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Induction of esophageal cancers by nitenpyram (NIT) in rats

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Abstract. – OBJECTIVE: This study was made to investigate and evaluate the safety and carcinogenicity of nitenpyram (NIT) in rats.

MATERIALS AND METHODS: A totally 50 male and 50 female SD rats were treated with NIT at 0, 800, 2400, and 7200 ppm, respectively, for 104 w. The growth, clinical signs, and survival rates, as well as the body and organ weights of these animals, were analyzed. Histopathological examination was also performed.

RESULTS: Compared with the control group, survival rates at 104 w were significantly decreased in the 7200 ppm dose group, for both the male and female animals. The occurrence of esophageal squamous papilloma (ESP) was significantly increased in the treated animals. The occurrences of ESP for the 0, 800, 2400, and 7200 ppm NIT treatment groups were 0/39, 0/39, 3/35, and 9/27 for the male animals, and 0/43, 0/43, 6/49, and 12/33 for the female animals, respectively. For pre-neoplastic lesion of ESP, the occurrences of esophageal squamous hyperplasia for the 0, 800, 2400 and 7200 ppm NIT treatment groups were 0/39, 1/39, 10/35, and 9/27 for the male animals, and 0/43, 2/43, 15/49, and 17/33 for the female animals, respectively. The basal cell hyperplasia from mild to severe degrees was observed in the treatment groups.

CONCLUSIONS: NIT exhibits carcinogenicity of ESP in the male and female rats after the two-year treatment.

Key Words:
Nitenpyram (NIT), Carcinogenicity, Esophageal squamous papilloma, Esophageal squamous hyperplasia, Squamous cell carcinoma of the esophagus.

Introduction

Nitenpyram (NIT) is a neonicotinoid insecticide acting on the nicotinic acetylcholine receptors to block the synaptic receptors in pests. Because of its broad insecticidal spectrum, quick effect, durable resistance, and no cross-resistance with conventional pesticides, NIT is widely used to control the aphids, rice planthoppers, whiteflies, and leafhoppers in crops like rice, fruit trees, vegetables, and teas[1]. In addition, NIT is capable of killing the cat flea (*Ctenocephalides felis*), and it is also used for the treatment of parasitic fleas on dogs and cats by veterinary[2-4].

NIT was brought to the market in 1995 by Japanese Takeda, as the second neonicotinoid insecticide after imidacloprid[5]. Compared with imidacloprid, NIT is considered to be of much lower toxicity against the mammals[6]. Later on, similar compounds (such as acetamiprid, thiamethoxam, thiacloprid, clothianidin, and dinotefuran) have been developed and widely applied in the plant protection. By 2010, neonicotinoids account for approximately one-quarter of the world insecticide market[7]. Although neonicotinoids have been considered to have low toxicity to mammals and humans, some reports[8,9] have shown that neonicotinoids have potential risk to mammals and even humans. In 2005, Green et al[10] found that thiamethoxam induces liver tumors in mice, which is the first public report suggesting that neonicotinoid insecticide could cause cancers in animals. In the safety evaluation, it is noted that thiacloprid could cause increased occurrences of malignant uterine adenocarcinomas and thyroid adenomas in rats, and ovarian luteomas in mice[11]. Therefore, as a widely used neonicotinoid compound, it is necessary to investigate the carcinogenicity of NIT.

It has been reported that NIT has low acute toxicity. The acute oral LD_{50} for NIT in rats is 1575-1680 mg/kg bw, and the acute dermal LD_{50} is greater than 2000 mg/kg bw[12]. A toxicokinetic study[13] in dogs has revealed that NIT has a quick oral absorption, with a half-life period of 2.8 h, and it takes 1.2 h to reach its C_{max}. NIT could

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undergo the N-demethylation to form NIT-dm, which can be detected in the brain, liver, and plasma of mice. A tentatively identified metabolite (NIT-dm-COOH) in the brain and liver is proposed to be able to have the nitromethylene carbon oxidized into the carboxylic acid, and 40% of prototype and its metabolites are excreted via urine. The short-term *in vitro* and *in vivo* tests for mutagenicity, chromosomal, and DNA damage have found that NIT is non-genotoxic[14]. However, there are several non-genotoxic substances that have been shown to be carcinogenic[15]. Hence, it is necessary to assess the long-term toxicity and/or carcinogenicity of NIT.

Although NIT has been widely used in the insect pest control in agriculture, differential findings on the toxicity and/or carcinogenicity of NIT have been reported in the previous literature. A previous research[12] in rats showed that NIT has no toxic reaction at the dose of 5000 ppm, which exhibits suppressing effects on body weight gain and induces decreased ingestion at the dose of 10000 ppm. However, in another study[16], at the dose of 1080 ppm, NIT causes loss of body weight in rats and induces pathological changes in the liver and lung. Moreover, in a 104-week rat carcinogenicity study[12], NIT does on show suppressing effects on body weight gain until the dose of 9000 ppm, and reports no other toxic reactions and carcinogenicity. Therefore, it is of great importance to evaluate the carcinogenicity of NIT. In the present investigation, to accomplish a more rigorous safety evaluation for NIT, a two-year carcinogenicity study in male and female SD rats was carried out under standard carcinogenicity bioassay protocols.

Materials and Methods

Experimental Animals

Male and female Sprague-Dawley (SD) rats, 4-w-old, were purchased from Sino-British SIPPR/BK Lab Animal Co., Ltd. (Shanghai, China). Animals were acclimated for 1 w prior to the commencement of testing. They were housed in groups of 2 in suspended-stainless steel cages, at 20-25°C and 35-70% humidity, on a 12-h light/dark cycle. Animals received sterilized powder diet with complete nutrition supplied by Keao Xieli Feed Co., Ltd. (Beijing, China). Diet and purified water were available to the animals *ad libitum*. The animals were fasted overnight before necropsy, but the water was still available that night. Animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of the Safety Evaluation Center, Shenyang Research Institute of Chemical Industry.

Chemicals

NIT with purity over 97% was obtained from the Agricultural Chemical Co., Ltd. NIT (brown crystalline powder) was mixed with sterilized powder diet. According to the design dosage, NIT was mixed with a small amount of feed, and added in blank feed gradually, followed by stirring for 30 min. Then, the mixture was transferred into stainless steel buckets and stored at room temperature.

Experimental Design

Animals were randomly divided into four groups, which were given 0 ppm, 800 ppm, 2400 ppm, and 7200 ppm formulation diets, respectively, once per day, for 104 w. The doses were selected based on the previously published subchronic toxicity studies[12,16]. Totally 80 animals per sex were used for each exposure level, i.e., 50 animals for the carcinogenicity phase of 104 w and 30 animals for the interim sacrifice at 26 w, 52 w, and 78 w, respectively. Grouping and dose designation were shown in Table I. These animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured once a week for the first 14 w, and once every 4 w between weeks 14 and 104. At 26 w, 52 w, and 78 w, interim sacrifice was conducted. At the end of 104 w, all surviving animals were anesthetized with ether, euthanized by exsanguination from the abdominal aorta, and then subjected to necropsy.

Histopathological Assessment

The brain, lungs, heart, liver, spleen, adrenal glands, kidneys, and testes or ovaries were removed from each animal and weighed. In addition, for all these animals, the pituitary, nasal cavity, tongue, trachea, aorta, thyroid gland, thymus esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, epididymis, prostate, seminal vesicles, ovaries, uterus, vagina, mammary gland, mesenteric and submandibular lymph nodes, mandibular salivary glands, sternum, femur, sciatic nerves, spinal cord (cervical, thoracic, and lumbar cords), eyes, and samples of skin and skeletal muscle were fixed in 10% buffered formalin. Slices of all the organs/

Table I. Animal grouping and dose designation.

		Interim sacrifice			Carcinogenicity
	NIT dose (ppm)	26 w	52 w	78 w	104 w
Males	0	10	10	10	50
	800	10	10	10	50
	2400	10	10	10	50
	7200	10	10	10	50
Females	0	10	10	10	50
	800	10	10	10	50
	2400	10	10	10	50
	7200	10	10	10	50

tissues were routinely processed for paraffin embedding, and the sections were prepared and stained with hematoxylin and eosin. Histopathological assessment was performed for all the tissues from the control and treated animals.

Statistical Analysis

Data were expressed as mean ± SD. Variances in data for body weights, food consumption, and absolute/relative organ weights were checked for homogeneity by the Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way ANOVA; if not, the Kruskal-Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple tests were employed for the group comparison. Data of clinical signs and survival rates were analyzed with the χ^2-test. For the histopathological changes, occurrences were compared using the Fisher's exact probability test. For a large number of animal deaths, the poly-3 survival-adjusted statistical test was selected to analyze the positive trend of dose-response relationship between hepatocellular adenoma and esophageal squamous papilloma[17-19,20]. $p<0.05$ was considered statistically significant.

Results

Growth, Clinical Signs, and Survival Rates

The animal growth, clinical signs, and survival rates after treatment were first analyzed. Our results showed that, compared with the control group, the mean body weights of male and female animals receiving 7200 ppm NIT were significantly lower, throughout the study (Figure 1). Compared with the control groups, there was a significant suppression of body weight gain at the study termination in these treated male and female animals ($p<0.01$) (Table II). During the first year of study, the body weight gain of the male and female animal receiving 2400 ppm NIT was significantly lower than the control groups (Table II). Moreover, for the high-dose treatment group, clinical signs such

Table II. Clinical findings after 2-year NIT treatment.

	NIT treatment (ppm)							
	Male rats				Female rats			
	0	800	2400	7200	0	800	2400	7200
Group size	50	50	50	50	50	50	50	50
To week 52 decedents	0	1	2	0	0	0	1	0
To week 78 decedents	4	5	6	6	3	4	1	3
To week 104 decedents	20	22	29	41	11	12	8	28
Survival rate (%)	60	56	42	18***	78	76	84	44**
Body weight gain (% of control)								
To week 52	-	98	95*	90**	-	101	94*	89**
To week 104	-	83**	81**	53**	-	99	91	52**

Note: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control group.

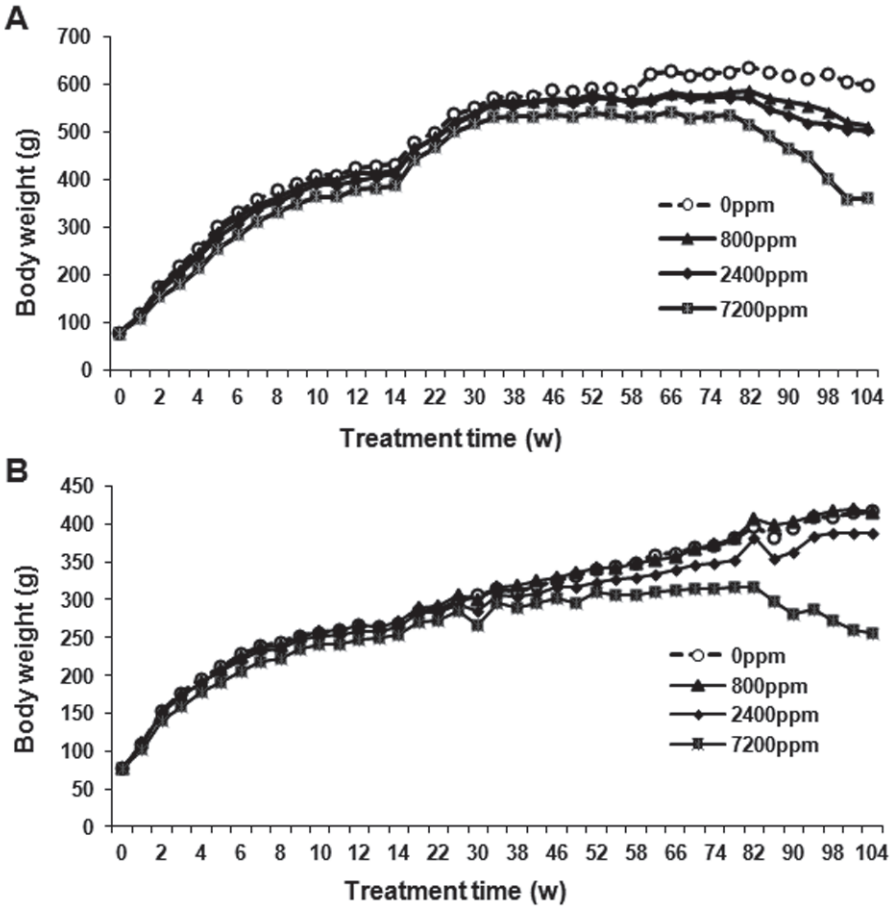

Figure 1. Growth curves for rats treated with NIT for 104 w. ***A,*** Male rats. ***B,*** Female rats.

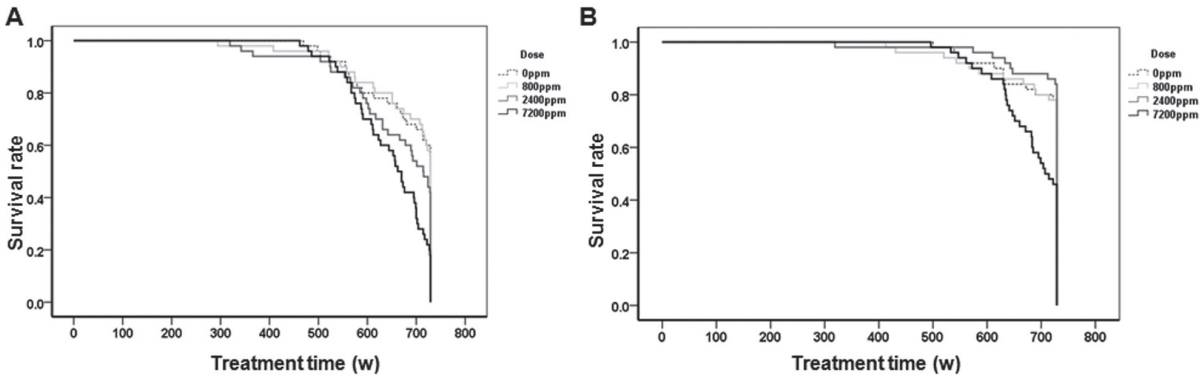

Figure 2. Survival curves for rats treated with NIT for 104 w. ***A,*** Male rats. ***B,*** Female rats.

as fluffy hair, marasmus, and abnormal breathing sounds were observed, and there was statistical significance (data not shown). Furthermore, the survival rates for the male animals receiving 0, 800, 2400, and 7200 ppm were 60%, 56%, 42%, and 18%, while for the female animals, were 78%, 76%, 84%, and 44%, respectively (Table II). For the 7200 ppm NIT treatment group, significantly decreased survival rates were observed in these male and female animals (both $p< 0.01$). Animal deaths mainly began at 78 w (Figure 2). These results suggest that high dose of NIT could induce systematic toxicity in animals, leading to decreased body weight and increased mortality.

Table III. Food consumption and NIT intake in rats over 104 w.

	NIT dose (ppm)	No. of animals examined	Food consumption (g/rat/day)[a]	Mean daily NIT intake (mg/kg bw/day)	Total NIT intake (g/kg bw)b
Males	0	50	28.4	0	0
	800	50	26.1	40.8	29.7
	2400	50	26.1	124.3	90.5
	7200	50	25.6	395.5	287.9
Females	0	50	21.9	0	0
	800	50	21.3	50.9	37.1
	2400	50	20.8	155.8	113.4
	7200	50	19.9	519.2	378.0

Note: [a]Food consumption was measured in five cages/group each including two animals throughout the experimental period; [b]Values were the sums of NIT administered during the experimental period, which were calculated by multiplying the mean daily NIT intake (mg/kg bw/day) by 728 days.

Food Consumption and NIT Intake

The food consumption and NIT intake of these animals were then investigated, and the results were shown in Table III. Compared with the control group, the food consumption per day for the 7200 ppm NIT treatment group was significantly lower. Moreover, the daily NIT intakes for the 800, 2400, and 7200 ppm NIT treatment groups were 40.8, 124.3 and 395.5 mg/kg bw/day for male animals, and 50.9, 155.8 and 519.2 mg/kg bw/day for female animals, respectively. The total NIT intakes over 104 w were 29.7, 90.5 and 287.9 g/kg bw for male animals, and 37.1, 113.4, and 378.0 g/kg bw for female animals, respectively. These results suggest that there is a good correlation between the NIT treatment dose and total intake for both the male and female animals.

Final Body and Organ Weights

To investigate the effects of NIT on the target organs in animals, the final body and organ weights were recorded and analyzed. As shown in Table IV, for the 7200 ppm NIT treatment group, the final body weights were reduced by 41% for the male animals, and by 39% for the female animals. Moreover, for the 7200 ppm NIT treatment group, significant increases were observed in the relative weights of hearts in the male animals (18%; $p<0.05$) and female animals (38%; $p<0.01$). In the high-dose treatment group, increased relative weights of kidneys were observed for the male animals (29%; $p<0.01$) and female animals (30%), respectively. As shown in Table IV, there were significant decreases in the absolute weights of brain, lungs, heart, liver, spleen, and kidneys in the male ($p<0.05$) and female ($p<0.01$) animals treated with different doses of NIT, respectively. However, these changes were not related to the NIT treatment. These findings suggest that NIT has no toxic effects on animal organs, and weight changes might be caused by reduced ingestion.

Histopathological Examination

To investigate the effects of NIT on the non-neoplastic and neoplastic lesions in animals, a histopathological examination was performed. The occurrences of neoplastic, pre-neoplastic, and non-neoplastic lesions were summarized in Tables V and VI. For the liver, significantly more cases of hepatocellular adenomas were observed for the female animals given 7200 ppm NIT (5/33), compared with the control animals (0/39). Pair-wise comparison suggested statistical significance between the control and 7200 ppm NIT treatment groups ($p=0.036$). Trend test indicated statistical significance when all groups were included ($p=0.0008$). Moreover, the occurrence of altered hepatocellular foci in the treated female animals (6/33) was also significantly increased ($p<0.05$). However, in the male animals, no significant increase was observed in the disease occurrence. Representative histopathological lesions of liver were shown in Figure 3. Although various non-neoplastic lesions (such as fatty degeneration and bile duct proliferation) were observed in the liver, the occurrence for these lesions was comparable between the control and treatment groups. For the esophagus, the occurrence of esophageal squamous papilloma (ESP) was significantly increased in the treated animals. The occurrences of ESP for the 0, 800, 2400, and 7200 ppm NIT treatment groups were 0/39, 0/39, 3/35, and 9/27 for the male animals, and 0/43, 0/43, 6/49, and 12/33 for the fema-

Table IV. Final body weights, and absolute and relative organ weights, for rats treated by NIT for 104 w.

	NIT treatment (ppm)							
	Male				Female			
	0	800	2400	7200	0	800	2400	7200
No. examined	30	28	21	9	39	38	42	22
Body weight (g)	568.61±78.02[a]	482.21±107.66	479.68±96.27	338.04±30.67	393.97±61.40	398.26±69.45	365.95±54.17	241.55±32.92
Absolute organ weights (g)								
Brain	2.24±0.08	2.21±0.09	2.24±0.10	2.12±0.13**	2.07±0.10	2.08±0.09	2.09±0.09	2.06±0.09
Heart	2.23±0.26	1.99±0.35*	1.95±0.27**	1.64±0.15**	1.44±0.24	1.43±0.30	1.41±0.23	1.21±0.15**
Lungs	2.76±0.40	2.48±0.31**	2.56±0.30**	2.93±0.61**	1.87±0.24	1.92±0.37	1.96±0.41	1.88±0.54
Liver	12.05±1.62	10.14±2.03**	10.15±1.90**	8.01±0.57**	10.15±2.90	9.40±1.85	9.13±2.95	6.19±1.38**
Spleen	1.18±0.62	0.10±0.29*	1.13±0.52**	0.70±0.33**	0.73±0.25	0.75±0.37	0.92±1.08	0.50±0.18**
Kidneys	3.63±0.45	3.30±0.55**	3.34±0.45**	2.84±0.20**	2.42±0.49	2.42±0.37	2.41±0.36	1.98±0.38**
Adrenals	0.065±0.036	0.064±0.013	0.068±0.028	0.066±0.016	0.626±1.866	0.111±0.082	0.119±0.091	0.205±0.539
Testes	3.61±0.99	3.77±2.25	3.47±1.80	2.59±1.17	-	-	-	-
Ovaries	-	-	-	-	0.21±0.45	0.41±1.13	0.13±0.13	0.10±0.07**
Relative organ weights (g/100 g bw)								
Brain	0.40±0.06	0.48±0.11	0.49±0.11	0.63±0.07	0.54±0.09	0.53±0.08	0.58±0.07*	0.86±0.12
Heart	0.40±0.06	0.42±0.08	0.42±0.05	0.47±0.03*	0.37±0.05	0.36±0.06	0.39±0.07	0.51±0.07**
Lungs	0.49±0.08	0.54±0.12	0.55±0.13	0.88±0.22	0.48±0.05	0.49±0.09	0.54±0.10**	0.80±0.29
Liver	2.14±0.25	2.13±0.25	2.13±0.25	2.38±0.19	2.61±0.74	2.37±0.27	2.49±0.62	2.57±0.46
Spleen	0.21±0.09	0.21±0.07	0.23±0.09	0.21±0.11	0.19±0.07	0.18±0.07	0.25±0.26	0.20±0.07
Kidneys	0.65±0.10	0.70±0.11	0.71±0.09	0.84±0.08**	0.63±0.15	0.61±0.06	0.66±0.10	0.82±0.12
Adrenals	0.012±0.008	0.014±0.004	0.014±0.005	0.020±0.006	0.178±0.536	0.029±0.022	0.033±0.024	0.077±0.182
Testes	0.65±0.21	0.81±0.52	0.72±0.39	0.76±0.34	-	-	-	-
Ovaries	-	-	-	-	0.06±0.14	0.09±0.24	0.04±0.04	0.04±0.03

Note: $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group.

Table V. Neoplastic findings at the end of investigation.

Lesions		NIT treatment (ppm)							
		Males				Females			
		0	800	2400	7200	0	800	2400	7200
	No. examined	39[a]	39	35	27	43	43	49	33
Pituitary gland	Adenoma	6	5	4	3	5	2	6	2
Lungs	Liver histiocytic sarcoma metastases	0	0	0	0	0	0	1	1
	Mammary gland adenocarcinoma metastases	0	0	0	0	0	0	1	0
	Suprarenal gland pheochromocytoma, malignant metastases	1	0	0	0	0	0	0	0
	Suprarenal gland carcinoma, cortical metastases	0	0	0	0	1	0	0	0
	Adenoma	0	0	0	0	1	0	0	0
	Thymus schwannoma, malignant metastases	0	1	0	0	0	0	0	0
Liver	Carcinoma, hepatocellular	0	0	0	0	1	0	0	1
	Adenoma, hepatocellular	0	0	2	1	0[##]	0	2	5[*]
	Histiocytic sarcoma	0	0	0	0	0	0	1	1
Thyroid gland	Carcinoma, C-cell	0	0	0	0	0	0	0	1
	Adenoma, C-cell	2	2	0	0	2	0	0	0
	Adenoma, follicular cell	1	1	1	0	0	0	1	0
	Adenoma	0	0	1	0	0	0	0	0
Lymph node	Mesenteric lymph node malignant lymphoma	1	1	2	0	1	1	0	0
	Malignant lymphoma	0	0	1	0	0	0	0	0
Skin /subcutaneous tissue	Keratoacanthoma, benign	1	1	0	0	0	0	0	0
	Papilloma, squamous cell	1	1	0	0	0	0	0	0
	Fibrous histiocytoma, malignant	0	0	1	0	0	0	0	0
	Schwannoma, malignant	1	0	0	1	1	0	0	0
	Osteosarcoma	0	1	0	0	0	0	0	0
	Basal cell tumor	1	0	0	0	0	1	0	0
	Keratoacanthoma, benign	0	0	1	0	0	0	0	0
	Trichoepithelioma, benign	0	0	1	1	0	0	0	0
	Fibroma	0	2	0	0	0	0	0	0
	Lipoma	1	0	0	0	0	0	1	0
Mammary gland	Fibroadenoma	0	0	0	0	20	19	22	5[**]
	Adenocarcinoma	0	0	0	0	0	0	2	0
	Adenoma	0	0	0	0	1	0	1	0
Adrenal gland	Pheochromocytoma, malignant	1	0	1	0	0	0	0	0
	Pheochromocytoma, benign	4	1	1	2	1	1	2	0
	Carcinoma, cortical	0	0	0	0	2	0	0	0
	Tumor, cortical	0	0	0	0	0	0	0	1
	Neuroblastoma, olfactory	0	0	1	1	0	0	0	0
Esophagus	Papilloma, squamous cell	0	0	3	9[**]	0	0	6[*]	12[**]
Small intestine	Malignant lymphoma	0	1	0	0	1	0	0	0
Great intestine	Malignant lymphoma	0	0	0	0	1	0	1	0
Heart	Endocardial schwannoma	1	1	0	0	1	0	0	0
Thymus	Schwannoma, malignant	0	1	0	0	0	0	0	0
	Thymoma, benign	0	0	0	0	0	0	1	0
	Epithelial thymoma	0	0	0	0	0	0	1	0
Pancreas	Adenoma, islet cell	1	1	0	0	1	0	0	0
	Adenoma	6	0	0	2	0	0	0	0
Brain	Astrocytoma	0	2	0	0	0	0	0	0
Ovary	Carcinoma, yolk sac	-	-	-	-	0	1	0	0
Uterus	Schwannoma, malignant	-	-	-	-	0	1	0	0
	Sarcoma	-	-	-	-	0	0	0	1
	Adenocarcinoma	-	-	-	-	0	1	0	0

Note: aEffective number of animals. ###statistically significant in the poly-3 trend test ($p = 0.0008$). *$p < 0.05$, **$p < 0.01$, compared with the control group (Fisher's exact probability test).

Table VI. Non-neoplastic histological findings.

Lesions		NIT treatment (ppm)							
		Male				Female			
		0	800	2400	7200	0	800	2400	7200
	Number Examined	39[a]	39	35	27	43	43	49	33
Urinary bladder	Hemorrhage	1	0	0	0	0	0	0	0
Pituitary	Hemorrhage	1	0	1	0	0	2	1	0
	Hyperplasia	9	1**	2*	2	11	4*	1**	5
Lungs	Pneumonia	0	0	0	2	0	0	0	0
	Abscess	0	0	0	2	0	0	0	1
Liver	Altered foci	0	0	0	0	1	0	1	6*
	Biliary cyst of liver	0	0	0	0	1	0	0	3
	Proliferation, bile duct	0	1	0	0	3	4	1	2
	Spongiosis hepatis (cystic degeneration)	0	0	1	1	0	0	0	0
	Necrosis	1	0	0	0	0	0	1	0
	Proliferation, oval cell	1	0	0	0	0	0	0	1
	Degeneration, fatty	3	6	5	5	8	6	6	2
Thyroid gland	Cell proliferation	4	0	1	1	2	0	0	0
Skin	Ulcer	0	0	0	0	0	2	0	0
	Erosion	0	0	0	0	0	0	1	0
	Skingland, sebaceous proliferation	1	0	0	0	0	0	0	0
Subcutaneous tissue	Inflammation, suppurative	1	0	0	0	0	0	0	0
	Necrosis	0	0	1	0	0	0	0	0
	Cyst	2	1	1	0	0	0	0	0
	Abscess	1	1	0	0	0	0	1	0
	Granuloma	0	0	0	0	0	0	0	1
Spleen	Hematopoiesis, extramedullary, increased	2	0	0	0	3	0	1	0
Forestomach	Hyperplasia, squamous cell	3	2	3	4	0	4	2	5*
	Hyperkeratosis	1	0	0	0	0	0	0	0
	Ulcer	0	0	0	1	0	0	0	4*
	Edema	0	1	0	0	0	0	0	3
Mammary gland	Degeneration, cystic	0	0	0	0	0	1	0	0
	Hyperplasia	0	0	0	0	2	1	1	0
Adrenal gland	Angiectasis	1	0	0	0	1	0	0	1
	Degeneration, cystic	2	0	1	0	18	12	5**	11
	Medulla proliferation	5	0	1	2	0	0	0	0
	Fatty change	1	0	0	0	0	0	0	0
	Lipidosis	0	0	0	1	0	0	0	0
Kidney	Nephrosis, chronic progressive	15	3**	4**	3*	3	3	3	0
	Cyst	0	1	0	0	0	0	1	0
	Transitional epithelium hyperplasia	1	0	0	0	0	0	0	0
Esophagus	Hyperplasia, squamous cell	0	1	10**	9**	0	2	15**	17**
Great intestine	Edema	0	0	0	1	0	0	0	0
Saliva gland	Inflammation	0	0	0	1	0	0	0	0
Heart	Endocardial hyperplasia	1	1	0	0	0	0	0	0
Eye ball	Cataract	0	0	2	0	1	0	1	1
	Dysplasia	0	0	0	0	0	0	1	0
Pancreas	Islet hyperplasia	1	0	0	0	1	0	0	0
	Hyperplasia	3	0	0	0	0	0	0	0
Epididymis	Atresia	0	1	1	0	-	-	-	-
Testis	Atresia	28	15**	20	24	-	-	-	-
Ovary	Cyst	-	-	-	-	10	8	7	8
	Abscess	-	-	-	-	0	0	0	1
Uterus	Tunica intima hyperplasia	-	-	-	-	3	0	0	4
	Polyp	-	-	-	-	2	1	0	0
	Inflammation	-	-	-	-	0	0	1	0
	Hyperplasia	-	-	-	-	0	0	0	1

Note: aEffective number of animals. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group (Fisher's exact probability test).

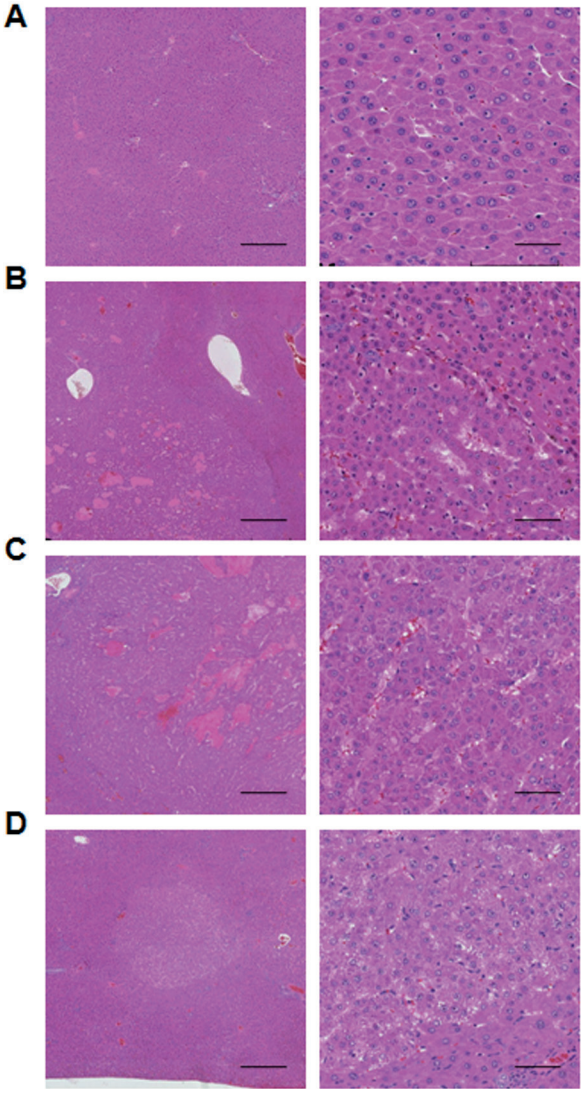

Figure 3. Representative histopathological lesions in the liver of rats treated with NIT for 104 w (HE staining). ***A,*** Normal liver tissue in a female rat treated with 0 ppm NIT. ***B,*** Hepatocellular adenomas in a female rat treated with 7200 ppm NIT. ***(C)*** Hepatocellular adenocarcinoma in a female rat treated with7200 ppm NIT. ***(D)*** Altered hepatocellular foci in a female rat treated with 7200 ppm NIT. Scale bar, left panel 500 μm, and right panel 100 μm.

le animals, respectively. Pairwise comparison suggested statistically significant differences between the control and 7200 ppm NIT treatment groups for the male (p = 0.01) and female (p = 0.01) animals, as well as between the control and 2400 ppm NIT treatment groups for the male (p = 0.036) and female (p = 0.01) animals, respectively. Trend test indicated statistical significance when all groups were included (p = 0.0000). For the pre-neoplastic lesion of ESP, the occurrences of esophagus squamous hyperplasia for the 0, 800, 2400, and 7200 ppm NIT treatment groups were 0/39, 1/39, 10/35, and 9/27 for the male animals, and 0/43, 2/43, 15/49, and 17/33 for the female animals, respectively. Pair-wise comparison suggested statistically significant differences between the control and 2400/7200 ppm NIT treatment groups for both the male and female animals. For the 800 ppm NIT treatment group, pair-wise comparison with the control did not indicate any statistical significance, for either male or female animals. Representative histopathological lesions of the esophagus were shown in Figures 4 and 5.

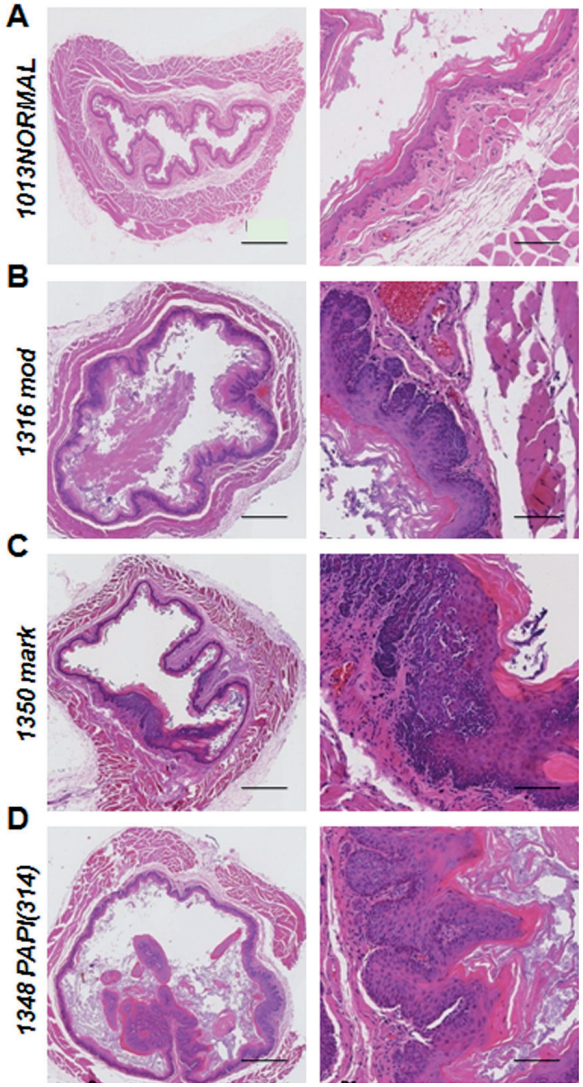

Figure 4. Representative histopathological lesions in the esophagus of male rats treated with NIT for 104 w (HE staining). ***A,*** Normal esophagus in a male rat treated with 0 ppm NIT. ***B-C,*** Esophagus squamous hyperplasia from moderate to severe degrees in a male rat treated with 7200 ppm NIT. ***D,*** Esophageal squamous papilloma in a male rat treated with 7200 ppm NIT. Scale bar, left panel 500 μm, and right panel 100 μm.

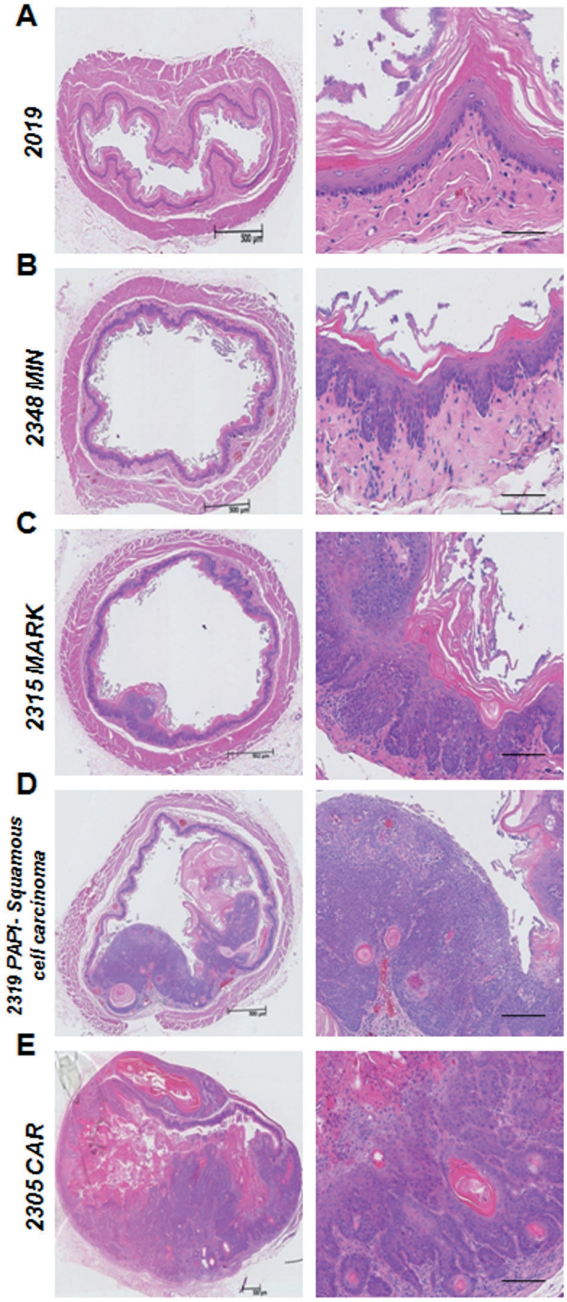

Figure 5. Representative histopathological lesions in the esophagus of female rats treated NIT for 104 w (HE staining). ***A*,** Normal esophagus in a male rat treated with 0 ppm NIT. ***B-C*,** Esophagus squamous hyperplasia in a female rat treated with 7200 ppm NIT. Note basal cell hyperplasia from mild to severe degrees. ***D*,** Esophageal squamous papilloma in a female rat treated with 7200 ppm NIT. ***E*,** Esophageal squamous cell carcinoma in a female rat treated with 7200 ppm NIT. Scale bar, left panel 500 μm, and right panel 100 μm.

For the forestomach, the occurrences of squamous cell hyperplasia (5/33) and ulcer (4/33) were significantly increased for the female animals from the 7200 ppm NIT treatment group. Pair-wise comparison suggested statistically significant differences between the control and 7200 ppm NIT treatment groups (p = 0.002). No neoplastic lesion of the forestomach was observed in the study. Representative histopathological lesions of the forestomach were shown in Figure 6. The occurrences of histopathological lesions in the esophagus and forestomach for animals at 104 w and interim sacrifice were summarized in Table VII. No carcinomas, nor hyperplasia in esophagus and forestomach were observed for the interim sacrifice at 26 w and 52 w (Table VII). At the 78-w interim sacrifice, one male rat with ESP, as well as two male rats with esophageal squamous cell hyperplasia, were found in the 7200 ppm NIT treatment group. Three female rats with esophageal squamous cell hyperplasia were found in the 7200 ppm NIT treatment group. A total of 23 rats with carcinomas were found in the final sacrifice, and other 7 rats with carcinomas died between 78 and 104 w. Compared with the control group, the incubation periods of carcinomas were ahead of time. There was an increased trend of the forestomach squamous cell hyperplasia in the animals with esophageal carcinogenicity pathologic changes. These results suggest that the drug administration might be associated with the animal liver tumors, anterior gastric hyperplasia, and esophageal tumors.

Discussion

In the present study, NIT treatment exhibited carcinogenic activity in the esophagus and liver, which was inconsistent with previous findings[12]. This investigation provided the first evidence for the carcinogenicity potential of NIT. In the 2-year study, the survival rates for the rats in the 7200 ppm NIT treatment groups were lower than the control groups. A major contributor of the early death in these groups was the esophagus tumor, which was the probable cause for these 7 cases of animal death.

In general, the occurrence of spontaneous esophageal tumor in rodents is so low that only a few evidence has been shown in previous background researches[21-25]. According to the standard background study, the occurrence of esophageal tumor is 0.08% in F344/N female rats, 0.0% in F344/N male rats, and 0.0% in SD rats[26,27]. The occurrence of esophageal tumor in SD rats has been reported as 0.05%, according to the Charles River Company[28]. In our laboratory, the occur-

Table VII. Histopathological lesions in the esophagus and forestomach of rats treated with NIT for 104 w and interim sacrifice.

		NIT dose (ppm)	Esophageal Squamous Papilloma	Esophagus squamous hyperplasia	Forestomach squamous hyperplasia	Lesions in Esophagus and Forestomach
104 w	Males	0	0### a	0	3	0
		800	0	1	2	0
		2400	3 (9%)	10	3	1
		7200	9 (33%)	9	4	4
	Females	0	0### b	0	0	0
		800	0	2	4	0
		2400	6 (12%)	15	2	1
		7200	12 (36%)	17	5	4
78 w	Males	0	0	0	0	0
		800	0	0	0	0
		2400	0	0	0	0
		7200	1 (10%)	2	0	0
	Females	0	0	0	0	0
		800	0	0	0	0
		2400	0	0	0	0
		7200	0	3	0	0
52 w	Males	0	0	0	0	0
	Females	7200	0	0	0	0
26 w	Males	0	0	0	0	0
	Females	7200	0	0	0	0

Note: astatistically significant in the poly-3 test was statistically significant ($p = 0.0000$); bstatistically significant in the poly-3 test was statistically significant ($p = 0.0000$).

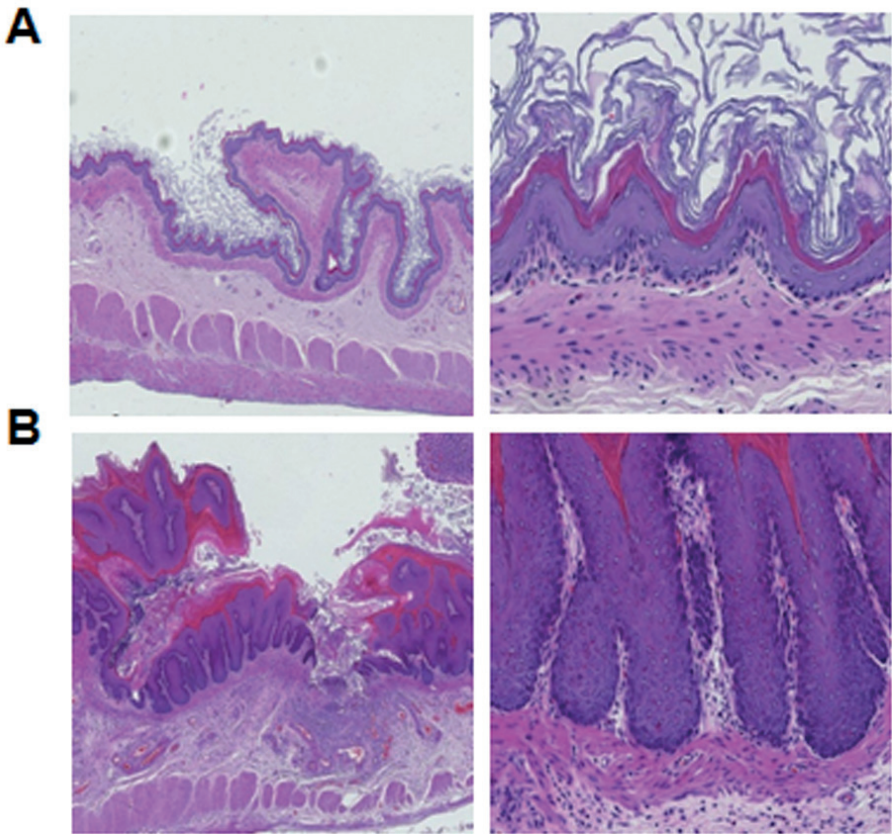

Figure 6. Representative histopathological lesions in the forestomach of female rats treated with NIT for 104 w (HE staining). ***A,*** Normal forestomach tissue in a female rat treated with 0 ppm NIT. **B,** Squamous cell hyperplasia and forestomach ulcer in a female rat treated with 7200 ppm NIT. Scale bar, left panel 500 μm, and right panel 100 μm.

rence of the esophageal tumor is 0.0% (unpublished data). In the present study, the occurrence of the esophageal tumor was up to 36% (for the female rats in the 7200 ppm NIT treatment group; Table VII), which should be induced by the NIT treatment.

Esophageal squamous cell papilloma is a kind of benign tumor, which usually causes no specific symptoms. However, it could deteriorate to the quite lethal esophageal squamous cell carcinoma (ESCC)[29]. In the present work, case of ESCC was found (Figure 3E), which may induce animal death. However, the eating difficulties induced by squamous cell papilloma may also contribute to the animal death.

A high ratio of basal cell hyperplasia in squamous cells in the esophagus was found at the NIT treatment doses of 2400 ppm and 7200 ppm (Figures 3 and 4). This mild, moderate, and severe hyperplasia led to the formation of squamous carcinomas. The development of ESCC is thought to be a multistage process, which progresses from the conversion of normal squamous epithelium to that with basal cell hyperplasia, intraepithelial neoplasia, and finally invasive ESCC[30,31]. Basal cell hyperplasia is the precursor lesion of esophageal squamous cell cancer. Our results revealed that NIT may lead to potential ESCC. Although an increased trend of forestomach squamous cell hyperplasia was observed, no carcinoma was found herein. The occurrence of forestomach carcinoma is generally believed to be much higher in rodents, and have no correlation with human beings[32]. In the present research, an increased trend was also observed in the animals with esophageal tumors and along with forestomach squamous cell hyperplasia. Since they were both pathological changes in the digestive system, the esophageal tumor and forestomach squamous cell hyperplasia might be induced by the same causing factors, which need to be further explored in the future. In the 7200 ppm NIT treatment group, forestomach ulceration was observed (Figure 5), which might be related to the inflammatory reaction and might contribute to the carcinoma formation.

The occurrence of hepatocyte adenocarcinoma in the female animals from the 7200 ppm NIT treatment group was significantly increased compared with the control group. However, there was only one case of hepatocyte carcinoma, indicating that the hepatocyte carcinoma had no correlation with NIT. Hepatocellular adenomas are benign tumors in the liver, which are caused by a benign proliferation of hepatocytes. Our results showed that the occurrence of altered hepatocellular foci was increased. Altered hepatocellular foci are the precursors of hepatocellular adenocarcinoma and hepatocellular carcinoma (HCC), which may deteriorate to hepatocellular carcinoma. These findings suggest that NIT can lead to liver carcinoma. The liver carcinogenicity was also found in the analogous compound thiamethoxam. Therefore, it is needed to explore whether carcinoma formation induced by these two compounds share the same mechanism. NIT has been reported to be of no genotoxicity, as it is shown[14] to exhibit negative reaction in the genic mutation and chromosome injury experiments, both *in vitro* and *in vivo*. Our results significantly revealed that NIT led to carcinoma formation. To further explore the carcinogenic mechanism of NIT, other genetic experiments are still needed to confirm whether NIT has genetic carcinogen. The latest data provided by Carcinogenic Potency Database (CPDB) show that, out of the already known 37 esophageal carcinogens (in rats)-inducing compounds, only one compound is of non-genetic carcinogen, accounting for 1/147 in the genetic carcinogenicity evaluations[33]. If NIT has non-genotoxic carcinogenesis, the epigenetic mechanisms may be attributed[34], and of course, further in-depth studies are still needed to address this issue. The metabolite of NIT may play a part in the process of its induction of carcinoma formation. It has been proven[10] that the liver carcinogenicity of thiamethoxam (with similar structure as NIT) is caused by its metabolites. In the *in vivo* environment, NIT is N-de methylated into NIT-dm, and oxidized into NIT-dm-COOH by CYP3A4. NIT-dm and NIT-dm-COOH, as well as their finale metabolites, are excreted[35]. NIT-CN is also detected in the liver, probably formed via the nitrosomethylene derivatives in the aldehyde oxidase (AOX) system[36]. AOX has the ability to reduce thiamethoxam, clothianidin, dinotefuran[35], and imidacloprid[37,38] into nitroso metabolites, and NIT-nitrate methylene is also reduced into nitroso compounds in *in vitro* rabbit cells[39]. Since the nitroso compounds are carcinogenic, NIT reduction through the AOX pathway and the possible carcinogenic products need to be further studied.

The dosage of NIT in this study was determined based on the previous toxicity study[12], in which not any toxic effect of NIT was found at 9000 ppm, except for the body weight suppression. However, the 7200 ppm (highest dose) used herein was actually somehow in excess, and at this dose level, animal survival rates of 2 years were

lower than 25%. At the dose level of 2400 ppm (moderate dose) here, less body weight loss and normal survival rates were observed. At the 800 ppm (low dose) level, no toxic effects, nor cancers, could be taken into account as the threshold for carcinogenicity. Daily NIT intake was 40.8 mg/kg bw/day for the male animals and 50.9 mg/kg bw/day for the female animals, respectively.

Conclusions

The present results clearly indicated that NIT exerted carcinogenic potential in the esophagus in rats. The data herein were sufficient to evaluate the carcinogenicity of NIT. Of course, further studies on the mechanism of carcinogenicity for NIT needs to be performed.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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