

## 別紙 1

### 論文の内容の要旨

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論文題目 Toxic effects and metabolism of aflatoxin B1 in rats neonatally exposed  
to estrogenic compounds

(新生時にエストロゲン様物質に暴露されたラットにおける  
アフラトキシン B1の毒性と代謝)

Mycotoxins are toxic metabolites produced naturally by fungi such as *Aspergillus*, *Penicillium* and *Fusarium*. The contamination with mycotoxins in agricultural commodities during pre- and post-harvest processes is a global problem since they cannot completely be decontaminated, leading to serious risks to human and animal health. Aflatoxin B1 (AFB1) is a potent hepatotoxin and hepatocarcinogen in human and several animal species. Contamination with AFB1 has widely been observed in a variety of foods and feed especially in grains, peanuts, corn, cottonseed and related products. Outbreaks of aflatoxicosis with a high mortality have been reported in tropical countries in Asia and Africa. Chronic exposure to aflatoxin B1 is associated with liver and lung cancer. Toxicity of AFB1 arises from its conversion to active form, AFB1-8, 9-epoxide (AFBO), by hepatic cytochrome P450 (CYP)

enzymes. This metabolite is highly reactive electrophile and covalently binds to cellular DNA to form AFB1-DNA adducts. The formation of AFB1-DNA adduct is generally considered as a critical step in tumor initiation.

Primary biotransformation of AFB1 by hepatic CYP also produces hydroxylated metabolites, such as aflatoxin Q1 (AFQ1), aflatoxin M1 (AFM1), and aflatoxin P1 (AFP1) on the one hand. Cytosolic reductases convert AFB1 to aflatoxicol (AFL) on the other. Metabolic pathways for conversion of AFB1 to these metabolites, which are less toxic and carcinogenic than the parent AFB1, are considered to be detoxification pathways. The conjugation of AFB1 to glutathione mediated by glutathione *S*-transferase (GST) is well known to be the other important detoxification pathway.

Genistein (GS), an isoflavone, is a phytoestrogen found abundant in soy and soy-based products. GS exhibits highly selective binding to estrogen receptors (ERs) particularly with a preferential binding to ER $\beta$  over ER $\alpha$ . Concerning to isoflavone consumed by infancy, a number of studies have focused on the relationship between neonatal exposure to phytoestrogens and the long-term effects in adulthood.

$\alpha$ -Zearalenol ( $\alpha$ -ZOL) is a mycotoxin produced with zearalenone (ZEA) by some species of *Fusarium* and a major hepatic metabolite of ZEA in various animal species.  $\alpha$ -ZOL is also a non-steroidal estrogen, its estrogenic activity being 3-100 times higher than ZEA. Although numerous studies have focused on environmental endocrine-disrupting chemicals, especially on phytoestrogens, little is known about the effects of neonatal administration of non-steroidal natural estrogens on long-term alteration of hepatic drug metabolizing enzyme system.

In order to gain an insight into the effects of neonatal exposure to GS and  $\alpha$ -ZOL on metabolism of AFB1 and its toxicity in rats, the author conducted a series of experiments.

In Chapter 1, the effects of neonatal exposure to GS and  $\alpha$ -ZOL on hepatic metabolic function toward AFB1 in adult rats were studied. Both sexes of rats received a single subcutaneous injection with either GS (0.08, 0.4 or 2 mg/pup) or  $\alpha$ -ZOL (0.02, 0.1 or 0.5 mg/pup) at two days of age, and sacrificed to collect the liver and kidney at 6 weeks or two months of age. The postmitochondrial, cytosolic and microsomal fractions of the liver and kidney tissues were prepared and used for determination of *in vitro* AFB1 metabolism.

The results showed that the level of AF-DNA adduct formation mediated by liver postmitochondrial fraction was significantly lower in both sexes of rats treated with GS at either dose of 0.08, 0.4 or 2 mg/pup than in the control rats at two months of their age. However, the level of AF-DNA adduct formation mediated by microsomal fraction was significantly higher in all GS-treated male rats than in the control rats. The liver cytosolic GST activity to inhibit the microsomes-mediated AF-adduct formation was significantly higher in both male and female rats treated with the highest dose of GS than in the control rats. The formation of AF-DNA adduct mediated by liver postmitochondrial fraction in both sexes of rats treated with  $\alpha$ -ZOL did not show any significant differences from control rats. However, the level of AF-DNA adduct formation mediated by liver microsomal fraction was significantly increased by neonatal treatment with  $\alpha$ -ZOL. The cytosolic GST activity to inhibit AF-DNA adduct formation was increased only in female rats treated with the highest dose of  $\alpha$ -ZOL.

The rate of microsomal conversion of AFB1 to AFQ1, AFM1 and AFP1 was increased by neonatal treatment with the highest dose of GS in adult male rats, while the rate of conversion of AFB1 to AFQ1 and AFM1 was significantly increased in some groups of GS-treated female rats. The rate of conversion of AFB1 to AFM1 was increased also by neonatal  $\alpha$ -ZOL treatment at doses of 0.02 and 0.1 mg/pup in male rats and by that at all doses in female rats. The formation of aflatoxicol (AFL) from AFB1 by cytosol fraction was enhanced in all groups of adult male rats by neonatal treatment with either GS or  $\alpha$ -ZOL, but it was not affected by the same treatment in female rats. In contrast to these alterations in AFB1 metabolism in the liver at two months of age, there were no observable effects of neonatal GS nor  $\alpha$ -ZOL on the metabolic activities of the liver at 6 weeks of age.

The GST activities of liver cytosol toward four model substrates, 1-Chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), trans-4-phenyl-3-buten-2-one (t-PBO) and cumene hydroperoxide (CPH), were increased remarkably in male rats at two months of age by neonatal treatment with the highest dose of GS. The specific activities of both liver and kidney tissues toward CDNB, a universal substrate, were increased in GS-treated female rats. In both sexes of  $\alpha$ -ZOL treated rats, an increase in specific GST activity toward CDNB was noted in liver and kidney tissues.

These results demonstrate that the exposure to GS in neonatal rats induces the increase in the liver microsomal CYP450 enzyme activity to form AF-DNA adduct in adulthood. On the other hand, neonatal GS induces the increase in the activity of cytosolic GST and reductase enzymes, both of which are responsible for detoxification of AFB1. Neonatal exposure to  $\alpha$ -ZOL also induces the increase not only in the liver microsomal CYP450 enzyme activity, but also in cytosolic reductase and GST activities toward AFB1. However, the alterations of these enzyme activities

noted in  $\alpha$ -ZOL treated rats were not so drastic as those in GS treated rats, suggesting that these substances may exert their effects, at least in part, through different mechanisms.

In Chapter 2, the effects of neonatal exposure to GS and  $\alpha$ -ZOL on acute toxicity of AFB1 in adult rats were studied. Both sexes of rats were neonatally treated with either GS (2 or 6 mg/pup) or  $\alpha$ -ZOL (0.5 or 2.5 mg/pup) in the same manner as in Chapter 1, and were given AFB1 at a dose of 0.5 or 1.5 mg/kg by gastric intubation at two months of age. Blood and liver samples were collected at 48 hours after AFB1 administration for measurement of blood biochemical parameters and histopathological examination, respectively.

The results of blood biochemical parameters indicated that AFB1 administration at a dose of 1.5 mg/kg caused severe damages in internal organs especially in the liver as was noted in marked increases in the levels of parameters such as gamma glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic acid dehydrogenase (LDH), and total bilirubin (TBIL). Administration of AFB1 also increased the plasma levels of urea nitrogen (BUN) and creatinine (CRE). However, GS and  $\alpha$ -ZOL treatment during neonatal period reduced the AFB1-induced elevation of the levels of biochemical parameters in adult rats, especially remarkably those of the activity of indicator enzymes for hepatic cell damages. The severity of the liver lesion in AFB1 treated rats was further confirmed by histopathological observations, which showed severe hepatocellular damages with diffuse hemorrhage and cell necrosis in the liver. However, neonatal GS and  $\alpha$ -ZOL reduced the severity of the AFB1-induced hepatic lesions in adulthood although the reduction was slight.

These findings demonstrate that exposure to GS and  $\alpha$ -ZOL during neonatal period suppresses AFB1-induced acute toxicity represented by hepatocellular damages in adult rats.

In Chapter 3, the effects of neonatal treatment with GS and  $\alpha$ -ZOL on chronic toxicity of AFB1 were studied. Male rats were neonatally treated with either GS (2mg/pup) or  $\alpha$ -ZOL (2.5 mg/pup) in the same manner as in Chapter 1. Control rats received vehicle alone. At two months of age, rats were given AFB1 (0.2 mg/kg) or its vehicle through gastric intubation once a day for five consecutive days per week for two weeks. Blood and liver samples were collected at 18 weeks after the last dose of AFB1 for measurement of blood biochemical parameters and determination of GST-P positive foci formation, respectively.

AFB1 administration caused a significant elevation of the plasma ALT activity, and induced the GST-P positive single cells and foci formation. However, the number of AFB1-induced GST-P positive single cells and foci containing 2-20 positive cells in adult liver were markedly decreased by neonatal treatment with GS. Neonatal GS also tended to reduce the number of foci containing 21-50 positive cells or the foci larger than 100  $\mu$ m. Furthermore, the elevated levels of plasma ALT after AFB1 administration in adult rats were also reduced by neonatal GS. In contrast, neonatal exposure to  $\alpha$ -ZOL induced formation of GST-P positive single cells without AFB1 administration. Moreover, the number of AFB1-induced GST-P positive single cells and foci containing 21-50 positive cells in the adult liver were significantly increased in rats neonatally treated with  $\alpha$ -ZOL compared with control. In addition, the plasma ALT level in adulthood was dramatically elevated by neonatal treatment with  $\alpha$ -ZOL regardless of whether they were given AFB1 or not. Thus, neonatal exposure to

$\alpha$ -ZOL does not suppress chronic toxicity of AFB1, but rather it tends to enhance AFB1-induced hepatocarcinogenesis.

In conclusion, all the results of this study show the importance of neonatal exposure to non-steroidal natural estrogens in the adverse health effects of AFB1 in adulthood. The neonatal exposure to GS and  $\alpha$ -ZOL induces alterations in hepatic metabolic functions involved in the activation and detoxification of AFB1 in adult rats. The neonatal GS- and  $\alpha$ -ZOL-induced enhancement of cytosolic GST and reductase activities toward AFB1 was associated with the decrease of AFB1-induced acute toxicity in adult rats. In contrast to similar effects on acute toxicity of AFB1, neonatal GS suppressed the AFB1-induced chronic toxicity in adulthood, whereas  $\alpha$ -ZOL enhanced it. This may indicate that not only common mechanisms involved in neonatal estrogen-induced alterations of endocrine function, but also irreversible alterations specifically induced by  $\alpha$ -ZOL or GS, might be involved in the effects of neonatal exposure to these compounds on the metabolism and toxicity of AFB1 in adult rats.