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Original Article

Effects of letrozole on rat placental development

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Abstract: We examined the morphological effects of letrozole on placental development in pregnant rats. Letrozole was orally administered at a repeat dose to pregnant rats at 0 mg/kg (control group) and 0.04 mg/kg (letrozole group) from gestation day (GD) 6 to GD 20. In the letrozole group, fetal mortality and placental weight increased from GD 15 onwards and GD 13 onwards, respectively. Fetal weights increased on GDs 15 and 17 but decreased on GD 21. Histopathologically, letrozole treatment induced multiple cysts lined with undifferentiated syncytiotrophoblasts in the trophoblastic septa on GD 13. These cysts then develop into dilated maternal sinusoids with congestive hyperemia, resulting in an enlarged placenta. In the metrial gland, there was a dilated lumen of the spiral artery and interstitial edema throughout the experimental period, resulting in thickened metrial gland. These changes are considered to be due to maternal blood circulation stagnation in the metrial gland, which is associated with dilated maternal sinusoids in the labyrinth zone. Thus, although letrozole induces an enlarged placenta due to congestive hyperemia of the labyrinth zone and transient increases in fetal weight, these placentas are thought to decline in function as the pregnancy progresses, leading to intrauterine growth restriction at the end of pregnancy. (DOI: 10.1293/tox.2024-0025; J Toxicol Pathol 2024; 37: 163–172)

Key words: enlarged placenta, intrauterine growth restriction, letrozole, multiple cysts, rat

Introduction

Although the placenta is a temporary organ during pregnancy, it plays a pivotal role in maintaining pregnancy and fetal growth, including nutrient uptake, waste elimination, gas exchange, mediation of maternal immune tolerance, and hormone production[1, 2]. The placenta is a major site of endocrine activity, including the synthesis of various steroid and peptide hormones, growth factors, cytokines, and other bioactive factors[3]. In addition, placental development is regulated by steroid hormone[4]. Changes in placental weight are important reproductive and developmental toxicity indicators[2]. An enlarged placenta is observed under various conditions such as an unfavourable maternal environment[5, 6], compensatory recovery of fetal growth retardation[7, 8], reduction in the number of corpora lutea[9], and ovariectomy with estrogen and progesterone treatment[10, 11]. In addition, an enlarged placenta is induced by exposure to chemicals such as ethanol[12, 13], indomethacin[14], methylhydrazine[15], estrogen[16], and some aromatase inhibitors[17–20].

Letrozole (4,4'-[(1H-1,2,4-triazol-1-yl) methylene] bisbenzonitrile) is a nonsteroidal third-generation aromatase inhibitor to treat breast cancer[21]. Letrozole is toxic to foetuses, with increased placental weight in rats when administered from gestation day (GD) 6 to GD 16, resulting in increased prenatal mortality and minor anomalies of the axial skeleton[22]. However, there have been no reports describing the detailed sequential histopathological changes in the placenta of rats exposed to letrozole. In the current study, we administered repeated oral doses of letrozole to pregnant rats from GD 6 to GD 20 and performed histopathological examination of the placentas on GDs 13, 15, 17, and 21 to elucidate letrozole-induced fetal toxicity and the sequential morphological effects on placental development.

Materials and Methods

Ethical considerations

This study was conducted per the Guidelines for Animal Experimentation, the Biological Research Laboratory, Nissan Chemical Corporation, and the Statement about sedation, anaesthesia, and euthanasia of rodent foetuses and newborns (2015) at the Japanese College of Laboratory Animal Medicine.

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Animals

Pregnant-specific pathogen-free Wistar Hannover rats (BrlHan: WIST@Jcl (GALAS); CLEA Japan, Tokyo, Japan) were purchased at approximately 11–12 weeks of age. 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 provided *ad libitum*.

Experimental design

A total of 32 pregnant rats (GD 6) were randomly allocated to two groups (n=16 per group) (Table 1). GD 0 was defined as the day on which the vaginal plug was identified. Letrozole (Tokyo Chemical Industries Ltd., Tokyo, Japan) was suspended in olive oil and administered as a repeat oral dose to the groups at doses of 0 mg/kg with olive oil (the control group) and 0.04 mg/kg (the letrozole group), with a volume of 0.5 mL/100 g body weight from GD 6 to 20. This dose was selected as letrozole at 0.04 mg/kg body weight, which has previously been reported to induce post-implantation loss and increase placental weight in pregnant rats[22]. All treatments were conducted between 9 AM and 11 AM. Maternal body weights were recorded from GD 6 to 21. Dams (n=4 for each time point per group) were sampled on GDs 13, 15, 17, and 21. The dams were euthanized by exsanguination under isoflurane anaesthesia and subsequently necropsied. All the foetuses were removed from the placenta. Approximately 1/3 of the placentas were separated between the basal zone and decidua basalis and removed from the uterine wall. The foetuses and removed placentas were weighed, and the individual fetal-placental weight ratio (BW:PW ratio) was calculated. The foetuses were macroscopically examined for external malformations on GDs 17 and 21. According to the criteria for intrauterine growth restriction (IUGR) fetal evaluation, foetuses were defined as having IUGR if their weight was less than −2 standard deviations (SD) below the mean of individual fetal weight in the control group on each GD[23] (<0.040 g on GD 13, <0.201 g on GD 15, <0.668 g on GD 17, and <4.485 g on each GD 21 in this study). Foetuses were defined as large for gestational age (LGA) if their weight was more than +2 SD over the mean of individual fetal weight in the control group for each GD (>0.094 g on GD13, >0.288 g on GD 15, >0.889 g on GD 17, and >6.018 g on GD 21). The IUGR (i.e., the actual number of IUGR foetuses as a percentage of the total number of foetuses) and LGA (i.e., the actual number of LGA foetuses as a percentage of the total number of foetuses) rates were then calculated. Finally, all fetal and placental samples were fixed in 10% neutral-buffered formalin.

Histopathological examination

Four placentas, randomly selected for each dam, were embedded in one paraffin block, and 4 μm thick sections were routinely stained with haematoxylin and eosin for histopathological examination. The thicknesses of the labyrinth zone, basal zone, decidua basalis, and metrial gland close to the central portion of the placenta were measured once per placenta using an image analyser (WinROOF, Mitani Co., Tokyo, Japan). Placentas were subjected to immunohistochemical staining for phospho-histone H3 (Ser10; Cell Signalling Technology, Boston, MA, USA) to evaluate cell proliferation, in situ TdT-mediated dUTP nick end labeling (TUNEL; *In Situ* Cell Death Detection Kit, POD; Roche Applied Science, Penzberg, Germany) to evaluate apoptosis, and glucose transporter 1 (GLUT1, Abcam, Cambridge, UK)[24]. With an aid of the image analyzer, the number 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 per placenta using light microscopy with a 40 × objective.

Statistical analysis

The means and SDs of individual litter values were calculated (Pharmaco Basic, Scientist Press Co., Ltd., Tokyo, Japan). Student's t-test for homoscedastic data or Aspin–Welch's t-test for non-homoscedastic data was performed after the F test. The Fisher's exact test was used to determine the incidence of IUGR, LGA, and external malformations. The significance level was set at $p<0.05$ and <0.01.

Table 1. Effects of Letrozole on Foetuses and Placentas

Autopsy	Group	No.of dams	Mean No. of implantations[a)]	Mean No. of live foetuses[a)]	Fetal mortality (%)[a)]	Foetus weight (g)[a)]	Placenta weight (g)[a)]	Foetus/ Placenta (g/g)[a)]	IUGR rates (%)	LGA rates (%)
GD13	Control	4	13.3 ± 1.0	11.3 ± 1.0	14.7 ± 9.7	0.07 ± 0.01	0.11 ± 0.00	0.61 ± 0.14	6.7	0.0
	Letrozole	4	13.3 ± 0.5	12.3 ± 1.0	7.4 ± 5.8	0.06 ± 0.01	0.13 ± 0.01**	0.51 ± 0.10	2.2	4.3
GD15	Control	4	13.3 ± 2.8	12.5 ± 2.4	5.2 ± 6.3	0.25 ± 0.02	0.21 ± 0.03	1.25 ± 0.16	4.0	0.0
	Letrozole	4	12.5 ± 1.3	9.8 ± 5.6	37.9 ± 33.6	0.29 ± 0.03*	0.32 ± 0.04**	0.94 ± 0.07*	0.0	34.4##
GD17	Control	4	13.5 ± 0.6	13.3 ± 0.5	1.8 ± 3.6	0.78 ± 0.03	0.30 ± 0.03	2.58 ± 0.20	5.9	2.0
	Letrozole	4	11.3 ± 1.5*	6.0 ± 1.6**	45.8 ± 16.0**	0.89 ± 0.08*	0.81 ± 0.11**	1.22 ± 0.28**	0.0	48.1##
GD21	Control	4	13.5 ± 1.0	13.0 ± 0.0	3.3 ± 6.7	5.25 ± 0.18	0.45 ± 0.02	12.12 ± 0.67	3.8	1.9
	Letrozole	4	13.0 ± 1.6	9.8 ± 1.5**	24.7 ± 10.4*	4.58 ± 0.41*	1.34 ± 0.29**	3.86 ± 0.88**	70.4##	4.5

Mean ± SD. a) Mean of individual litter values. *, ** Significantly different from control at $p<0.05$, $p<0.01$, respectively (Student's t-test/ Aspin-Welch's t-test). ## Significantly different from control at $p<0.01$ (Fisher exact test). IUGR: intrauterine growth restriction; LGA: large for gestational age; SD: standard deviation.

Results

Effects on dams

The body weight gain (%) of dams (based on the body weight on GD 6 as 100%) decreased or trended downwards from GD 10 onwards in the letrozole group compared to that in the control group (Fig. 1). In both groups, no maternal mortality or clinical signs were observed in any dams during the experimental period.

Effects on foetuses and placentas

Table 1 shows the effects of letrozole on the foetuses and placentas. In the letrozole group, the number of live foetuses decreased or trended downwards, and fetal mortality increased or trended upwards from GD 15 onwards. The number of implantations decreased on GD 17, which was not considered to be due to letrozole treatment because of a transient change. The fetal weights increased on GDs 15 and 17 but decreased on GD 21, and the placental weights increased from GD 13 onwards. The BW:PW ratio decreased from GD 15 onwards. The LGA rates on GDs 15 and 17, and the IUGR rate on GD 21 increased. Macroscopically, the placenta showed marked enlargement with a well-defined white peripheral rim from GD 17 onwards in the letrozole group (Fig. 2). No macroscopic external fetal abnormalities on GD 17 or 21 in either group.

Histopathological observations

Labyrinth zone

In the letrozole group, the thickness of the labyrinth zone increased from GD 15 onwards (Figs. 3, 4). On GD 13, multiple small cysts were detected in the syncytiotrophoblast masses in the trophoblastic septa (Fig. 5a). These cysts contained serous fluid but not maternal blood, and were

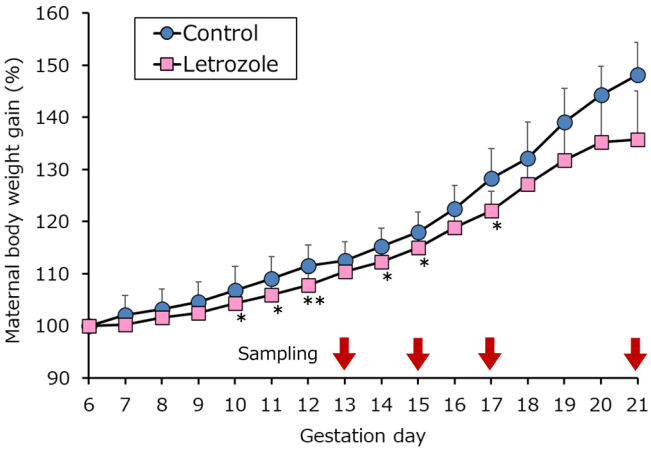

Fig. 1. Maternal body weight changes. *, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively (Student's t-test). Error bar: standard deviations. Blue: control group; Pink: letrozole group.

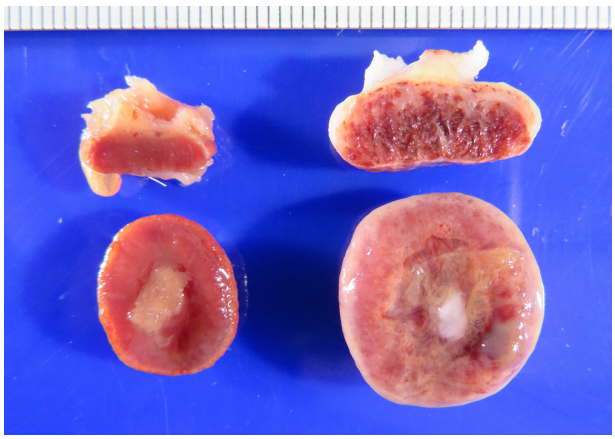

Fig. 2. Gross appearance of the placenta on gestation day 21. Marked enlarged placenta with a well-defined white peripheral rim in the letrozole group. Left: control group; Right: letrozole group.

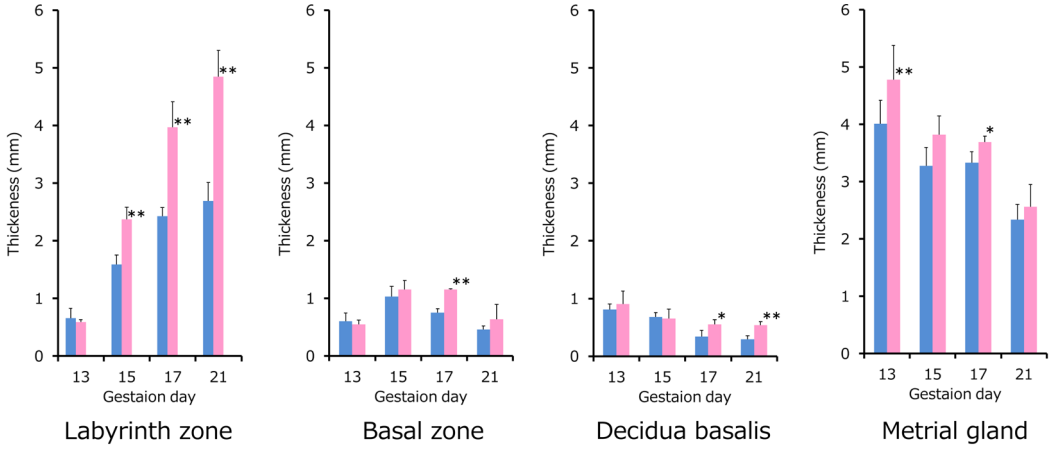

Fig. 3. Thickness of the labyrinth zone, basal zone, decidua basalis, and metrial gland. Blue: control; Pink: letrozole group. Each value represents mean ± standard deviation. *, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively (Student's t-test).

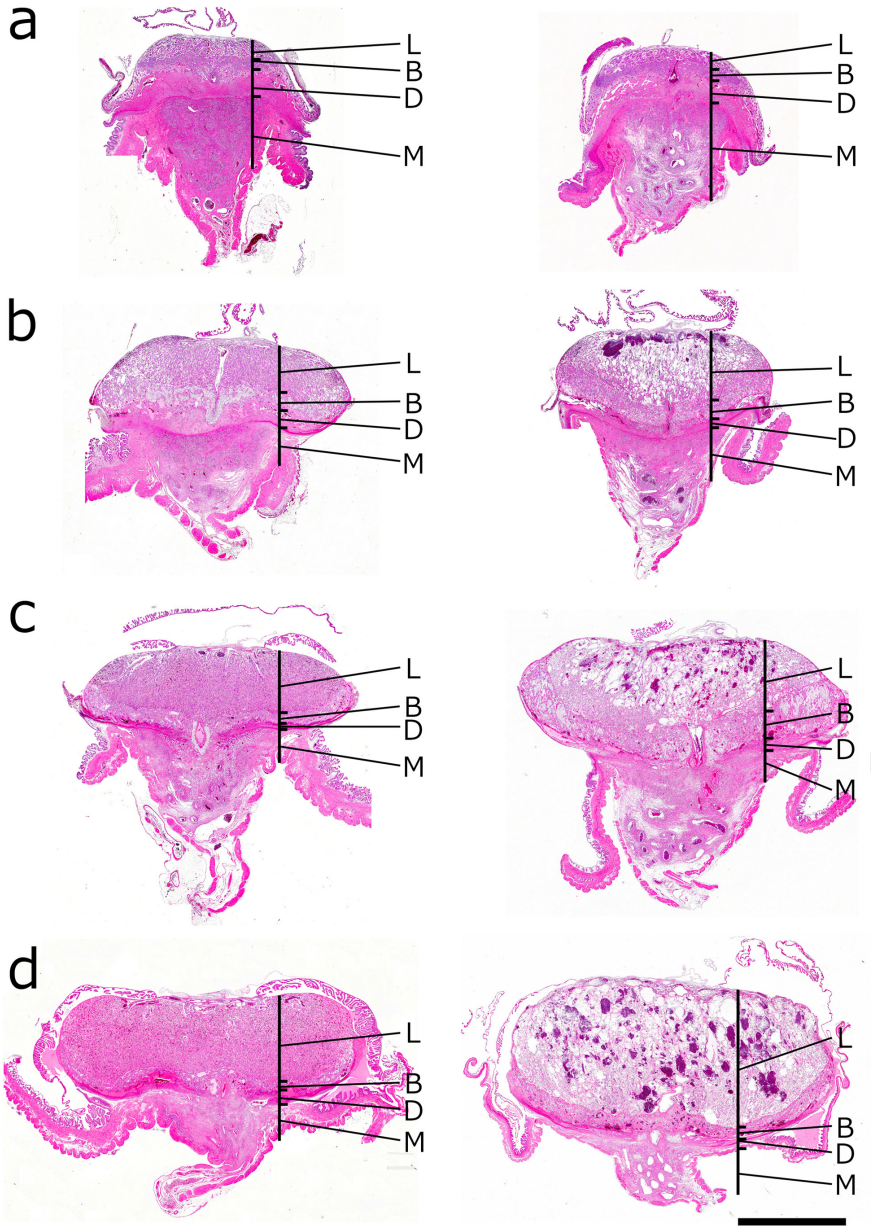

Fig. 4. Low magnification images of placenta. In the labyrinth zone, dilated maternal sinusoids from gestation day (GD) 15 onwards resulting in a thickened labyrinth zone. Expansion from the superficial to deeper parts of the labyrinth zone as pregnancy progresses. In the metrial gland, a dilated lumen of spiral artery and interstitial edema from GD 13 onwards. a, GD13; b, GD 15; c, GD 17; d, GD 21. Left, control group; Right, letrozole group. L, labyrinth zone; B, basal zone; D, decidua basalis; M, metrial gland. Haematoxylin and eosin staining. Bar=4,000 μm.

lined with cuboidal- or flat-shaped syncytiotrophoblasts, some of which showed no GLUT1 expression (Fig. 5a, 5b). On GD 15, the multiple cysts which were dilated with containing maternal blood, were lined with GLUT1-positive syncytiotrophoblasts (Fig. 5c, 5d), and the trophoblastic septa were thinning; therefore, it was impossible to distinguish between the multiple cysts and maternal sinusoids. From GD 17 onwards, these cysts and maternal sinusoids were markedly dilated with congestive hyperemia and interspersed with thrombi. Moreover, apoptosis and calcification were scattered throughout the thinning of trophoblastic septa (Fig. 5e). These dilated cystic lesions expanded from the superficial to the deeper parts of the labyrinth zone as the pregnancy progressed (Fig. 4). A decrease in the number of phospho-histone H3-positive cells was observed on GDs 15 and 17 and an increase in the number of TUNEL-positive cells on GDs 17 and 21 in the letrozole group (Figs. 6, 7).

Basal zone

In the letrozole group, the thickness of the basal zone increased on GD 17 (Fig. 3). On GD 15, only a few glycogen cell islands developed, and there was little interstitial trophoblast invasion from the basal zone to the metrial gland,

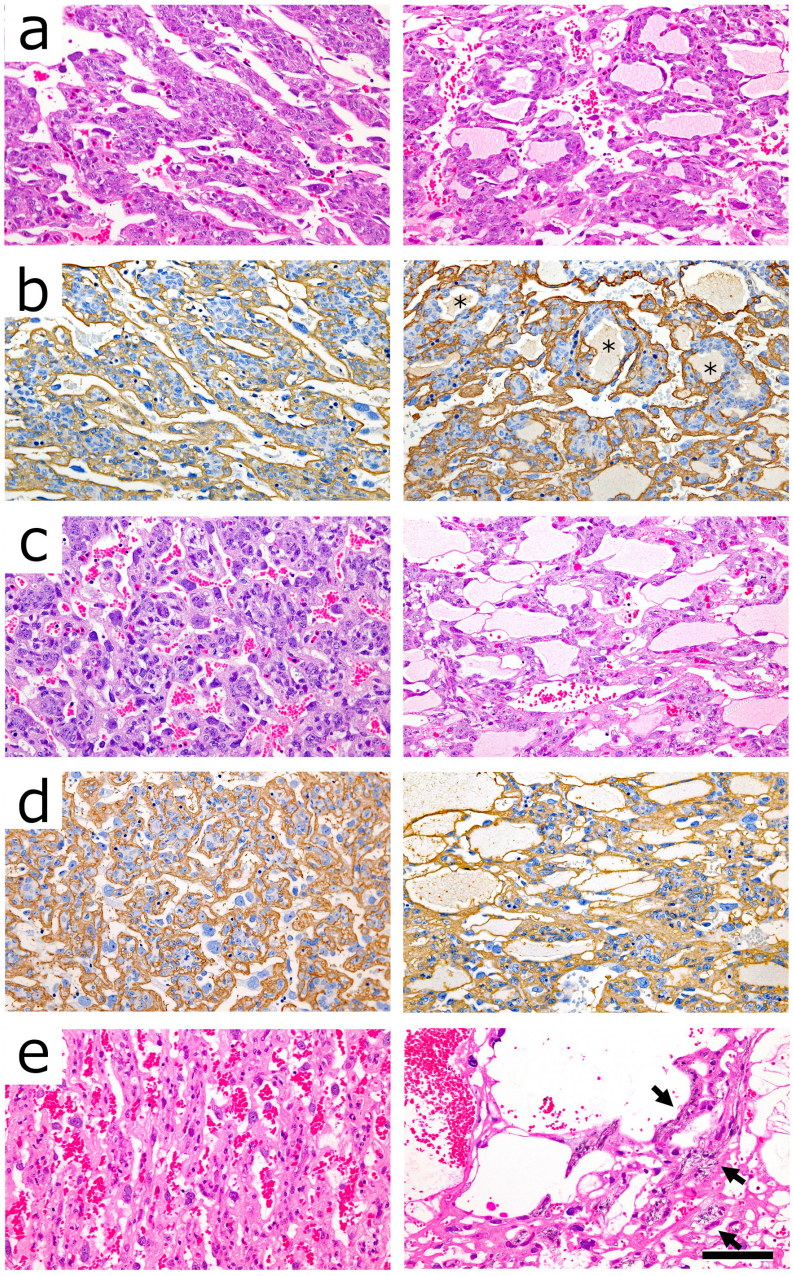

Fig. 5. Histopathological changes of labyrinth zone. a, b. Gestation day (GD) 13. Formation of multiple small cysts containing serous fluid, but not maternal blood in the letrozole group. Cysts lined with cuboidal- or flat-shaped syncytiotrophoblasts without glucose transporter 1 (GULT1) expression (*). Haematoxylin and eosin (HE) staining; b, GULT1 immunohistochemical staining. c, d. GD 15. Dilated maternal sinusoids lined with syncytiotrophoblasts with GLUT1 expression. c, HE staining; d, GULT1 immunohistochemical staining. e. GD 17. Thinning trophoblastic septa with calcification (arrow) and marked cystic dilatation of maternal sinusoids with containing maternal blood. HE staining. Left: control group; Right: letrozole group. Bar=100 μm.

compared to the control group (Fig. 8a). On GD 17, the basal zone was thickened due to the many remaining glycogen cell islands (Figs. 4c, 8b), and these changes corresponded to an enlarged white peripheral margin on the placenta in macropathology (Fig. 2). Hyaline droplet degeneration of spongiotrophoblasts and cystic degeneration of glycogen cells containing erythrocytes were scattered. On GD 21, cystic degeneration of glycogen cells and necrosis were observed at the decidua basalis border (Figs. 4d, 8c). No differences were observed in the numbers of phospho-histone H3-positive and TUNEL-positive cells between the control and letrozole groups (Figs. 6, 7). In both groups, GLUT1 expression was detected in spongiotrophoblasts in the marginal part of the basal zone on GD 13 and subsequently spread to spongiotrophoblasts in the central part of the basal zone as the placenta developed.

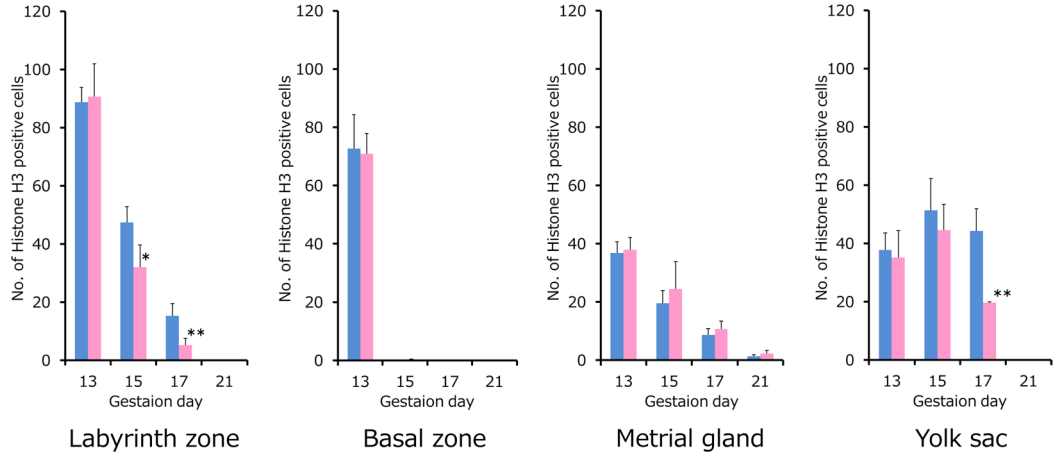

Fig. 6. Number of phospho-histone H3-positive cells. Each value represents mean ± standard deviations. *, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively (Student's t-test). Blue: control; Pink: letrozole group.

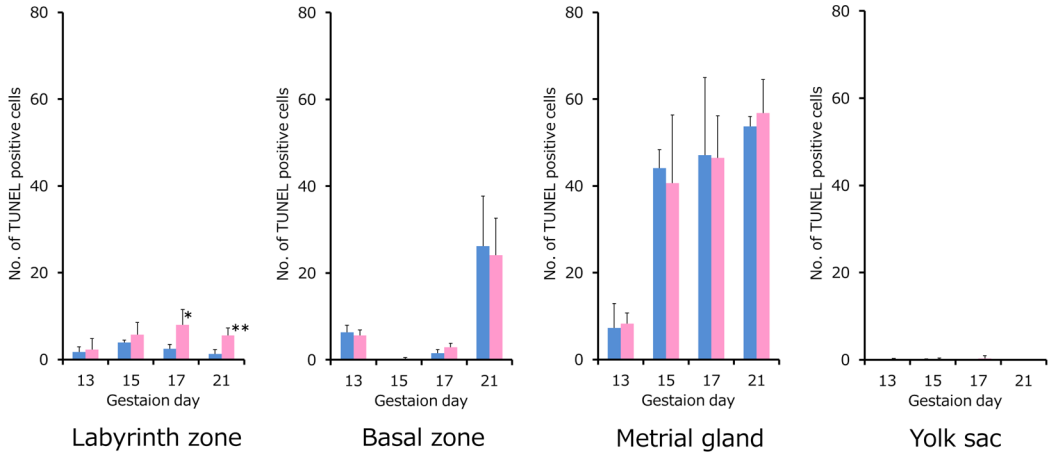

Fig. 7. Number of TdT-mediated dUTP nick end labeling (TUNEL)-positive cells. Each value represents mean ± standard deviations. *, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively (Student's t-test). Blue: control; Pink: letrozole group.

Decidua basalis

In the letrozole group, the thickness of the decidua basalis increased on GDs 17 and 21 (Fig. 3). This thickening was due to edema and haemorrhage with slight neutrophil infiltration, which corresponded to basal zone damage. In both groups, GLUT1 expression was slightly scattered in decidual cells just beneath the basal zone on GD 13.

Metrial gland

In the letrozole group, the thickness of the metrial glands increased or trended upwards throughout the study period (Fig. 3). Dilated spiral artery lumens and interstitial edema with periodic acid-Schiff stain-positive serous exudations were observed throughout the study period (Figs. 4, 9). No differences were observed in the numbers of phospho-histone H3-positive and TUNEL-positive cells between the control and letrozole groups (Figs. 6, 7). In both groups, GLUT1 expression was detected in some endometrial stromal cells from GD 13 onwards and in interstitial trophoblasts on GDs 15 and 17.

Yolk sac

No histopathological changes were observed in the yolk sacs of either group. The number of phospho-histone H3-positive cells decreased on GD 17 in the letrozole group (Fig. 6). Our analysis revealed no difference in TUNEL-positive cells between the control and letrozole groups (Fig. 7). In both groups, GLUT1 expression was detected in the epithelium on GD 13 and then decreased and disappeared by GD 17.

Discussion

In this study, letrozole histologically induced dilated maternal sinusoids with congestive hyperemia in the labyrinth zone, resulting in an enlarged placenta. These lesions have been reported in rats exposed to other aromatase inhibitors such as ketoconazole[17], epoxiconazole[18], tebucon-

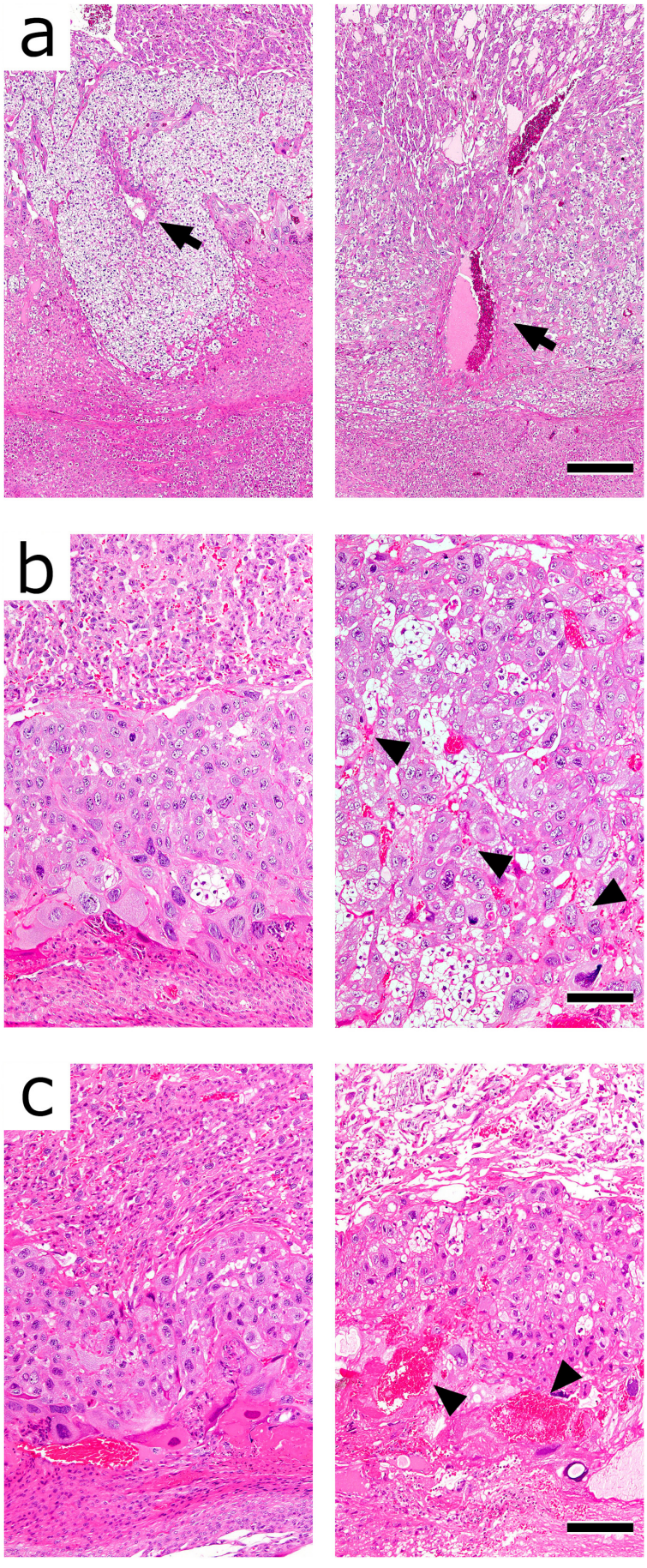

Fig. 8. Histopathological changes of basal zone. a. Gestation day (GD) 15. Decreased interstitial trophoblast invasion around maternal arterial channel (arrow) in the letrozole group. Haematoxylin and eosin (HE) staining. Bar=600 µm. b. GD 17. Thickened basal zone with hyaline droplet degeneration of spongiotrophoblasts (arrowhead) in the letrozole group. HE staining. Bar=150 µm. c. GD 21. Cystic degeneration of glycogen cells containing blood (arrowhead) and necrosis at the border with the decidua in the letrozole group. HE staining. Bar=150 µm. Left: control group; Right: letrozole group.

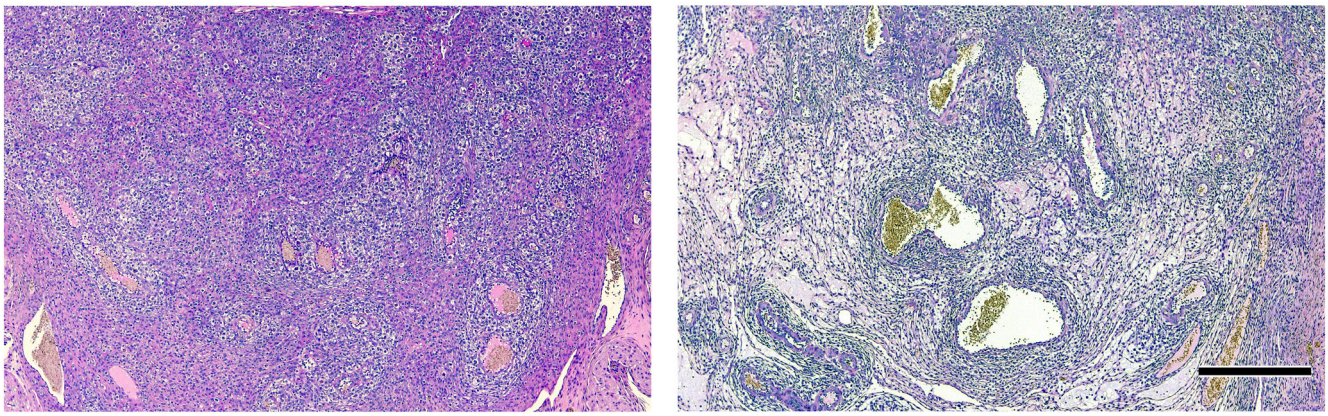

Fig. 9. Histopathological changes of metrial zone. Dilated spiral artery lumen and interstitial edema with periodic acid-Schiff (PAS) stain-positive serous exudation on gestation day 13 in the letrozole group. PAS staining. Bar=500 μm. Left: control group; Right: letrozole group.

azole[19], and triadimefon[20]. Therefore, an enlarged placenta in letrozole-exposed rats is considered a common lesion induced by aromatase inhibition. In contrast, the letrozole-induced increase in placental weight is normalized by the concomitant exposure to estrogen[22]. However, estrogen induces dilated maternal sinusoids[25], resulting in an enlarged placenta[16]. Thus, aromatase inhibitor-induced placental enlargement may be more closely related to estrogen imbalance than to estrogen deficiency.

Histologically, the trophoblastic septa in the labyrinth zone are composed of one cytotrophoblast layer, two syncytiotrophoblast layers (I and II), and fetal vessels in rodents[26]. These two syncytiotrophoblast layers are closely apposed and form a placental barrier. GLUT1, a facilitated-diffusion glucose transporter isoform, is localized on the membrane of syncytiotrophoblast layer I facing the maternal blood side, and on the membrane of syncytiotrophoblast layer II facing the fetal vessels[27]. While maintaining the basic morphological structure of the trophoblastic septa, the trophoblastic septa are thinning with decreased cellular density, and the maternal sinusoid develops in the labyrinth zone, as the pregnancy progresses[2, 28]. In this study, since multiple cysts contained no maternal blood and some of the syncytiotrophoblasts surrounding them did not express GLUT1 on GD 13, these syncytiotrophoblasts were considered undifferentiated. Therefore, although the cause is unknown, it is speculated that letrozole precociously formed multiple small cysts inside immature syncytiotrophoblast masses in the trophoblastic septa, which subsequently develop into dilated maternal sinusoids in the labyrinth zone.

Placental weight has a strong relationship with placental functions that qualitatively and quantitatively affect O_2/CO_2 exchange, providing nutrients for the foetus, and removing waste products[29]. A strong correlation has been reported between fetal and placental weight in humans[30] and rats[2]. In addition, the BW:PW ratio is used as a proxy for placental efficiency, which is how placental development or function has adapted to meet fetal nutritional requirements. A decreased BW:PW ratio indicates functional inefficiency of the placenta[31]. Therefore, in this study, transient increases in fetal weight on GDs 15 and 17 in the letrozole group are closely related to a placental enlargement, and it is speculated that congestive hyperemia in the labyrinth zone contributes to the increase in fetal weight. However, these fetal weights did not increase commensurately with the placental weight throughout the examination period from the viewpoint of the BW:PW ratio. In addition, the trophoblastic septa were conspicuously thinned and degenerated from GD 15 onwards. These results suggest that the function of letrozole-exposed placentas is progressively reduced due to damage as pregnancy progresses and is unable to accommodate the rapid development of LGA foetuses, leading to IUGR at the end of pregnancy. Furthermore, because more than half of the foetuses survived and the surviving foetuses on GDs 15 and 17 were LGA despite increased fetal mortality from GD 15 onwards, letrozole-induced fetal death is speculated to be caused by accidental placental damage, such as trophoblastic septa disruption, rather than a direct effect of letrozole.

Embryologically, the basal zone is transiently thickened by the formation of a glycogen cell island around GD 15. Then, glycogen cells regress and disappear before parturition, resulting in thinning of the basal zone[2]. In this case, it is speculated that letrozole induces the delayed formation of glycogen cells and the failure of them to regress at the end of pregnancy, leading to thickening of the basal zone on GD 17 and cystic degeneration and necrosis on GD 21. In addition, the fact that injured lesions in the decidua basalis were observed in response to lesions in the basal zone suggests that both changes are closely related. The same lesions in the basal zone have been observed in rats exposed to the aromatase inhibitors described above[18–20] and in rats exposed to 6-mercaptopurine[32], chlorpromazine[33], testosterone[34], and cyclophosphamide[35], and therefore, these lesions are considered to be non-specific changes that occur independently of their own mode of action. Ketoconazole induces a

markedly thickened basal zone, but this lesion is caused by spongiotrophoblast hypertrophy but not cystic degeneration of glycogen cell islands[17]. Ketoconazole inhibits the steroid aromatase and CYP3A[36], which are located primarily in the basal zone. Therefore, the thickened basal zone induced by ketoconazole is speculated to be due to compensatory changes in CYP3A inhibition, and it is revealed that letrozole and ketoconazole has different effects on the basal zone.

In this study, dilated the spiral artery lumens were observed with interstitial edema in the metrial gland during the experimental period. Therefore, it is suggested that maternal blood circulation in the metrial gland is stagnant. Letrozole is known to inhibit the conversion of testosterone to estrogen, thus increasing the serum testosterone concentrations in rats[37]. However, the lesions in the labyrinth zone and metrial gland in letrozole-exposed placentas are not observed with testosterone[34]. Anatomically, in the maternal blood circulation of the rat placenta, the spiral arteries traverse the metrial gland and decidua basalis and converge to form a small number of centrally located arterial canals, which turn around at the surface of the placenta and lead to maternal sinusoids in the labyrinth zone[38]. Accordingly, it is speculated that the congestive lesions in the metrial gland in the current study are closely related to the dilated maternal sinusoids with congestive hyperemia in the labyrinth zone but not to the testosterone imbalance attributable to aromatase inhibition.

In conclusion, the most distinctive change due to letrozole exposure from GD 6 to GD 20 was the formation of multiple small cysts lined with undifferentiated syncytiotrophoblasts in the trophoblastic septa. And then, these cysts developed into dilated maternal sinusoids with congestive hyperemia, resulting in an enlarged placenta. Although letrozole induces an enlarged placenta and transient increases in fetal weight, these placentas are thought to decline in function as the pregnancy progresses, leading to IUGR at the end of pregnancy.

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

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