal toxicity testing including the single dose oral acute and repeated dose subacute toxicity, and genotoxicity. At 5 g/Kg dosage, no lethality and obvious acute toxicity were observed. At repeated treatment with NE 1 g/Kg for 28 days, no subacute toxicity was noticed. Genotoxicity was accessed by the Ames test in *Salmonella typhimurium* strains TA97, TA98, TA100, TA102 and TA1535. Result indicated that NE did not induce revertant increase in bacteria.

Results of the current study support the safety of the use of NE, and the acute, subacute toxicity and genotoxicity are not observed in this study.

Key words: Nutria, Chemical analysis, Muscone, Safety Assessment

INTRODUCTION

The general pharmacological properties of musk were studied for hundreds years. From the first report regarding the male sex hormonal modification in 1936[1,2], there are numerous pharmacological actions listed in the literatures, such as, cardiovascular stimulation[3,4], anti-inflammatory action[5-9], protection of CCl_4-induced acute hepatitis[10], inhibition of leukocyte migration[9] and the platelet aggregation induced by collagen but not ADP and AA in rats[11] and the potentiation of β-adrenergic effect [12].

Since the consumption of musk cannot be met by natural musk, and the increasing use of this substance for medicinal proprietaries has made this animal near the edge of extinction. From 1973, its usage has been limited by the Convention on International Trade in Endangered Species of Wild Fauns and Flora. Hence it is necessary to find a substitute for the same or similar use. Nowadays, for commercial purposes, large amount of synthetic musks are produced worldwide, due to the high cost and uncertainty of supply of the natural musk. In medicinal proprietaries, the gland from nutria (*Myocastor coypus*) has been used as a musk substitute in Traditional Chinese Medicine in Taiwan, in order to protect the endangered species.

From chemical analysis, Muscone ((*R*)-3-methylcyclopentadecanone), an odoriferous secretion, is believed to be the active ingredient. In addition, small amount of muscopyridine is also found in the secretion. Common counterfeit ingredients are musk xylene, musk ambrette, musk ketone, and diphenhydramine[13]. To study the therapeutic effects of the series of musky compounds became the main stream for searching the substitute of natural musk since 1980's. Muskone has significant therapeutic effect on myocardial infarction[14], estrogenic like activity[15], induction of cytochrome P450[16,17] and positive psychological effects on human[18].

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Here we evaluted the safty of a substitute of musk from nutria (*Myocastor coypus*), which has been used as a musk substitute in Traditional Chinese Medicine in Taiwan. Unlike other classes of synthetic musks currently on the market, there is no toxicological study of nature musk compounds available in literature. For the safety evaluation, we have completed the animal toxicity tests for the musk from nutria including the single dose oral acute and repeated dose subacute toxicity, and genotoxicity.

MATERIALS AND METHODS

I. *Preparation of NE (Nutria extraction)*

The nutria gland extract (NE) was prepared from the gland of *Myocastor Coypus*. Its identity was comfirmed by Dr. Shoei-Sheng Lee (School of Pharmacy, National Taiwan University, Taipei, Taiwan). The GC-MS technique was applied to analyze the volatile ingredients in the gland secretion or the gland extract. The good resolution of gas chromatography coupled with the VOC MS data bank allowed efficient identification of the ingredients. For confirmation of major ingredients and some structural isomers, six authentic samples including two prepared were subjected to the same GC-MS system. Stock solutions of the NE were prepared daily by dissolving the concentrated NE in corn oil for gastric gavage, or in DMSO for Ames test. The DMSO was kept in 0.1%.

II. *Chemicals*

D(+)-glucose and sodium chloride were obtained from Merck (Merck, Darmstadt, Germany). Glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (β-NADP), potassium phospate dibasic anhydrous (K_2HPO_4), colcemid, benzo(a)pyrene, Alcolor 1254, and the positive control chemicals for Ames test, 9-Aminoacridine, 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2AA), mitomycin C and sodium azide were obtained from Sigma Chem. Co. (St. Louis, MO, USA) All other chemicals were of reagent grades and obtained from Sigma Chemical Co. (St. Louis, MO). *Salmonella* strains TA97, TA98, TA100, TA102 and TA1535 were purchased from MOLTOX (Molecular Toxicology, Annapolis, MD, USA).

III. *S-9 Fraction Preparation*

Rat liver S9 used for metabolic activation was prepared according to the method of Maron and Ames (1983)[19] and Matsuoka *et al.,* (1979)[20]. In order to obtain liver microsomal fraction, each rat was injected i.p. with 30 mg Acrolor 1254/kg body weight every day and four days later they were killed by cervical dislocation. The livers were homogenated, diluted × 4 with 0.15 M KCl and centrifuge for 10 min at 900 g. The supernatant was collected and diluted to give a protein concentration of 30 mg/mL, frozen in small aliquots and stored at -70 to -80°C until use. The final preparation of the metabolizing system (S9 mixture) was made in accordance with the protocol of Ames *et al.,* (1975)[21]. Three mL of S9 was mixed with 7 mL of co-factor solution which consists of 2 mL of 20 mM HEPES buffer (pH 7.2), 1 mL of 50 mM $MgCl_2$, 1 mL of 330 mM KCl, 1 mL of 50 mM glucose-6-phosphste, 1 mL of 40 mM nicotinamide adenine dinucleotide phosphate, and 1 mL of distilled water. The S9 solution was prepared separately for each experiment, and it was discarded if the duration exceeded 4 h.

IV. *Ames Salmonella/Microsome Test*

The method used followed the recommendations by Maron and Ames, (1983)[19] and the Organization for Economic Cooperation and Development (OECD) guideline (1997)[22]. The *Salmonella typhimurium* bacteria, histidine auxotrophic strains TA97 (hisD6610/rfa/ΔuvrB/pKM101), TA98 (hisD3052/rfa/△uvrB/pKM101), TA100 (hisG46/rfa/ΔuvrB/pKM101), TA102 (hisG428/rfa/pKM101/pAQ1) and TA1535 (hisG46/rfa/ΔuvrB) were purchased from MOLTOX (Annapolis, MD, USA) and grown for 14 h at 35 ± 2°C with continuous shaking. Bacteria were grown to a density of 1-2 × 10^8 cells/mL with OD600 absorbance between 0.2 - 0.3. Top agar containing 2 mL of heated agar, 0.1 mL of test chemical, 0.1 mL of bacteria, and 0.5 mL of S9 solution were mixed and added to three different minimal glucose agar plates. All plates were incubated at 37°C for 48 h, and the number of bacteria colonies was determined. The entire experiment was replicated again on a different day for a total of six plates with each concentrations of nutria gland (NE) with and without S9. S9 liver cell extracts contain enzymes that may activate the potential mutagen. Each tester strain was checked routinely to confirm their features for optimal response to known mutagenic chemicals as follow: 4-NQO (0.5 μg/plate), mitomycin c (0.5 μg/plate), AAF (5 μg/plate). The test compounds, 100 μL portions, were pre-incubated with the S9-mix at 37°C before addition of the bacteria and top agar. A test compound was judged to be mutagenic in the plate test if it produces, in at least one concentration and one strain, a response equal to twice (or more) the control incidence with a dose-response relationship was considered as positive mutagen[23,24]. The only exception is strain TA 102, which has a relatively high spontaneous revertant number, where an increase by a factor of 1.5 above the control level is taken as an indication of a mutagenic effect.

V. *Animal Treatment*

Actue and subacute toxicity study of NE were conducted based on the test guideline of US Environmental Protection Agency (EPA, 2000)[25], and OECD guideline (OECD, 2001)[26]. All procedures involving the use of animal were undertaken with the Animal Ethics

Committee, Taipei Medical University. Five weeks old ICR mice (20 g) were divided into 8 groups of 5 animals each (Male and Female). They were housed in individual Health Guard-cages in a 12-h light/dark cycle, 22 ± 1°C, 50 - 70% humidity room and were given food (Rodent Laboratory Chow 5001, Purina, St. Louis, MO, USA) and water (Ion reverse water, Millipore, Billerica, MA, USA) *ad libitum*. After 1 week of acclimatization, the groups were administered, by gavage, Nutria gland (NE) in corn oil at three of the following doses: 0.1 g/kg body weight (low), 0.5 g/kg body (medium), and 1.0 g/kg body weight (high). The oral administration was performed daily, 7 days/week, for 4 consecutive weeks. Control animals were administered corn oil (1.0 mL/100 g body weight) only. Body weight and food consumption were measured weekly. Overnight urine was collected from individual animals at the end of the fourth week. All urine analyte concentrations were measured with AMES Reagent Strips combined with URISYS 2400 analyzer (Roche Diagnostics, USA & Gemany). At termination, the animals were anesthetized with inhaled ether. Blood was withdrawn from the abdominal aorta for hematological analysis and serum clinical chemistry. The brain, heart, thymus, liver, kidneys, spleen, adrenal and testis/ovary were excised and weighed, and were preserved in phosphate-buffered formalin. The fixed tissues were dehydrated with graded alcohol, cleared, and impregnated in paraffin. The paraffin blocks were sectioned to 5 μm thickness and stained with hematoxylin and eosin for microscopic examination (Opticphot-2, Nikon, Tokyo, Japan).

VI. *Hematological and Biochemical Analysis*

A Sysmec-450 hematology analyzer (Tokyo, Japan) was used for the determination of the following hematological parameters: erythrocyte count (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin, platelet count (PLT), white blood cell count (WBC). Differential leukocyte count (percentages of lymphocytes), such as neutrophil, monocyte, eosinophil and basophil were calculated with Weigert's Iron Hematoxylin Stain Kit (A.J.P. Scientific Inc., Clifton, NJ, USA). An automated coagulated analyzer (CA-550, System Corporation, Kobe, Japan) was used to detected the clotting time (sec.) and amount (mg/dl) of fibrinogen (Fbg). Express Plus Automatic Clinical Chemistry Analyzer (Chiron Diagnostics Corporation, Oberlin, OH, USA) was used to obtain the following serum chemistry parameters: aspartate aminotransferase activity (AST), alinine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine.

VII. *Urinalysis*

Urinalysis was performed before allocation cages without food and water, and 24-h urine outputs were collected. Glucose (Glu), Bilirubin (Bil), Ketone (Ket), Specific gravity (S.G), pH, Protein (Pro), Urobilinogen (Uro), Nitrite (Nit), Occult blood (Occ), Leukocyte (Leu), Appearance (apr) and urine volume were detected with URISYS 2400 analyzer (Roche Diagnostics, USA & Gemany). Leukocyte, white blood cells, crystal and parasite were detected with technician.

VIII. *Statistical Analysis*

The significance of the results was analyzed by Student's *t*-test for unpaired observations. The results were expressed as Mean ± standard error of mean (S.E.M.), and one-way ANOVA. Duncan's multiple range test were used to identify groups that were significantly different from the control at the $p < 0.05$ level. The values of statistical significance being set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ levels.

RESULTS

I. *Acute Toxicity*

No death was recorded in the 14 days of observation period in the male and female animals given 5 g/kg bw of the NE orally. The animals did not show any changes in the general appearance during the observation period. Table 1 shows similar body weight gain at 7 and 14 days in control and NE (5 mg/kg bw)-treated male and female mice.

Table 1. The body weight and body weight gain of mice treated with NE by gastric gavage.

	Dose	Body weight (g)			B.W. gain (%)	
	(g/Kg)	0-day	7-day	14-day	(7-day)	(14-day)
Male	0	24.4 ± 0.3	26.8 ± 0.7	30.0 ± 1.0	9.6 ± 2.8	22.6 ± 4.0
Female	0	24.5 ± 0.3	26.3 ± 0.5	29.0 ± 1.0	7.5 ± 2.4	18.4 ± 4.2
Male	5	23.9 ± 0.6	25.7 ± 0.7	28.1 ± 0.9	7.2 ± 2.4	17.5 ± 2.0
Female	5	23.4 ± 0.2	25.0 ± 0.4	27.4 ± 0.5	7.2 ± 2.6	17.0 ± 1.3

Data were expressed as mean ± S.E. (n = 10)

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Table 2. The food consumption of mice treated with NE by gastric gavage daily for 28 days

	Dose (g/kg)	Food consumption per day (g)			
		1st week	2nd week	3rd week	4th week
Male	0	3.86 ± 0.21[1)]	4.44 ± 0.49	5.37 ± 0.26	4.56 ± 0.18
	0.1	3.95 ± 0.83	3.99 ± 0.20	5.15 ± 0.61	3.28 ± 1.13
	0.5	4.18 ± 0.61	4.27 ± 0.86	4.55 ± 0.59	3.27 ± 0.62
	1.0	5.35 ± 0.03*	4.84 ± 0.61	5.66 ± 0.01	4.08 ± 0.46
Female	0	5.18 ± 0.31	4.22 ± 0.09	4.86 ± 0.34	3.30 ± 0.70
	0.1	4.08 ± 0.21	5.20 ± 0.37	4.39 ± 0.09	3.45 ± 0.29
	0.5	5.44 ± 0.18	4.52 ± 0.04	4.13 ± 0.01	3.19 ± 0.18
	1.0	4.92 ± 0.11	3.84 ± 0.02	4.61 ± 0.53	3.64 ± 0.28

[1)] Data were expressed as mean ± SE (n = 10).
[2)] * Significant difference between control treated group at *: $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student *t*-test.

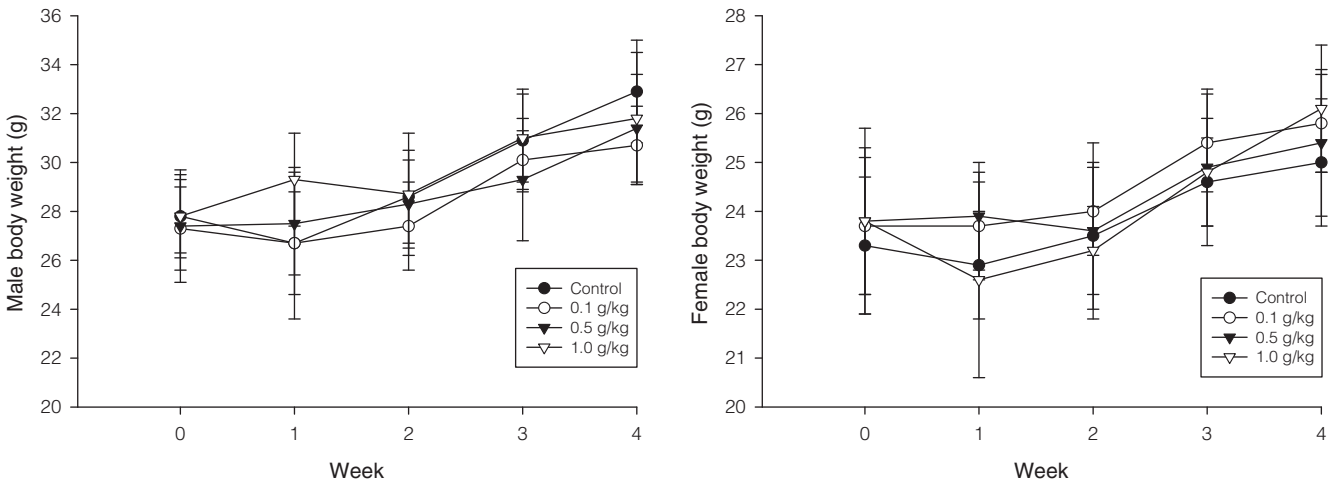

Figure 1. The body weight change of of mice treated with NE by gastric gavage daily for 28 days. (A) Male, (B) Female. Data were expressed as mean ± SE (n = 10). Significant difference between control treated group at *: $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control by Student *t*-test.

II. *Subacute Toxicity*

General observation, effect on body weight, organ weight and food consumption

Food consumption and body weight gain of animals treated daily with NE for 4 weeks did not differ significantly from the control animals both in male and female mice (Table 2; Figure 1). None of the treated animals exhibited clinical signs of toxicity. The relative weights of the brain, heart, liver, spleen, and kidneys, thymus, adrenal, and testes/ovary were unaffected by treatment (Table 3). Averaged daily food consumption significantly increased in male mice treated with 1 g/kg of NE at first week (Table 2).

III. *Hematological and Biochemical Analysis*

Lymphocyte count was significantly decreased when male animals receiving the medium (0.5 g/kg bw) and high (1.0 g/kg bw) dose of NE. The monocyte count, however was significantly increased in male animals receiving the medium (0.5 g/kg bw) and high (1.0 g/kg bw) dose of NE (Table 4). RBC, HGB, HCT, MCV, MCH, MCHC PLT and WBC were not significantly different from those of control. Eosinophil and basophil were non-detectable. No treatment-related changes were observed in serum creatinine, BUN, AST, ALT and fibrogen (Table 5).

IV. *Urinalysis*

There were no significant treatment-related changes in urinary sediments, glucose, Bil, Ketone, S.G, pH, Pro, Uro, Nit Occ, Leu, apr and urine volume (Table 6).

V. *Histopathology Fundings*

Table 3. The organ weight change of mice treated with NE by gastric gavage daily for 28 days

	Dose (g/Kg)	Organ Weight (%)							
		Brain	Thymus	Heart	Liver	Spleen	Adrenal	Kidney	Testes
Male	0	1.24 ± 0.09	0.060 ± 0.006	0.44 ± 0.01	3.76 ± 0.08	0.55 ± 0.14	0.028 ± 0.003	1.22 ± 0.03	0.59 ± 0.02
	0.1	1.32 ± 0.09	0.060 ± 0.006	0.44 ± 0.02	3.73 ± 0.16	0.49 ± 0.09	0.022 ± 0.002	1.19 ± 0.03	0.60 ± 0.02
	0.5	1.44 ± 0.04	0.057 ± 0.010	0.45 ± 0.02	3.68 ± 0.12	0.47 ± 0.12	0.033 ± 0.003	1.26 ± 0.06	0.58 ± 0.01
	1.0	1.34 ± 0.03	0.081 ± 0.011	0.45 ± 0.02	3.65 ± 0.14	0.44 ± 0.07	0.032 ± 0.004	1.21 ± 0.05	0.61 ± 0.03
									Ovary
Female	0	1.79 ± 0.05	0.218 ± 0.014	0.47 ± 0.01	3.54 ± 0.07	0.33 ± 0.02	0.036 ± 0.003	1.02 ± 0.05	0.057 ± 0.000
	0.1	1.67 ± 0.04	0.185 ± 0.020	0.50 ± 0.03	3.38 ± 0.12	0.28 ± 0.02	0.049 ± 0.005	0.94 ± 0.04	0.089 ± 0.021
	0.5	1.78 ± 0.05	0.199 ± 0.012	0.49 ± 0.01	3.65 ± 0.04	0.36 ± 0.08	0.048 ± 0.005	1.03 ± 0.02	0.055 ± 0.007
	1.0	1.74 ± 0.03	0.173 ± 0.026	0.45 ± 0.01	3.58 ± 0.16	0.29 ± 0.03	0.038 ± 0.005	1.05 ± 0.02	0.048 ± 0.005

Data were expressed as mean ± S.E. (n = 10)
*Significant difference between control treated group at *: $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student *t*-test.

Table 4. The changes of hematological parameter in mice treated with NE by gastric gavage daily for 28 days.

	Dose (g/Kg)	RBC (10^6/μL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (10^3/μL)
Male	0	7.06 ± 0.32	12.04 ± 0.57	39.52 ± 1.98	55.95 ± 0.78	17.09 ± 0.39	30.53 ± 0.37	754.5 ± 48.8
	0.1	6.59 ± 0.40	11.23 ± 0.72	38.39 ± 2.37	58.16 ± 0.59	17.00 ± 0.19	29.22 ± 0.16	794.8 ± 65.4
	0.5	6.82 ± 0.17	11.31 ± 0.32	38.60 ± 0.84	56.57 ± 0.69	16.57 ± 0.37	29.28 ± 0.55	793.8 ± 124.0
	1.0	6.60 ± 0.23	11.45 ± 0.57	37.80 ± 1.11	57.34 ± 0.67	16.87 ± 0.28	29.44 ± 0.26	817.1 ± 114.1
Female	0	6.92 ± 0.21	12.06 ± 0.34	38.61 ± 1.21	55.42 ± 0.34	17.32 ± 0.11	31.27 ± 0.17	582.3 ± 45.0
	0.1	6.30 ± 0.27	11.75 ± 10.1	36.12 ± 1.85	57.16 ± 0.82	18.15 ± 0.21	31.77 ± 0.27	549.4 ± 49.9
	0.5	7.01 ± 0.19	12.54 ± 0.41	39.80 ± 1.38	56.69 ± 0.77	17.89 ± 0.42	31.60 ± 0.48	684.1 ± 65.1
	1.0	6.40 ± 0.24	11.15 ± 0.41	36.53 ± 1.53	56.96 ± 0.45	17.43 ± 0.21	30.59 ± 0.38	458.4 ± 66.2

	Dose (g/Kg)	WBC (× 10^3)	Lymphocyte	Neutrophil		Monocyte	Eosinophil	Basophil
				Band	Segment			
Male	0	6.25 ± 1.74	70.90 ± 6.81	1.10 ± 0.27	23.70 ± 6.74	2.80 ± 0.32	0	0
	0.1	6.22 ± 1.62	47.80 ± 9.22	3.30 ± 1.05	44.00 ± 8.54	5.20 ± 1.16	0	0
	0.5	5.97 ± 0.37	48.13 ± 8.04*	1.37 ± 0.53	35.13 ± 6.13	6.87 ± 1.30**	0	0
	1.0	4.81 ± 0.53	43.77 ± 5.89**	1.66 ± 0.47	47.88 ± 6.52*	6.77 ± 1.23**	0	0
Female	0	3.31 ± 0.46	77.70 ± 4.36	1.00 ± 0.14	16.80 ± 3.84	4.50 ± 0.63	0	0
	0.1	3.22 ± 0.28	77.88 ± 2.68	0.89 ± 0.39	15.56 ± 2.08	5.67 ± 0.75	0	0
	0.5	5.25 ± 0.68*	74.20 ± 3.57	0.80 ± 0.24	19.40 ± 3.18	5.20 ± 1.06	0	0
	1.0	5.87 ± 0.98*	76.10 ± 3.21	0.40 ± 0.22	16.40 ± 3.82	7.00 ± 1.36	0.10 ± 0.00	0

RBC: Red blood cell; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelet. Data were expressed as mean ± S.E. (n = 10); Significant difference between control treated group at *: $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student *t*-test.

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Table 5. Serum biochemical change in mice treated with NE by gastric gavage daily for 28 days.

(A) Male

	Dose (g/Kg)	Fbg[1)]		ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
		(sec)	(mg/dL)				
Male	0	13.17 ± 0.89	378.37 ± 25.47	36.78 ± 4.32	117.42 ± 9.87	19.48 ± 0.96	0.24 ± 0.02
	0.1	17.90 ± 11.1	468.94 ± 60.93	25.58 ± 3.47	166.9 ± 47.84	19.20 ± 1.67	0.20 ± 0.01
	0.5	11.60 ± 1.87	447.42 ± 61.18	33.24 ± 3.31	170.1 ± 27.54	19.00 ± 1.49	0.20 ± 0.01
	1.0	12.94 ± 2.20	399.90 ± 44.49	53.18 ± 14.2	260.7 ± 79.88	21.08 ± 1.65	0.16 ± 0.02
Female	0	17.82 ± 0.97	276.16 ± 15.23	30.78 ± 4.01	153.04 ± 23.15	17.28 ± 1.33	0.20 ± 0.01
	0.1	22.10 ± 1.91	228.16 ± 19.73	49.70 ± 14.9	232.42 ± 35.78	20.12 ± 0.25	0.08 ± 0.03
	0.5	19.26 ± 3.11	287.32 ± 56.80	53.06 ± 7.75*	275.56 ± 67.00	22.12 ± 1.40	0.14 ± 0.02
	1.0	23.85 ± 4.81	225.72 ± 34.00	56.26 ± 16.3	238.38 ± 38.16	23.08 ± 2.24	0.12 ± 0.04

[1)] Fbg: Fibrinogen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen.

[2)] Data were expressed as mean ± S.E. (n = 5); Significant difference between control treated group at *: $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student *t*-test.

Morphological changes in the spleen were detected in animals receiving the medium and high doses of NE. The changes consisted of spleen swelling which might be due to myeloid and megakaryocytic hyperplasia, and are the consequence of traumatic inflammation on the dorsal skin in male mice treated with high dose of NE (1.0 g/kg). (Figure 2). In the liver, a mild to moderate degree of swelling and glycogen infiltration in hepatic cell were found in the animals treated NE at all dose levels. Although number of lesions were found in both sexes but these were also present in the controls. Due to the random distribution and low incidence, it was considered to be spontaneous in nature.

VI. *Mutagenicity test of NE*

The mutagenicity of NE was examined by the Ames method (Maron and Ames, 1983)[19]. The assay was carried out *in vitro* using five histidine requiring strains of *Salmonella* typhimurium (TA97, TA98, TA100 and TA102 and 1535) with and without metabolic activation enzyme (S9). 9-Aminoacridine for TA97 (50 μg/plate), 4-nitro-o-phenylenediamine for TA98 (2 μg/plate), sodium azide for TA100 and TA1535 (5 μg/plate) and mitomycin C for TA102 (0.5 μg/plate) were used as postitive control in without S9 mix. In the present of S9, 2-aminoanthrene (5 μg/mL) was used as positive control in five strains. All positive control both with or without S9 all showed significant increase in the colony formation relative to control (Table 7). Each strain of Salmonella was treated with NE 0.005, 0.05, 0.5 5 mg/plate, respectively. The result of Ames test demonstrated the NE had no mutagenicity effect under the tested concentration range.

DISCUSSION

Obviously, not much pharmacological evidances supported the same effect of muskone and musk, but lots of researches suggested the potential effect of musk fragrances and synthetic musk[27,28]. Synthetic musks not only present in fine fragrances but also included in less-expensive consumer products such as cosmetics, soaps, and laundry detergents[29,30]. Musk xylene, musk ketone and other nitro musks were the principal substances to be synthesized for commercial purposes. Gradually they have been replaced by the polycyclic musk compounds and became dominating in the global market[31] recently. The widespread use of synthetic musk fragrances and their lipophilic property make them as the well known environmental pollutants. Nitro musk occurrence in the environment was first reported in adipose fish tissue in Japan[32], later was identified in river water, sewage, marine muscles and oysters[33]. Following, polycyclic musks were found in the aquatic environment[34] and the most commonly found synthetic musks in environmental samples are musk ketone, Galaxolide and Tonalide[35].

Although, lot of synthetic musks were widely used in the world, but no one can replace their medical purpose. The use of this substance has made this male deer near the edge of extinction, it is necessary to find a substitute with similar pharmacological function. In Taiwan, the gland from nutria (*Myocastor coypus*) has been used as a musk substitute for hundred years. Up to now, the toxicological study of the gland from nutria still unclear. In the present study, the safety assessment of nutria gland was performed with acute, subacute and genotoxicity test. In the acute toxicity test, mice were randomly divided into two groups of five animals per sex, according to the

Table 6. Urinary analysis in rats treated with NE by gastric gavage daily for 28 days[1)].

(A)Male

Dose	Glu.	Bil.	Ket.	S.G.	pH	Pro.	Uro.	Nit.	Occ.	Leu.	Apr.	Vol.	Urinary sediments (10^3)				
(g/kg)						(mg/dL)	(EU/dL)					(mL)	R	W	E	Ca	Cr	Pa
0																		
	-	-	±	>1.03	6.5	100	0.2	-	-	-	Y	6	0	0	4.4	7.7	±	-
	-	-	±	1.025	6.5	30	0.2	-	-	-	Y	6	0	0	2.2	24.4	±	-
0.1																		
	-	-	±	>1.03	6.5	300	0.2	-	+	±	Y	4	0	0	1.1	2.7	±	-
	-	-	±	1.025	7.5	30	0.2	-	-	-	Y	8	0	2.7	5.5	9.9	++	-
0.5																		
	-	±	±	1.02	7.5	30	0.2	-	-	±	Y	8	0	0.5	2.2	3.3	±	-
	-	-	±	1.025	7.0	100	0.2	-	-	±	Y	4	0	0	1.1	5.5	+++	-
1.0																		
	-	-	±	>1.03	6.5	30	0.2	-	-	-	Y	6	0	1.1	1.6	4.9	±	-
	-	-	±	>1.03	7.0	100	0.2	-	-	±	Y	8	0.5	0.5	6.1	19.4	+++	-

(B) Female

Dose	Glu.	Bil.	Ket.	S.G.	pH	Pro.	Uro.	Nit.	Occ.	Leu.	Apr.	Vol.	Urinary sediments (10^3)				
(g/kg)						(mg/dL)	(EU/dL)					(mL)	R	W	E	Ca	Cr	Pa
0																		
	-	-	±	>1.03	6.5	30	0.2	-	-	-	Y	4	0	6.1	3.8	4.9	++	-
	-	-	±	>1.03	7.0	100	0.2	+	-	++	Y	6	0	3.3	3.3	5.5	+	-
0.1																		
	-	-	±	>1.03	6.0	30	0.2	-	-	-	Y	4	0.5	0	2.2	12.2	++	-
	-	±	±	1.025	6.0	30	0.2	-	-	-	Y	4	0.5	2.2	8.3	1.1	+	-
0.5																		
	-	-	±	>1.03	6.5	30	0.2	-	-	-	Y	6	0	0.5	3.3	12.7	+	-
	-	-	±	1.025	6.5	30	0.2	+	-	-	Y	4	0	1.6	1.1	4.4	++	-
1.0	-	-	±	>1.03	6.0	30	0.2	-	-	-	Y	2	0.5	10.5	4.4	2.7	++	-
	-	-	±	1.025	6.5	<30	0.2	-	-	-	Y	4	0.5	0	0.5	6.6	++	-

1. Appearance: C-Colorless; P-Pale yellow; Y-Yellow; YB-Yellow brown; B-Brown6 (N = 10).
2. Glu.: Glucose; Bil.: Bilirubin; Ket.: Ketone; S.G.; Specific gravity; Pro.: Protein; Uro.: Urobilinogen; Nit.: Nitrite; Occ.: Occult blood; Leu.: Leukocyte; Apr.: Appearance; Vol.: Volume
3. Grades for glucose, ketone, Protein, Nitrite, occult blood and urobilinogen:
   Glucose (mg/dL): -; 10 (±); 25 (+); 50 (++); 100 (+++); 200 (++++)
   Ketone (mg/dL): -; 5 (±); 15 (+); 40 (++); 80 (+++); 160 (++++)
   Protein: -; <30 (±); 30 (+); 100 (++); 300 (+++); 1000 (++++)
   Urobilinogen (Ehrlich unit/dL): 0 (-&±); 1 (+); 2 (++); 4 (+++); 8 (++++)
   Nitrite: normal (-); Positive (+); -: Negative; ±: Trace; +: Mild; ++: Moderate; +++: Remarkable
4. Urinary sediments: R-Red blood cells; W-White blood cells; E-Epithelial cells; Ca-Casts; Cr-crystals: abnormal crystals in cholesterol, tyrosine, leucine, cystine or test substance, etc.
5. Grades for observation:

-	Nil	±	A few in a few fields examined
+	A few in some fields examined	++	A few in all fields examined
+++	Many in all fields examined		

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Organization for Economic Co-operation and Development (OECD) guildline for testing of chemicals TG420 (OECD, 2001)[26]. Oral treatment of mice with the NE up to 5 g/kg showed no observious behavioral changes after administration up to 14 days (Table 1). No lethal dose or LD50 could be determined since the highest dose tested caused no mortality. In the subacute toxicity test, NE did not produced any marked changes in both male and female mice, no changes in water and food ingestion (Table 2), or weight gain (Figure 1). No statistical significant changes in organ weight (Table 3) as evidenced by the absence of organ swelling, atrophy or hypertrophy. As to the hematological analysis, neutrophil segmented form and monocyte were significant increased and lymphotocyte was significant decreased compared with control group (Table 4). Other parameters evaluated which include RBC, HGB, HCT, MCV, MCH, MCHC, PLT, and WBC were statistically equal to those of the control group. Eosinophil and basophil were not detectable. These changes were not considered to have toxicological significance since the significant changes of neutrophil segmented form, monocyte and lymphocyte count did not demonstrate dose-dependence, and no related alteration were observed in clinical symptoms of toxicity, moreover, these effects were not observed in female mice. The biochemical results in control and NE treated groups were consistent with the morphological analysis, include ALT, AST, BUN, creatine and fibrinogen (Table 5). Although the data in ALT, AST and BUN have slightly increased in dose-dependent manner, but most of them did not show significant difference, and no abnormal histopathological finding were observed in the cardiovascular, respiratory digestive, urinary or nervous systems, at least in these three dosage of 0.1 g/kg, 0.5 g/kg and 1 g/kg of NE treatment. The higher dosage might cause adverse effect but need futher investigation. However, the treated male mice became aggressive and bit the back each other, causing some infections. The reason for this effect remains to be explored (Figure 2). The urinalysis in the control and NE treated groups of both male and female mice were negative for glucose, ketone, RBC, bilirubin, specific gravity, pH, protein, urobilinogen, nitrite, occult blood,

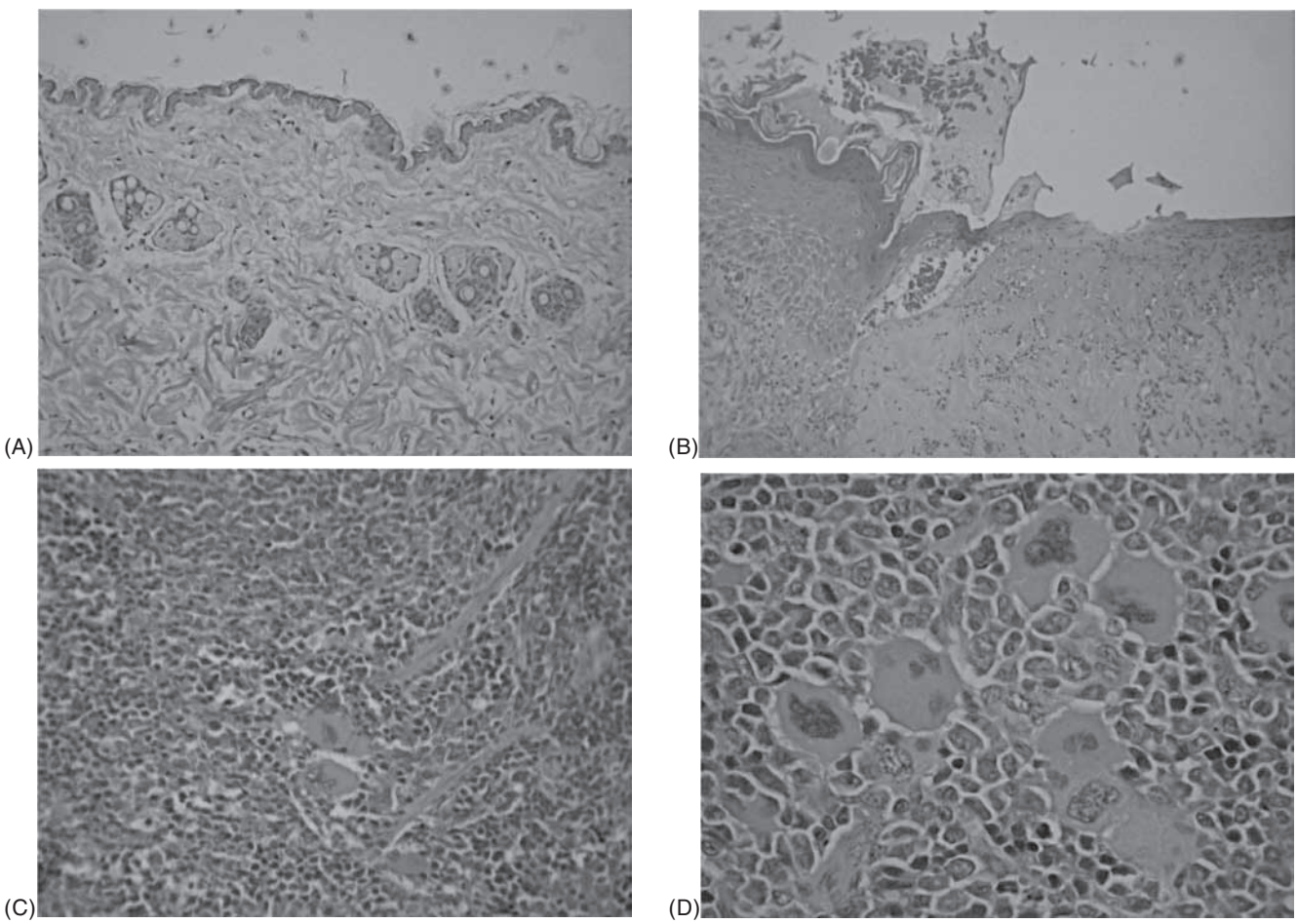

Figure 2. Histopathological examinations of male mice treated with NE by gastric gavage daily for 28 days. A. Control skin, B. Necrotic dermatitis of hyper-, hypokeratosis and spongiosis with inflammatory cells infiltration in the subcutaneous tissues and lost of squamous epithelia layers were found on the dorsal skin (1 g/kg group). C. Control skin, D. Myeloid and megakaryocytic hyperpalsia are mostly seen together and are the consequence of traumatic inflammation on the dorsal skin (1 g/kg group).

Table 7. The revertants in five strains of *Salmonella typhimurium* treated with different concentrations of NE with and without metabolic activating enzyme (S9).

(A) Without S9

Strains	His-/plate (- S9)					
			(NE)			
	Negative	Positive	0.005 mg/plate	0.05 mg/plate	0.5 mg/plate	5 mg/plate
TA 97	162.0 ± 16.9	773.3 ± 56.4***	132.3 ± 20.7	186.3 ± 6.2	162.7 ± 42.8	179.0 ± 12.1
TA 98	18.7 ± 3.2	417.2 ± 144.3***	18.3 ± 3.1	16.0 ± 1.1	18.7 ± 2.80	19.5 ± 1.6
TA 100	76.3 ± 16.8	859.3 ± 224.0***	104.8 ± 14.1	93.7 ± 16.4	90.7 ± 12.1	101.0 ± 11.0
TA 102	193.2 ± 19.3	1399.0 ± 116.0***	208.8 ± 61.5	193.5 ± 16.4	190.7 ± 32.6	158.3 ± 18.0
TA 1535	12.3 ± 0.9	476.2 ± 60.1***	10.3 ± 2.3	8.7 ± 0.7	10.8 ± 1.6	9.3 ± 1.3

(B) With S9

Strains	His$^-$/plate (+ S9)					
			(NE)			
	Negative	Positive	0.005 mg/plate	0.05 mg/plate	0.5 mg/plate	5 mg/plate
TA 97	113.0 ± 9.8	1589.1 ± 101.3***	182.0 ± 7.5	162.0 ± 11.7	156.0 ± 0.0	102.3 ± 8.1
TA 98	24.2 ± 5.8	3418.2 ± 807.5***	26.3 ± 3.5	27.5 ± 2.7	22.7 ± 3.7	21.0 ± 3.1
TA 100	75.7 ± 4.9	1901.0 ± 530.8***	78.5 ± 2.7	82.7 ± 8.3	92.8 ± 14.0	100.4 ± 16.3
TA 102	166.3 ± 42.4	1683.1 ± 158.8***	237.3 ± 59.5	254.2 ± 42.1	255.0 ± 32.0	238.5 ± 30.6
TA 1535	15.0 ± 1.3	428.9 ± 120.7***	12.2 ± 1.9	10.4 ± 2.4	12.6 ± 3.3	11.5 ± 2.0

1. The values were presented as Mean ± S.E.($N \geq 6$).
2. 2.5 μL DMSO/plate was used as negative control.
3. Positive control in –S9 plate: TA 97, 9-Aminoacridine 50 μg/plate; TA 98, 4-Nitro-o-phenylenediamine 2.5 μg / plate; TA 100 and TA 1535, Sodium azide 5 μg / plate; TA 102, Mitomycin C 0.5 μg / plate.
4. Positive control in +S9 plate: TA 97、TA100、TA102 and TA1535, 2-aminoanthrene 5 μg/plate; TA 98, benzo(a)pyrene 5 μg/plate.
Significant difference between control treated group at *: $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student *t*-test.

leukocyte, appearance, urine volumn and urinary sediments (Table 6).Since genotoxic potential is one of the most important criteria for prediction of adverse health effects, for example, carcinogenicity, the musk substitute NE was tested for genotoxicity in the present investigation. Ames bacterial test was used for gene mutation test with Salmonella strains TA97, TA98, TA100, TA102 and TA1535. The Ames test was negative to NE treated groups compared to control group. The same results were seen with or without metabolic activation (Table 7).

Results of the current study support the safety of the use of NE, and the acute, subacute toxicity and genotoxicity are not observed in this study. In spite of the safety assessment of NE, using nutria gland as musk substitute in medicines still awaits further pharmacological support.

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REFERENCES

1. Sano, T. 1936. Uber die maennlichscxualhormonartige Wirkung des Moschus. Yakugaku Zasshi 56: 913-914.
2. Sano, T. 1937. Uber die maennlich sexualhormonartige Substanz des Moschus. Yakugaku Zasshi 57: 851-852.
3. Kimura, M., Osada, E. and Waki I., 1968a. Fundamental research for the pharmacological activity of oriental drugs. VII. On the combined actions of several crude drugs included in “Rokushingan” on the function of heart. Yakugaku Zasshi 88: 125-129.
4. Mukhopadhyay, A., Seth, S. D. S. and Bagchi, N. 1973. Cardiac and CNS actions of musk. Indian J. Pharm. 36: 169-170.

5. Mishra, R., K., Arora, R. B., Seth, S., 1962. Anti-inflammatory activity of musk. J. Pharm. Pharmaco. 14: 830-831.
6. Kimura, M., Waki, I. and Ikeda H. 1968b. Fundamental research for the pharmacological activity of oriental drugs. VIII. The potentiation of crude drug "Moschus" for catecholamines. Yakugaku Zasshi 88**:** 130-134.
7. Taneja, V., Siddiqui, H. H. and Arora, R. B. 1973. Studies on the anti-inflammatory activity of Muschus Moschiferus (musk) and its possible mode of action. Indian J. Physiol. Pharmacol. 17: 241-247.
8. Seth, S. D. S., Mukhopadhyay, A., Bagchi, N., Pcabhakar, M. C. and Arora, R. B. 1973. Antihistaminic and spasmolytic effects of musk. Jpn. J. Pharmacol. 23: 673-679.
9. Kimura, M., Waki, I. and Ishida, M. 1978. Fundamental research for the pharmacological activity of oriental drugs. XI. Combined effects of animal-origin drugs on the inhibition of leukocyte migration and their effective components. Yakugaku Zasshi 98: 442-447.
10. Lee, K. K. H., Kwong, W. H., Chau, F. T., Yew, D. T. and Chan, W. Y. 2002. Pien Tze Huang protects the liver against carbon tetrachloride-induced damage. Pharmacol. Toxicol. 91: 185-192.
11. Kubo, M., Matsuda, H. and Arichi, S. 1984. Pharmacological study on animal crude drugs (I) On Bufonis venenum, Bezoar bovis and Moschus. Shouyakugaku Zasshi 38: 59qM.
12. Kimura, M. and Waki, I. 1966. Potentiation of β-adrenergic receptor to musk. Jpn. J. Pharmacol. 16: 129-130.
13. Lin, D. L., Chang, H. C. and Huang, S. H. 2004. Characterization of allegedly musk-containing medicinal products in Taiwan. J. Forensic Sci. 49(6): 1187-1193.
14. Zhang, Y. T., Ma, K., Zhang, J. Y., Li, Y. K., Wu, L. M., Li, X. R., Pan, L., Gao, H. L., and Li, L. D. 2007. Effects of series of Muskone on heart hemodynamics and myocardial consumption of oxygen in experimental dogs. Zhongguo Zhong Yao Za Zhi. 32(9): 827-30. Chinese.
15. Bitsch, N., Dudas, C., Korner, W., Failing, K., Biselli, S., Rimkus, G. and Brunn, H. 2002. Estrogenic activity of musk fragrances detected by the E-screen assay using human mcf-7 cells. Arch. Environ. Contam. Toxicol. 43(3): 257-264.
16. Peng, R., Zhu, X. Y. and Yang, C. S. 1986. Induction of rat liver microsomal cytochrome P-450 by muscone (3-methylcyclopentadecanone). Biochem. Pharmacol. 35(8): 1391-1394.
17. Lehman-McKeeman, L. D., Stuard, S. B., Caudill, D. and Johnson, D.R. 1997. Induction of mouse cytochrome P450 2B enzymes by amine metabolites of musk xylene: contribution of microsomal enzyme induction to the hepatocarcinogenicity of musk xylene. Mol. Carcinog. 20(3): 308-316.
18. Jacob, S., Garcia, S., Hayreh, D., and McClintock, M. K. 2002. Psychological effects of musky compounds: comparison of androstadienone with androstenol and muscone. Horm. Behav. 42(3): 274-283.
19. Maron, M. and Ames, B.N. 1983. Revised methods for the Salmonella mutagenicity tests. Mutat. Res. 113: 173-215.
20. Matsuoka, A., Hayashi, M. and Ishidate, M. 1979. Chromosomal aberration tests on 29 chemicals combined with S9 mix in vitro. Mutat. Res. 66: 277-290.
21. Ames, B. N., McCann, J. and Yamasaki, E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31: 347-364.
22. Organization for Economic Cooperation and Development (OECD) Test Guideline 471, 1997. Bacterial Reverse Mutation Test. In: OECD Guideline for Testing of Chemicals. Paris, Organization for Economic Cooperation & Development.
23. Suter, W., Hartmann, A., Poetter, F., Sagelsdorff, P., Hoffmann, P. and Martus, H. J. 2002. Genotoxicity assessment of the antiepileptic drug AMP397, and Ames-positive aromatic nitro compound. Mutat. Res. 518: 181-194.
24. De Serres., F. J. and Shelby, M. D. 1979. The Salmonella mutagenicity assay: Recommendations. Science 203: 563-565.
25. Environmental Protection Agency (EPA), 2000. Repeated dose 28-day oral toxicity study in rodents. In: OPPTS Harmonized Test Guidelines Series 870:3050, EPA712-C-00-366, Washington, DC, 10pp.
26. Organization for Economic Cooperation and Development (OECD)., 2001. Acute Oral Toxicity-Fixed Dose Procedure. In: OECD guildline for the Testing of Chemicals. Section 4: Health Effects, No: 420, 14 pp. Adopted: 17th December, 2001.
27. Gooding, M. P., Newton, T. J., Bartsch, M. R. and Hornbuckle, K. C. 2006. Toxicity of synthetic musks to early life stages of the freshwater mussel Lampsilis cardium. Arch. Environ. Contam. Toxicol. 51(4): 549-558.
28. Apostolidis, S., Chandra, T., Demirhan, I., Cinatl, J., Doerr, H. W. and Chandra, A. 2002. Evaluation of carcinogenic potential of two nitro-musk derivatives, musk xylene and musk tibetene in a host-mediated in vivo/in vitro assay system. Anticancer Res. 22(5): 2657-2662.
29. Betts, T. J., Tai, G. M. and Turner, R. A. 1982. Evaluation of toiletries for possible allergenic concentrations of nitro musks using electron-capture gas chromatography. J. Chromatogr. 244: 381-384.
30. Welie, R. T. H. 1995. Eel with parfume. Consumentengids 7: 443-445.
31. Rimkus, G. G. 1999. Polycyclic musk fragrances in the aquatic environmnet. Toxicol. Lett. 111: 37-56.
32. Yamagishi, T., Miyazaki, T., Horii, S. and Kaneko, S. 1981. Identification of musk xylene and musk ketone in freshwater fish collected from the Tama River, Tokyo.

Bull. Environ. Contam. Toxicol. 26: 656-662.
33. Yamagishi, T., Miyazaki, T., Horii, S. and Akiyama, K. 1983. Synthetic musk residues in biota and water from Tama River and Tokyo Bay (Japan). Arch. Environ. Contam. Toxicol. 12(1): 83-89.
34. Eschke, H. D., Traud, J. and Dibowski, H. J. 1995. Untersuchungen zum Verkommen polycyclischer Moschus-Duftstoffe in verschiedenen Umweltkompartimenten. UWSF-Z Umweltchem. Ökotox. 7: 131-138.
35. Gatermann, R., Biselli, S., Huhnerfuss, H., Rimkus, G. G., Hecker, M. and Karbe, L. 2002. Synthetic musks in the environment. Part 1: Species-dependent bioaccumulation of polycyclic and nitro musk fragrances in freshwater fish and mussels. Arch. Environ. Contam. Toxicol. 42: 437-446.