Original Article

The effects of β-naphthoflavone on rat placental development

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Abstract: The morphological effects of β-naphthoflavone (β-NF) on placental development in pregnant rats were examined. β-NF, administered to pregnant rats intraperitoneally at 15 mg/kg bw from gestation day (GD) 9 to GD 14, had no effect on maternal body weight gain, mortality, or clinical sign. In the β-NF-exposed rats, intrauterine growth retardation (IUGR) rates increased on GDs 17 and 21, although there was no effect on fetal mortality rate, fetal or placental weight, or external fetal abnormality. Histopathologically, β-NF induced apoptosis and inhibition of cell proliferation of the trophoblastic septa in the labyrinth zone, resulting in its poor development. In the basal zone, β-NF induced spongiotrophoblast apoptosis and delayed glycogen islet regression, resulting in their cystic degeneration. β-NF-induced CYP1A1 expression was detected in the endothelial cells of the fetal capillaries in the labyrinth zone and in the endothelial cells of the spiral arteries in the metrial gland, but not in any trophoblasts. This indicates that CYP1A1 is inducible in the endothelial cells of the fetal capillaries in the labyrinth zone, and that these cells have an important role in metabolizing CYP1A1 inducers crossing the placental barrier. (DOI: 10.1293/tox.2019-0047; J Toxicol Pathol 2019; 32: 275–282)

Key words: apoptosis, β-naphthoflavone, CYP1A1, intrauterine growth retardation (IUGR), placenta, rat

Introduction

The placenta forms an interface between the dam and the developing embryo/fetus. It has multiple functions that are essentially important for normal fetal growth, including the mediation of maternal immune tolerance, hormone production, nutrient uptake, waste elimination, and gas exchange[1]. The placenta is able to metabolize many xenobiotics, altering their concentration and the metabolites to which the fetus is exposed[2]. However, the spectrum of metabolized substrates and the metabolic activities of the placenta are somewhat restricted in comparison with the liver[3]. Xenobiotics which cross the placenta are modulated by the actions of phase I and phase II drug metabolizing enzymes in the placenta. Phase I cytochrome P450 (CYP) enzymes have been well characterized in the placenta. CYP1A1, 2E1, 3A4, 3A5, 3A7, and 4B1 have been detected in human placenta[4], and their members and quantities of vary depending on placental development, length of gestation, and maternal health. CYP1A1 is the most important xenobiotic-metabolizing enzyme of the placenta. Elevated CYP1A1 activity in the placenta has been associated with pregnancy complications in humans, such as premature birth, intrauterine growth retardation (IUGR), fetal death, placental abruption, risk of low birth weight, low birth length, and decreased head circumference[5, 6], indicating that placental CYP1A1 induction contributes to fetal developmental toxicity.

β-Naphthoflavone (β-NF) is one of the most potent flavonoid compounds, and is a strong CYP1A inducer, regulated by the aryl hydrocarbon receptor (AhR). β-NF induces an impairment of feto-placental growth and placental CYP1A1 expression in rats[7], although this has no teratogenic potential in mice[8]. In β-NF-exposed rats, the activity of ethoxyresorufin-O-deethylase, a CYP1A marker, is markedly higher (approximately 100-fold) in the labyrinth zone than in the basal zone[9]. CYP1A1 is detected in the maternal and fetal livers, and in the placentas of β-NF-exposed pregnant rats, whereas CYP1A2 is detected in the maternal livers, but not in the placentas or fetal livers[10]. However, there are no reports describing detailed sequential placental histopathology in β-NF-exposed pregnant rats. In this study, we examined the sequential histopathological changes in placentas, including CYP1A1 immunohistological expression, after intraperitoneal exposure to β-NF from gestation day (GD) 9 to GD 14 in order to elucidate the morphological effects of β-NF on the development of rat placenta.

Materials and Methods

Animals

Pregnant (GD 6) specific pathogen-free Wistar Hannover rats (Japan Laboratory Animals, Inc., Hanno, Japan) were purchased at approximately 11–12 weeks of age. GD 0 was designated as the day when the presence of the vaginal plug was identified. The animals were single-housed in plastic cages on softwood chip bedding in an air-conditioned room (22 ± 2°C; 55 ± 10% humidity; 12 h/day light cycle). Food (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*.

Experimental design

This study was conducted according to the Guidelines for Animal Experimentation, Biological Research Laboratory, Nissan Chemical Corporation, and the Statement about sedation, anesthesia, and euthanasia in a rodent fetus and newborn (2015) in Japanese College of Laboratory Animal Medicine. Thirty-two pregnant rats were randomly allocated into two groups of 16 rats each (Table 1). β-NF (Wako Pure Chemical Industries, Ltd., Osaka, Japan), suspended in olive oil, was administered intraperitoneally to one group at a dose of 15 mg/kg bw in a volume of 0.5 ml/100 g bw from GD 9 to GD 14. This dose was selected as β-NF at 15 mg/kg bw has previously been reported to have effects on both the fetuses and placentas of pregnant rats[8]. Animals in the control group were dosed similarly with olive oil alone. All treatments were administered between 9 and 11 a.m. Maternal body weight was recorded on GD 0, 6, and 14–21. Dams (n=4/each time point/group) were sampled on GD 13, 15, 17, and 21.

The dams were euthanized by exsanguination under anesthesia with isoflurane, and necropsied. All fetuses were removed from the placentas. A third of the placentas were separated between the basal zone and the decidua basalis, and removed from the uterus wall. The fetuses and removed placentas were weighed, and the individual fetal-placental weight ratios were calculated. The fetuses were examined for external malformations with the aid of a dissecting microscope on GD 21. According to the criteria for IUGR evaluation, the fetuses were defined as having IUGR if their weight was −2 standard deviations (SD) of the mean of the fetuses in the control group on each gestation day[11], which in the present study was <0.78 g on GD 17 and <4.62 g on GD 21. The IUGR rate (i.e. the actual number of fetuses exhibiting IUGR as a percentage of the total number of fetuses[12]) was calculated. At each sampling point (except on GD 13), fetal and maternal livers were randomly obtained from two litters in both groups. All fetal and placental samples, and maternal and fetal liver samples were fixed in 10% neutral buffered formalin.

Histopathological examination

The placentas and livers were embedded in paraffin, and 4 μm thick sections were stained routinely with hematoxylin and eosin (H&E) for histopathological examination. All selected placentas were subjected to phospho-histone H3 (Ser10; Cell Signaling Technology, Boston, MA, USA) immunohistochemical staining and *in situ* TdT-mediated dUTP nick end labeling (TUNEL; *In Situ* Cell Death Detection Kit, POD, Roche Applied Science, Penzberg, Germany)[13] to evaluate cell proliferation and apoptosis, respectively. All selected placentas and livers were subjected to CYP1A1 analysis (EMD Millipore, Billerica, MA, USA). The thicknesses of the labyrinth zone, basal zone, decidua basalis, and metrial gland close to the central portion of the placentas were measured using an image analyzer (Win-ROOF, Mitani Co., Tokyo, Japan). With the aid of the image analyzer, the numbers of phospho-histone H3-positive cells and TUNEL-positive cells in the labyrinth zone, basal zone, metrial gland, and yolk sac were counted in 20 sections using light microscopy with a 40× objective.

Statistical analysis

Means and SD of the individual litter values were calculated (Pharmaco Basic, Scientist Press Co., Ltd., Tokyo, Japan). For comparisons between two groups, either the Student's *t*-test for homoscedastic data, or the Aspin-Welch's *t*-test for non-homoscedastic data, was performed after the F test. The level of significance was set at P<0.05 and <0.01, respectively.

Table 1. Effects of β-naphthoflavone on Placentas and Fetuses

Autopsy	Group	No. of dams	Mean No. of live fetuses[a]	Fetal mortality (%)[a]	Fetus weight (g)[a]	Placenta weight (g)[a]	Fetus / Placenta (g/g)[a]	IUGR rate (%)
GD13	Control	4	11.6 ± 1.5	5.2 ± 4.7	0.08 ± 0.01	0.08 ± 0.01	1.01 ± 0.06	ND
	Treated	4	11.0 ± 1.4	2.1 ± 4.2	0.09 ± 0.00	0.05 ± 0.01*	0.05 ± 0.01	ND
GD15	Control	4	11.8 ± 0.5	5.8 ± 7.4	0.27 ± 0.00	0.19 ± 0.02	1.52 ± 0.13	ND
	Treated	4	9.4 ± 1.5*	14.6 ± 8.1	0.31 ± 0.02*	0.20 ± 0.03	1.50 ± 0.19	ND
GD17	Control	4	10.5 ± 0.6	2.1 ± 4.2	0.90 ± 0.07	0.29 ± 0.01	3.10 ± 0.13	9.5
	Treated	4	10.3 ± 1.0	8.4 ± 9.8	0.81 ± 0.07	0.26 ± 0.03	3.11 ± 0.13	34.1##
GD21	Control	4	10.3 ± 2.4	0.0 ± 0.0	5.42 ± 0.43	0.53 ± 0.03	10.33 ± 0.71	0.0
	Treated	4	11.3 ± 1.0	0.0 ± 0.0	5.13 ± 0.09	0.48 ± 0.05	11.13 ± 0.93	11.4#

Mean ± SD. [a] Mean of individual litter values. *Significantly different from the control group at P<0.05 (Student's *t*-test/Aspin-Welch's *t*-test). #, ##Significantly different from the control group at P<0.05, <0.01, respectively (χ^2 test). IUGR, intrauterine growth retardation; GD, gestation day; ND, Not done.

Results

Effects of β-NF administration on dams

β-NF administration had no effect on body weight gain (%) of the dams (based on the body weight on GD 6 which was 100%), compared with the control group. No mortality or clinical signs were observed in any dams in both groups during the experimental period.

Effects of β-NF administration on embryos/fetuses and placentas

The effects of β-NF administration on embryos/fetuses and placentas are shown in Table 1. β-NF administration had no effect on the fetal mortality rate, compared with the control group. Transiently, an increase in fetal weight on GD 15, caused by fetal loss, and a decrease in placental weight on GD 13 were observed in the β-NF-treated group. IUGR rates significantly increased in the β-NF-treated group on GDs 17 and 21. There were no obvious macroscopic external fetal abnormalities on GD 21 in either group.

Histopathological observation

Labyrinth zone: The labyrinth zone thickness diminished from GD 15 onward in the β-NF-treated group, resulting in its poor development (Fig. 1 and 2). Apoptotic cells, characterized by pyknosis or karyorrhexis, phagocytosis, and cell debris, and positively stained by the TUNEL method, were slightly scattered in the trophoblastic septa in the β-NF-treated group. There was an increase in the number of TUNEL-positive cells from GD 13 to GD 17, and a decrease in the number of phospho-histone H3-positive cells on GDs 13 and 15 in the β-NF-treated group in comparison with that in the control group (Fig. 3 and 4). CYP1A1 expression was detected in the endothelial cells of fetal capillaries in the trophoblastic septa from GD 13 to GD 17 in the β-NF-treated group, but not in the control group (Table 2, Fig. 1).

Basal zone: The basal zone thickness increased from GD 17 onward in the β-NF-treated group in comparison with that in the control group (Fig. 1 and 2). This change occurred due to the presence of glycogen cell island remains on GD 17, and the cystic degeneration of glycogen cells on GD 21 (Fig. 1), although the glycogen cell islands regressed

from GD 17 onward in the control group. The number of TUNEL-positive cells increased on GD 13 in the β-NF-treated group in comparison with that in the control group (Fig. 3), and these apoptotic cells seemed to be spongiotrophoblasts. However, there was no change in the number of phospho-histone H3-positive cells and no CYP1A1 expression in either group (Table 2, Fig. 4).

Decidua basalis: There was no change in the decidua basalis thickness or histopathological lesions in the β-NF-treated group (Fig. 1 and 2). CYP1A1 expression was minimally detected in the endothelial cells of the spiral arteries on GD 13 in the β-NF-treated group (Table 2).

Metrial gland: There was no change in the metrial gland thickness, or the number of TUNEL-positive or phospho-histone H3-positive cells in the β-NF-treated group (Fig. 1, 2, 3 and 4). CYP1A1 expression was detected in the endothelial cells of the spiral arteries from GD 13 to GD 17 in the β-NF-treated group (Table 2, Fig. 1). In contrast, there was no CYP1A1 expression in the interstitial trophoblasts, which penetrated through the decidua basalis, or the invaded endovascular trophoblasts in the spiral arteries (Fig. 1).

Yolk sac: There was no difference between the number of TUNEL-positive or phospho-histone H3-positive cells in the two groups, and no CYP1A1 expression in either group (Table 2).

Other tissues: CYP1A1 expression was detected in the endothelial cells of the maternal capillaries in the endometrium, and in the peripheral lobule of the maternal livers, from GD 13 to GD 17, and was minimally present in fetal livers only on GD 15 in the β-NF-treated group (Table 2, Fig. 1).

Discussion

CYP1A1 is responsible for metabolically activating, and detoxifying, numerous polycyclic aromatic hydrocarbons (PAHs) and aromatic amines present in combustion products[14]. In humans, CYP1A1 is the most important xenobiotic-metabolizing enzyme of the placenta, and its inducible activity has been demonstrated throughout pregnancy[5]. CYP1A1 is detected in the syncytiotrophoblasts in human placenta[15]. In addition, CYP3C4 and other CYP types are

Table 2. Expression of CYP1A1 in Placenta, Uterus and Liver

| Autopsy | Group | Placenta | | | | | Uterus | Liver | |
		Labyrinth zone	Basal zone	Decidua basalis	Metrial gland	Yolk sac		Fetus	Dam
GD13	Control	−	−	−	−	−	−	ND	−
	Treated	++	−	+/−	++	−	++	ND	+++
GD15	Control	−	−	−	−	−	−	−	−
	Treated	++	−	−	++	−	+	+/−	++
GD17	Control	−	−	−	−	−	−	−	−
	Treated	+/−	−	−	+/−	−	+/−	−	++
GD21	Control	−	−	−	−	−	−	−	−
	Treated	−	−	−	−	−	−	−	−

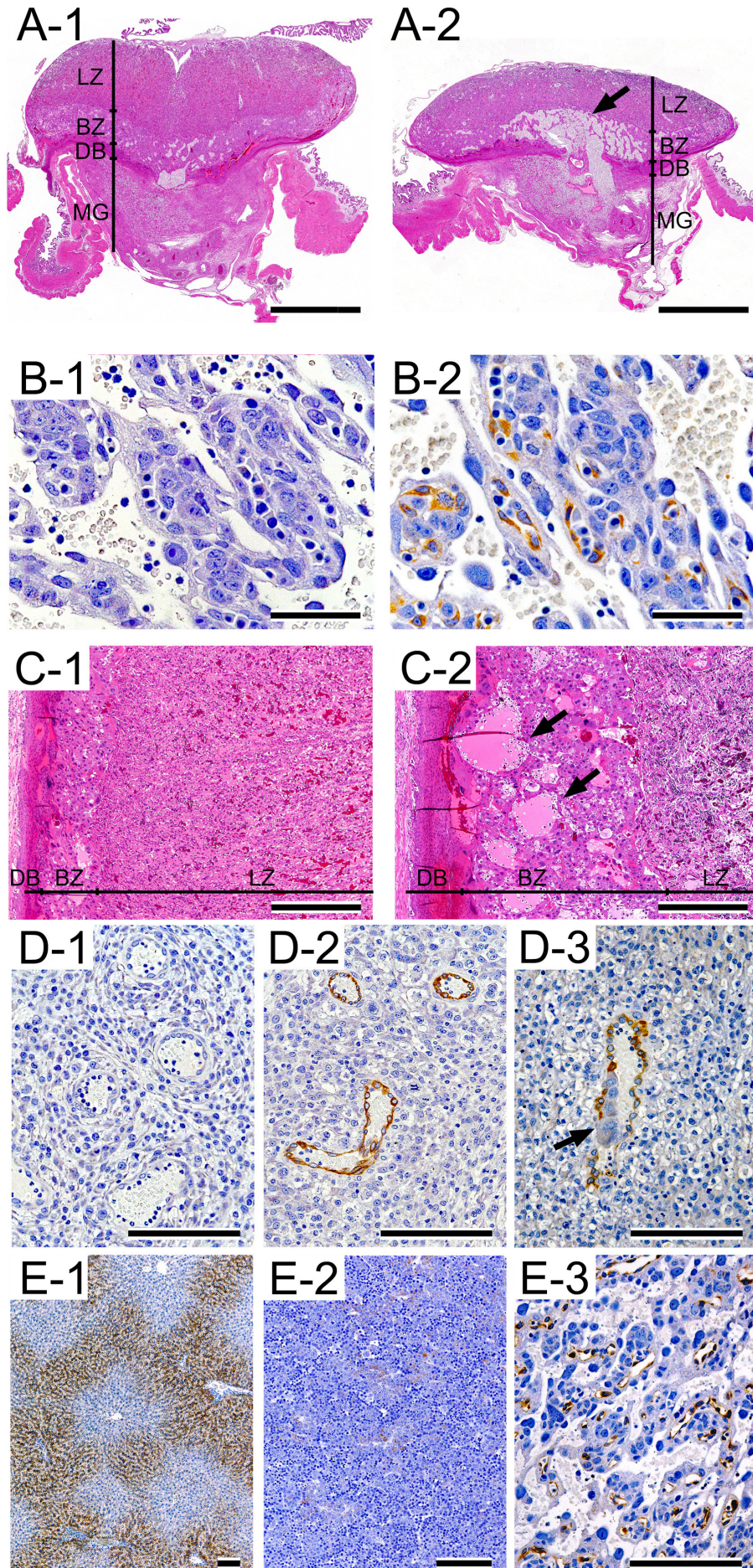−, Negative; +/−, Minimal; +, Slight; ++, Moderate; +++, Severe; GD, gestation day; ND, Not done.
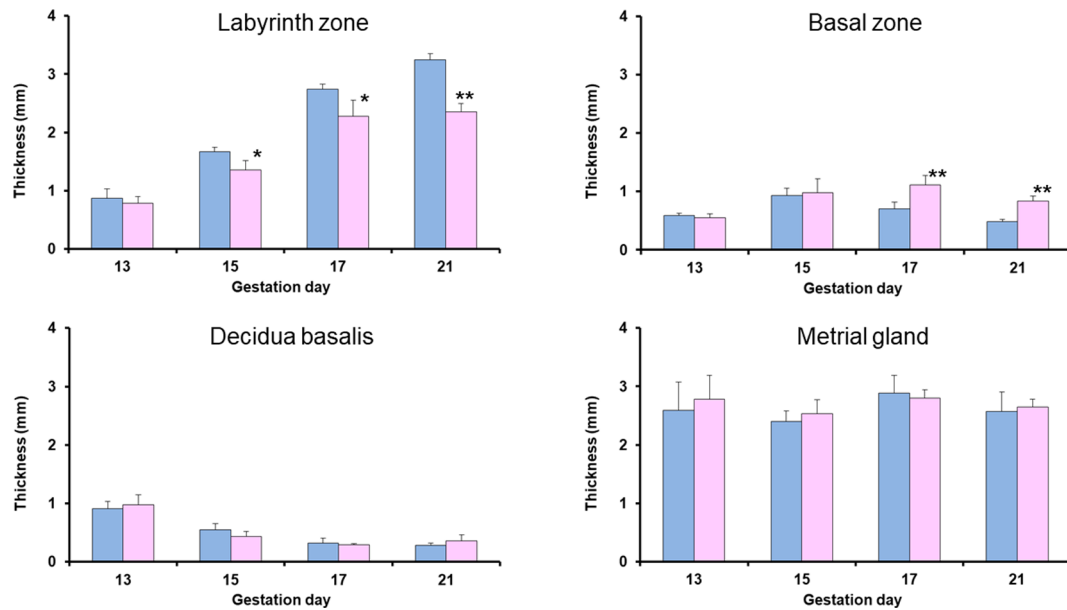
Fig. 1.

Fig. 2. The thickness of the labyrinth zone, basal zone, decidua basalis, and metrial gland. Pink bar, Control; Blue bar, β-naphthoflavone-treated group. Each value represents mean ± SD. *, **Significantly different from the control group at P<0.05, <0.01, respectively (Student's *t*-test/Aspin-Welch's *t*-test).
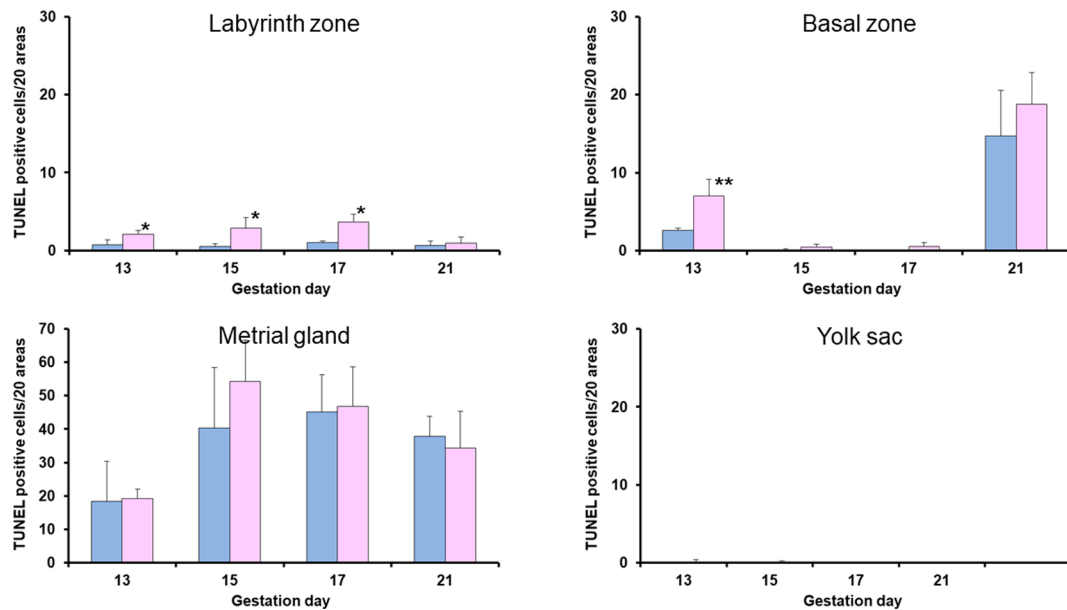

Fig. 3. TdT-mediated dUTP nick end labeling (TUNEL) positive cells in the labyrinth zone, basal zone, metrial gland, and yolk sac. Pink bar, Control; Blue bar, β-naphthoflavone-treated group. Each value represents mean ± SD. *, **Significantly different from the control group at P<0.05, <0.01, respectively (Student's *t*-test/Aspin-Welch's *t*-test).

Fig. 1. Histopathological placenta findings. A. Histological changes in the placenta on gestation day (GD) 17. Bar=2,500 μm. HE stain. 1. Control group. 2. β-naphthoflavone (β-NF)-treated group. Poor development of the labyrinth zone and thickening of the basal zone with delayed glycogen islet regression (↑). B. CYP1A1 expression in the labyrinth zone on GD13. Bar=50 μm. CYP1A1 immunohistochemical stain. 1. Control group. 2. β-NF-treated group. CYP1A1 expression in the endothelial cells of fetal capillaries in the trophoblastic septa. C. Histological changes in the basal zone on GD 21. Bar=600 μm. HE stain. 1. Control group. 2. β-NF-treated group. Thickening of the basal zone with cystic degeneration of glycogen cells (↑). D. CYP1A1 expression in the metrial gland on GD13. Bar=150 μm. CYP1A1 immunohistochemical stain. 1. Control group. 2 and 3. β-NF-treated group. CYP1A1 expression in the endothelial cells of the spiral arteries, but not in the invaded endovascular trophoblasts (↑) from the labyrinth zone. E. Comparison of CYP1A1 expression in the liver and placenta on GD 15. Bar=150 μm. CYP1A1 immunohistochemical stain. 1. Maternal liver. 2. Fetal liver. 3. Placenta (labyrinth zone). CYP1A1 expression in the placenta is lower than that in the maternal liver, but is much higher than that in the fetal liver. LZ, Labyrinth zone; BZ, Basal zone; DB, Decidua basalis; MG, Metrial gland.
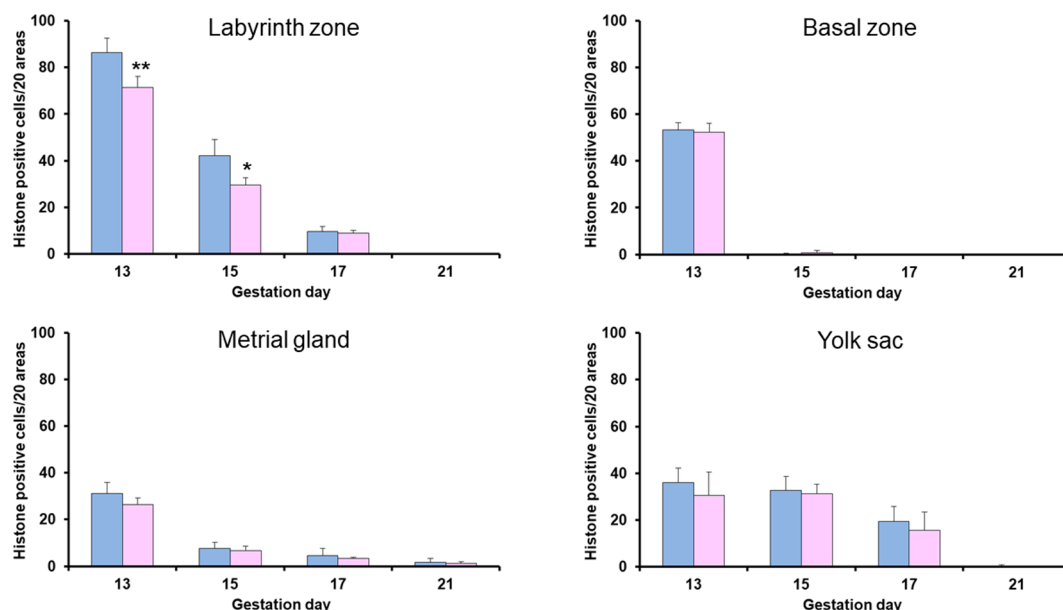
Fig. 4. Phospho-histone H3 positive cells in the labyrinth zone, basal zone, metrial gland, and yolk sac. Pink bar, Control; Blue bar, β-naphthoflavone-treated group. Each value represents mean ± SD. *, **Significantly different from the control group at P<0.05, <0.01, respectively (Student's *t*-test/Aspin-Welch's *t*-test).

expressed in placental trophoblast cell lines *in vitro*[16, 17]. In rats, it is reported that the activities of CYP1A, 2B, 2C, and 3A in the placenta are below the limit of enzyme assay detection[18]. However, only CYP3A1 is detected in the placenta by Western blot analysis, but not CYP1A1, 2B1, 2C6, 2C12, 2D1, 2D4, 2E1, and 4A1 at all stages of gestation[19]. Immunohistochemical analysis indicated that CYP3A1 is located in trophoblastic giant cells in the basal zone[19]. It is considered to be a major component of the CYP system in the rat placenta. CYP1A1 activity in the normal rat placenta is extremely low, although CYP1A1 mRNA expression can be detected. In contrast, CYP1A1 activity in rat placenta is elevated by β-NF[9], hexachloronaphthalene[20], nicotine[21], and tobacco smoke[10]. Although CYP1A1 is known to be mainly induced in the labyrinth zone[20], there have not been any previous studies investigating its specific localization within this zone. The results of the present study indicate that CYP1A1 expression in the rat placenta is induced in the endothelial cells of both fetal capillaries in the labyrinth zone and spiral arteries in the metrial gland, but that it is not expressed in any trophoblasts in the labyrinth or basal zones. CYP1A1 expression is known to be induced in the endothelial cells in veins of the heart, skeletal muscle, and uterine smooth muscle, in addition to hepatocytes in the β-NF-exposed non-pregnant rats[22]. This study also demonstrated that CYP1A1 expression in the placenta is lower than that in the maternal liver, but is much higher than that in the fetal liver. Therefore, it is revealed that the endothelial cells of both fetal capillaries and spiral arteries in rat placenta have CYP1A1-induction potential in the present study. The endothelial cells of the fetal capillaries in the labyrinth zone, in particular, have an important role in metabolizing CYP1A1-

inducers crossing the placental barrier. CYP1A1 and CYP3A1 expression is induced in different CYP-expressing cell types in the rat placenta. Additionally, CYP1A1 expression is induced in different cell types in rat and human placenta, indicating that there is a difference between rats and humans in the cell types which contribute to drug metabolism in the fetal-maternal placental interface. Therefore, caution should be taken when extrapolating placental toxicity in rats to human risk evaluation of CYP1A1-inducing xenobiotics in developmental toxicity. However, we could not determine, from the results of the present study, whether this cell type difference in CYP induction would result in a difference in CYP1A1-related toxicities between the species.

Elevated CYP1A1 activity in the placenta is thought to be involved in adverse birth outcomes, such as IUGR, premature birth, structural abnormalities, and mortality observed in tobacco-exposed rats[23]. It has been reported that β-NF administration from GD 7 to GD 14 is associated with fetal mortality[24], and that its administration from GD 11 to GD 14 impairs late feto-placental growth in rats[8]. In this study, the IUGR rate increased in the β-NF-treated group, although no effect on either fetal or placental weight was observed. However, histopathologically, β-NF induced apoptosis and/or inhibition of cell proliferation in trophoblastic septa in the labyrinth zone and in spongiotrophoblasts in the basal zone, resulting in poor development of the fetal part of the placenta. These changes were considered to be induced by AhR-mediated changes in the expression of genes related to apoptosis and cell cycle arrest by the xenobiotic response element-dependent mechanism[25, 26], but not by either oxidative metabolism of endogenous or xenobiotic CYP1A1 substrates[27]. The labyrinth zone plays a role

in O_2/CO_2 exchange, providing nutrients for the fetus, and removing waste products. Its damage is highly correlated with IUGR[28]. In addition, β-NF induced delayed glycogen islet regression and the cystic degeneration of glycogen cells in the basal zone. The glycogen cells are the storage sites for glycogen produced from maternal glucose[29]. Glycogen islet regression in normal placental development is thought to meet the increased demand for glycogen as an energy substrate for fetal growth in the final period[30, 31]. Cystic degeneration is a non-specific lesion in the basal zone, which is induced, in association with IUGR, by chemicals, including chlorpromazine[28], 6-mercaptopurine[32], and dibutyltin[33]. These alterations in the basal zone are expected to affect fetomaternal homeostasis and fetal development[30]. Thus, it is suggested that the β-NF-induced IUGR in rats is not only related to the elevated CYP1A1 activity in the placenta, but also to the poor development and dysfunction of the fetal part of the placenta.

In conclusion, this study demonstrates that β-NF induces apoptosis and/or inhibition of cell proliferation in the labyrinth and basal zones of rat placenta, resulting in poor development of the fetal part of the placenta. CYP1A1 induction in the placentas of β-NF-treated rats occurred in the endothelial cells of the fetal capillaries in the labyrinth zone and the spiral arteries in the metrial gland, but not in any trophoblasts.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

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