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**Original**

# $\alpha$-Naphthylisothiocyanate Induces Intrahepatic Bile Duct with Greater Proliferation in Female Rats than in Males

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**Abstract:** The present study was conducted with the original purpose of investigating the possibility that $\alpha$-naphthylisothiocyanate (ANIT) might induce intrahepatic cholangiocellular neoplasms in rats after appropriate carcinogenesis-initiating treatments, based on its known effect of causing intrahepatic bile duct proliferation. Fischer 344 rats (6 weeks old) were given 3 weekly intraperitoneal administrations of vehicle (female and male), *N*-nitrosobis(2-oxopropyl)amine (BOP) (20 mg/kg body weight, female and male) or *N*-nitrosodimethylamine (DMN) (10 mg/kg body weight, male only), and fed a basal diet with or without 200 ppm of ANIT from the commencement for up to 24 weeks. Animals were sequentially sacrificed at the ends of weeks 8, 16 and 24 to examine morphological changes in the liver. ANIT caused proliferation of intrahepatic bile duct epithelial cells with no atypia when administered alone or in combination with BOP (female and male) or DMN (male only), while neither BOP nor DMN caused bile duct proliferation *per se* or altered the magnitude of the effect of ANIT. The degree of bile duct proliferation caused by ANIT was greater in females than in males. No hepatocellular or liver (pre)neoplastic changes were observed. These results indicate that although ANIT does not induce any neoplastic changes in the liver even after initiation with BOP (female and male) or DMN (male), it causes non-neoplastic intrahepatic bile duct proliferation with a clear sex difference.
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**Key words:** $\alpha$-naphthylisothiocyanate, sex difference, intrahepatic bile duct proliferation, rat

## Introduction

Intrahepatic cholangiocellular carcinoma (ICC) is a malignancy with high morbidity[1]. Whereas chronic injury, inflammation and cholestasis are postulated as risk factors, the underlying mechanisms of ICC still remain largely obscure[2]. It is therefore necessary to assess detailed mechanisms in appropriate animal models to establish mechanism-based strategies to control human ICC. A number of animal models of ICC have been developed in hamsters using combinations of chemical carcinogens, *Opisthorchis* infection and bile duct ligation[3–8]. However, only limited information is available about the genetic background of hamsters. In contrast, no mouse models are available, and in rats the only well-established model uses the long-term repeated injection of furan, a highly flammable and toxic agent which is difficult to handle[9–11].

$\alpha$-Naphthylisothiocyanate (ANIT) is a compound that causes injury to intrahepatic bile ducts but is not carcinogenic *per se* in rats[12–15]. Bile duct epithelia selectively proliferate in response to injury induced by ANIT, characterized by enhanced epithelial apoptosis[15]. Therefore, this compound might be able to generate ICC in rats in combination with appropriate initiating stimuli. In this context, the present study was performed to investigate the effects of ANIT on the liver with or without initiating treatment with *N*-nitrosobis(2-oxopropyl)amine (BOP) or *N*-nitrosodimethylamine (DMN). BOP and DMN were selected, because both of them have been used in hamster ICC models[3–8]. Male hamsters have been used in previous experiments to produce ICC[4,7], so we used only male animals in investigations using DMN. On the other hand, female hamsters have been used in the BOP models for

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ICC[5,6]. Moreover, the carcinogenic effects of BOP have been reported to differ between the sexes in rats[16,17]. The present study therefore used both female and male rats for groups administered BOP and the control groups, whereas only male animals were used for groups administered DMN.

## Materials and Methods

*Ethical considerations*

The experimental protocols were approved by the Animal Experimentation Committee of the Sasaki Institute prior to commencement, and the experiments were conducted under monitoring by the committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105, and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

*Animals*

A total of 80 female and 120 male Fischer 344 rats, 4 weeks old, were purchased from Charles River Japan, Atsugi, Kanagawa, Japan, and housed 3 to a plastic cage in an air-conditioned animal room at 24 ± 2°C, 55 ± 10% humidity, and a 12 hour dark/light cycle. After a 2-week acclimation period, 6-week-old animals were divided into 10 (4 female and 6 male) groups each consisting of 20 rats for the experimentation. Diet and tap water were freely available throughout the acclimation and experimental periods. Body weight, food consumption and water intake were monitored weekly.

*Chemicals*

ANIT was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and admixed into the basal diet (CRF-1 from Oriental Yeast Co., Ltd., Tokyo, Japan). A preliminary study was performed to determine an appropriate dose of ANIT. Male Fischer 344 rats were fed a basal diet containing 0, 100, 200, 500 or 1000 ppm of ANIT from 6 weeks of age for 2 weeks. In summary, the preliminary study revealed that ANIT induced intrahepatic bile duct proliferation in a dose-dependent manner. While no changes were detected with 100 ppm of ANIT, severe jaundice and marked suppression of body weight gain were observed with 500 or 1000 ppm of ANIT. On the other hand, 200 ppm of ANIT caused moderate changes in the liver without suppression of body weight gain. We thus decided to set the dose of ANIT as 200 ppm.

BOP and DMN were purchased from Nacalai Tesque, Inc., Kyoto, Japan, and were diluted with 0.9% NaCl solution at concentrations of 20 and 10 mg/ml, respectively, to achieve a unified injection volume of 1 ml/kg body weight.

*Animal experiment*

Figure 1 illustrates the experimental protocol. Females were used in groups 1–4, while males were used in the other groups. Groups 1/5 and 2/6 were given 3 weekly intraperitoneal administration of vehicle without and with the dietary administration of ANIT, respectively. Groups 3/7 and 4/8 were given 3 weekly intraperitoneal administration of BOP at a dose of 20 mg/kg body weight without and with ANIT, respectively. Groups 9 and 10 were given 3 weekly intraperitoneal administration of DMN at a dose of 10 mg/kg body weight without and with ANIT, respectively. In every group, 5 animals each were sacrificed at the ends of weeks 8 and 16, and 10 animals were sacrificed at the end of week 24 under light ether anesthesia. At autopsy, whole body of animals was macroscopically examined. Blood was collected by bifurcation of the abdominal aorta, and the serum samples were prepared to measure activities of alanine aminotransferase (ALT) and contents of total bilirubin. The liver (weighed), lungs, pancreas, thyroid glands, trachea, and kidneys were excised, fixed in 10% buffered formalin and embedded in paraffin. Then 4-$\mu$m thick sections were prepared, and at least one section from each organ was processed by routine hematoxylin and eosin staining for histological assessment.

The degree of bile duct proliferation in the liver was light microscopically evaluated. Under a magnification of ×200, numbers of bile ducts were counted in 4 different portal areas for each animal. In addition, proliferative activity of intrahepatic bile duct epithelia was assessed by means of immunohistochemical staining with an anti-proliferating cell nuclear antigen (PCNA) antibody (DakoCytomation Ltd., Kyoto, Japan). Over 1000 bile duct epithelial cells were counted under a light microscope at a magnification of ×200 in 4 different fields for each animal, and percentages of PCNA-positive cells were calculated.

*Statistical analysis*

The statistical significance of intergroup differences of means was analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test, and results were considered significant when the $p$ value was less than 0.05.

## Results

*General findings*

During the experiment, none of the rats in any of the groups died. There were no significant intergroup differences in body weights among groups within the same sex throughout the study (data not shown).

Relative liver weights are summarized in Table 1. In females, relative liver weights were heavier in groups 2 (ANIT) and 4 (ANIT + BOP) than in group 1 (control) at the end of week 8. In males, relative liver weights were heavier in groups 8 (ANIT + BOP) and 10 (ANIT + DMN) than in group 5 (control) at the end of week 8, and heavier in group 6 (ANIT) than in group 5 at the end of week 24.

*Serum ALT activity and total bilirubin content*

Serum ALT activities are summarized in Table 2.

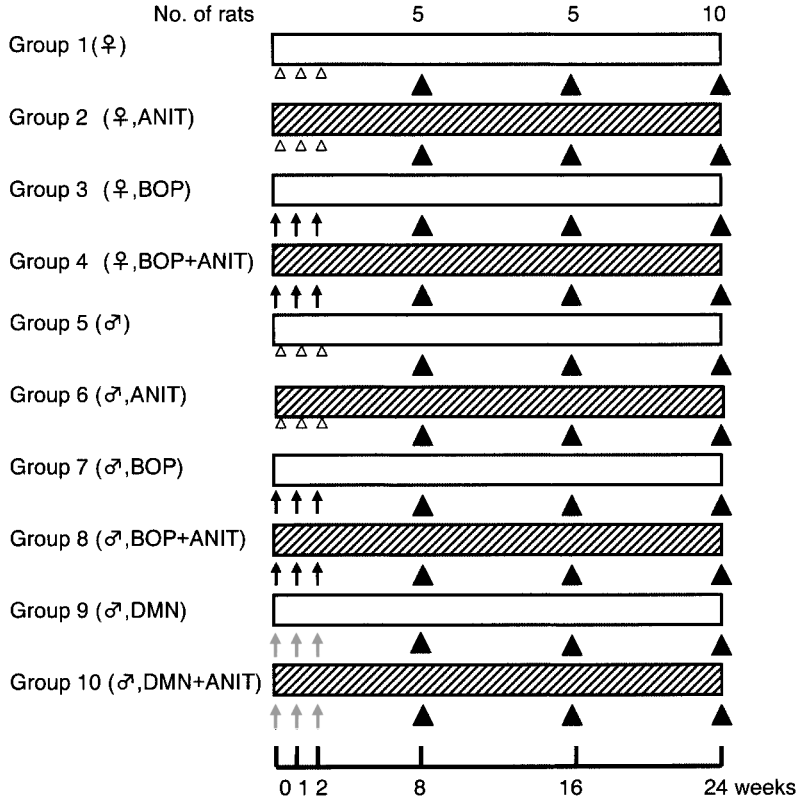


**Fig. 1.** Experimental protocol. Open bar, basal diet; hatched bar, ANIT; open arrowhead, vehicle; black arrow, BOP; gray arrow, DMN; closed arrowhead, sacrifice.

**Table 1.** Relative Liver Weights (g/100 g body weight )[a]

| Group | Treatment period (weeks) | | |
|---|---|---|---|
| | 8 (n=5) | 16 (n=5) | 24 (n=10) |
| Group 1 (♀) | 3.0 ± 0.2 | 2.9 ± 0.3 | 2.9 ± 0.2 |
| Group 2 (♀, ANIT) | 3.4 ± 0.1[b] | 2.9 ± 0.2 | 3.0 ± 0.2 |
| Group 3 (♀, BOP) | 3.2 ± 0.1 | 2.7 ± 0.1 | 2.8 ± 0.2 |
| Group 4 (♀, ANIT+BOP) | 3.7 ± 0.2[b] | 3.1 ± 0.2 | 3.1 ± 0.3 |
| Group 5 (♂) | 3.4 ± 0.1 | 2.9 ± 0.0 | 2.9 ± 0.1 |
| Group 6 (♂, ANIT) | 3.6 ± 0.1 | 3.0 ± 0.5 | 3.3 ± 0.2[c] |
| Group 7 (♂, BOP) | 3.3 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.2 |
| Group 8 (♂, ANIT+BOP) | 3.7 ± 0.1[c] | 3.0 ± 0.2 | 3.1 ± 0.1 |
| Group 9 (♂, DMN) | 3.6 ± 0.2 | 2.9 ± 0.3 | 2.9 ± 0.2 |
| Group 10 (♂, ANIT+DMN) | 3.9 ± 0.3[c] | 3.0 ± 0.2 | 3.0 ± 0.1 |

a: Values are given as means ± SDs.
b: Significantly different from group 1.
c: Significantly different from group 5.

Serum ALT activities gradually increased with age in both female and male control animals (group 1 for females and group 5 for males). The only significant change was obtained for group 9 (DMN) at the end of week 24, the value being higher than the group 5 (control) value. Serum total bilirubin contents were not altered among groups.

*Histological and immunohistochemical findings*

No particular changes were observed in any extrahepatic assessed organs in any group. In the liver, whereas no notable hepatocellular abnormalities were observed, portal bile duct proliferation was observed in the ANIT-administered groups. Our microscopic observation also gave the impression that bile duct proliferation was more prominent in females than in males (Fig. 2). No epithelial atypia or fibrosis, however, accompanied the ANIT-induced portal bile duct proliferation (Fig. 2). Figure 3 summarizes numbers of bile ducts per portal area at the end

**Table 2.** Serum ALT Activity (U/l)[a]

| Group | Treatment period (weeks) | | |
|---|---|---|---|
| | 8 (n=5) | 16 (n=5) | 24 (n=10) |
| Group 1 (♀) | 41.0 ± 3.2 | 58.4 ± 11.8 | 57.6 ± 7.5 |
| Group 2 (♀, ANIT) | 42.2 ± 7.4 | 46.6 ± 4.3 | 55.6 ± 12.6 |
| Group 3 (♀, BOP) | 44.2 ± 3.1 | 47.2 ± 4.9 | 49.6 ± 6.0 |
| Group 4 (♀, ANIT+BOP) | 50.6 ± 6.7 | 51.2 ± 3.3 | 50.6 ± 8.8 |
| Group 5 (♂) | 43.8 ± 4.7 | 57.0 ± 6.8 | 62.6 ± 7.4 |
| Group 6 (♂, ANIT) | 48.0 ± 5.1 | 46.6 ± 3.8 | 62.4 ± 11.8 |
| Group 7 (♂, BOP) | 48.2 ± 7.9 | 61.0 ± 10.3 | 66.6 ± 17.0 |
| Group 8 (♂, ANIT+BOP) | 60.4 ± 19.0 | 59.0 ± 5.1 | 65.2 ± 5.6 |
| Group 9 (♂, DMN) | 55.0 ± 3.4 | 61.8 ± 8.5 | 87.8 ± 16.9[b] |
| Group 10 (♂, ANIT+DMN) | 50.8 ± 16.0 | 49.0 ± 3.9 | 60.5 ± 17.5 |

a: Values are expressed as means ± SDs. b: Significantly different from group 5.

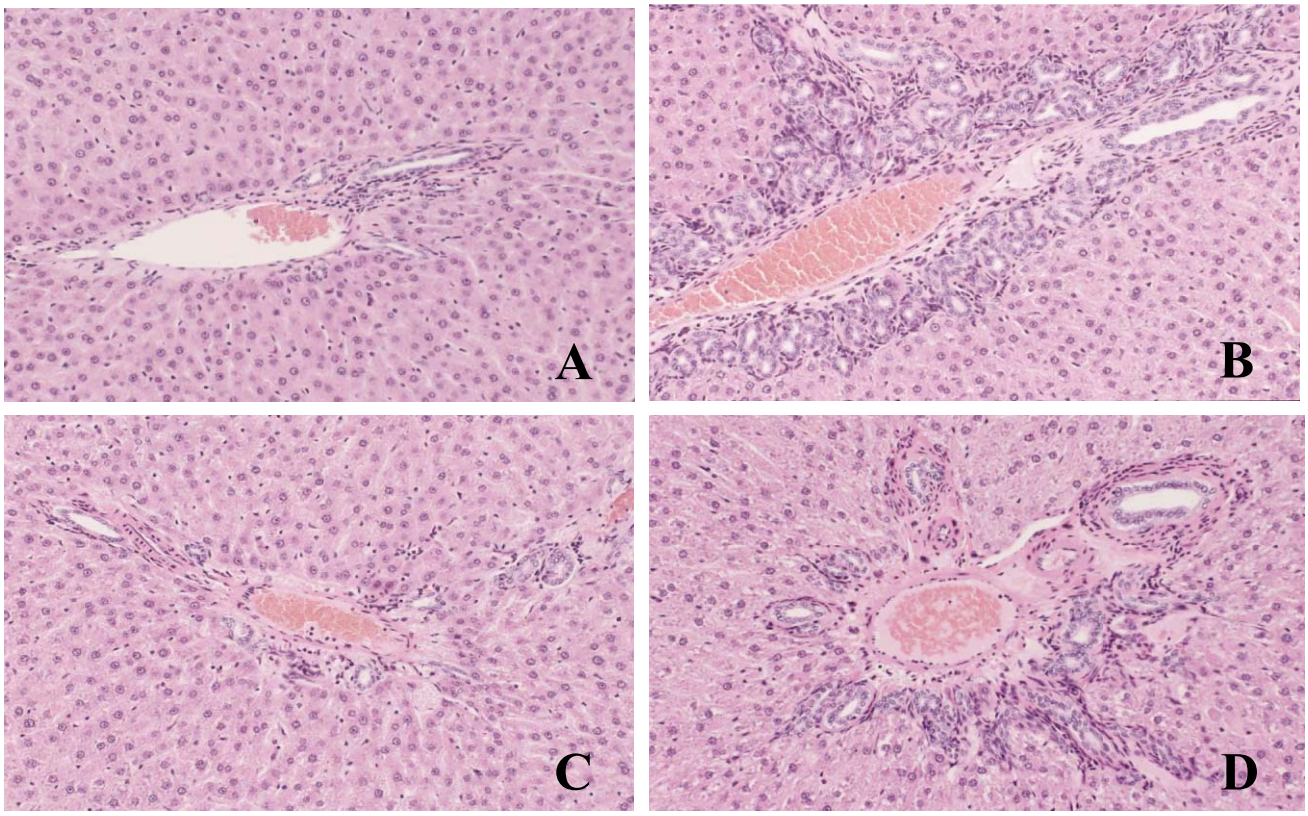


**Fig. 2.** Representative histology of the liver of groups (A) 1, (B) 2, (C) 5 and (D) 6 at the end of week 24; hematoxylin and eosin staining, ×200.

of weeks 8, 16, and 24. The degree tended to be enhanced by aging in each group. It became clear that ANIT induced marked and significant proliferation of intrahepatic bile ducts at each time-point in both sexes (compare the data for groups 2, 4, 6, 8 and 10 with those for groups 1, 3, 5, 7 and 9, respectively). Neither BOP (females and males) nor DMN (males) affected bile ducts with or without the concomitant treatment with ANIT (compare the data for groups 3, 4, 7/9 and 8/10 with those for groups 1, 2, 5 and 6, respectively). When comparing the data for groups 2 and 4 with those for groups 6 and 8, respectively, the effect of ANIT was significantly greater in females than in males at the end of weeks 16 and 24. It was thus confirmed that ANIT causes intrahepatic bile duct proliferation and that neither BOP nor DMN affect the degree of proliferation. In addition, the effect of ANIT was significantly greater in females than in males. Probably reflecting the lack of epithelial atypia in ANIT-induced proliferating portal bile ducts (Fig. 2), percentages of PCNA-positive bile duct epithelial cells were not different among groups (Fig. 4).

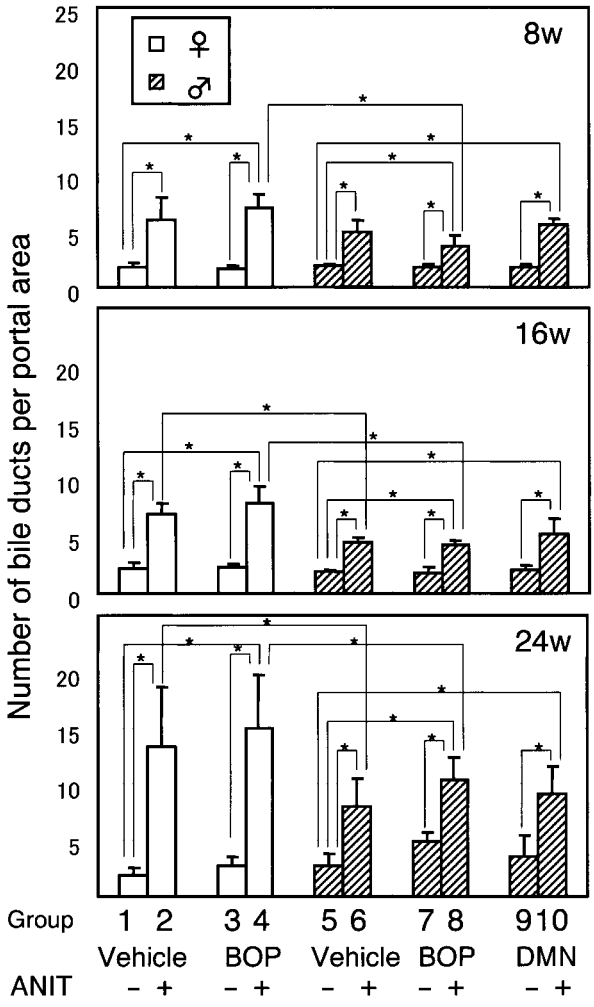


**Fig. 3.** Number of bile ducts per portal area at the end of weeks 8, 16 and 24. Asterisks represent statistical significance obtained for the inter-group differences of the paired group data.

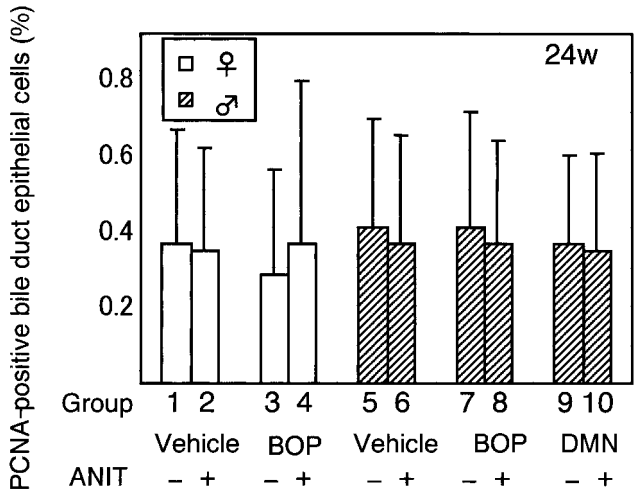


**Fig. 4.** Cell proliferating activity of bile duct epithelia of the liver at the end of week 24. Asterisks represent statistical significance obtained for the inter-group differences of the paired group data.

## Discussion

The present results demonstrate that ANIT *per se* induces intrahepatic bile duct proliferation in both female and male rats at 200 ppm. This change was not accompanied, however, by morphological evidence of malignancy such as cellular atypia and active stroma. Although it has been reported that a 2-week dietary administration of ANIT at a dose of 1000 ppm increases PCNA-positive portal bile duct epithelial cells[15], the suitability of such a high concentration for a long-term study was not indicated by our preliminary study, in which severe jaundice and suppression of gain of weight occurred. The results indicate that ANIT *per se* cannot induce neoplastic proliferation of intrahepatic bile ducts within a dose range allowing its long-term administration. Furthermore, neither BOP (in females and males) nor DMN (in males) caused any neoplastic (or even non-neoplastic) liver cholangiocellular lesions with or without the subsequent ANIT administration. This may be due to the lack of initiating activity of BOP or DMN in rats, in contrast to hamsters[3–8], and/or the lack of promoting activity of ANIT. At the dose employed here, 200 ppm, PCNA-positive bile duct epithelial cells did not increase in spite of bile duct proliferation. The reason is unclear, but because our doses were lower than usually used in short-term studies[12–15], the rate of proliferation may have been relatively slow, and increases in PCNA in bile duct epithelial cells could have been below detection levels. Taken together, we concluded that, at least under the present experimental conditions, ANIT cannot develop ICC or its preneoplastic lesion. Another strategy to develop a rat model for ICC will therefore be necessary.

In this study, a difference in the severity of intrahepatic bile duct proliferation was observed between female and male animals, the former appearing more sensitive to ANIT. This phenomenon was seen both in the absence and presence of the BOP treatment. ANIT is a well-known intrahepatic bile duct toxicant and has been extensively employed in animals to study the functions of intrahepatic bile ducts in physiological and pathological states[12–15,18–20], but to the best of our knowledge, there have been no reports that indicate sex differences in the effects of ANIT. ANIT feeding in experimental animals results in damage to bile duct epithelial cells and subsequent proliferation[18]. It also alters hepatic glutathione levels[19,20]. The mechanisms of bile duct proliferation still remain obscure, but the proliferation is accompanied by enhanced apoptosis. The accumulation of reactive oxygen species is suggested to be a cause of ANIT-induced apoptosis[15]. It has also been reported that ANIT causes mitochondrial dysfunction in the liver[21,22], which may be involved in the hepatic effects of this compound, and mitochondria-related cellular dysfunctions are frequently affected by the activities of sex hormones[23,24]. Because ANIT itself reduces serum progesterone level and affects hepatic progesterone status in female rats[25], we suggest that

the sex difference in the sensitivity of the intrahepatic bile duct to ANIT may be a sex hormone-dependent event. The mechanisms underlying this phenomenon are still largely obscure, and further studies will be required to elucidate them. Whereas this study failed to give positive results for the original purpose regarding ICC, the novel finding of the sex difference for the effects of ANIT is important from the viewpoint of toxicologic pathology.

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