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Ketoconazole-induced estrogen deficiency causes transient decrease in placental blood flow associated with hypoxia and later placental weight gain in rats

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ABSTRACT

This study investigated the relationship among estrogen, placental blood flow and placental weight gain in rats treated with ketoconazole. Oral administration of ketoconazole (25 mg/kg/day) on Days 12 to 14 of pregnancy induced reduction of plasma estradiol-17β (E₂) concentration and transient decrease in placental blood flow and an increase in the intensity of a hypoxia index on Day 14 of pregnancy. On Day 20 of pregnancy, placental weights of ketoconazole-treated rats increased when compared to controls. Histologically, maternal sinusoidal area of the placenta decreased on Day 14 of pregnancy and the total area of maternal and fetal sinusoids increased on Day 20. All the changes disappeared by concomitant subcutaneous infusion of E₂. These results indicate that ketoconazole-induced E₂ deficiency causes transient decrease in placental blood flow associated with hypoxia and later placental weight gain in rats.
1. Introduction

The placenta is a pivotal organ that synthesizes several growth and angiogenic factors for the maintenance of pregnancy [1-3] as well as playing critical roles in immunological and transport functions between dams and fetuses. Although changes in placental morphology and function induced by chemicals or drugs cause pregnancy loss or fetal damage [4], their etiology is poorly understood.

Estrogen is known as one of the factors involved in the development of the placenta. In pregnant rats, injection of estradiol-17β (E2) retarded placental growth [5], and the reduction of blood E2 concentrations following ovariectomy with exogenous hormonal replacement induced excessive placental hypertrophy [6-7]. Furthermore, treatment with the antibody to E2 caused increases in placental weights [8]. These findings suggest that a deficiency of E2 could be involved in the hypertrophic responses of the rat placenta during pregnancy. The placenta produces estrogen during pregnancy in some mammalian species [9-11], while slight or negligible production of estrogen was detected in rat placentas [2, 12]. During the second half of pregnancy estrogen is produced mainly in the ovary from androgen, which is generated in the placenta in rats [13, 14]. It has been assumed that placental hypertrophy by estrogen deficiency may be a compensatory response related to an effective ‘luteo-placental shift’ by steroid production in the support of the maintenance of pregnancy [7, 15].

Concerning the other factors regulating placental growth, hemorrhage [16], uterine vessel ligation [17], or treatment with indomethacin [18] or nifedipine [19], which reduces placental blood flow, has been reported to increase placental weights. A reduction of oxygen transport as a result of maternal anemia, iron deficiency or high
altitude also causes increased placental weights [20-26]. From these findings, it has been assumed that oxygen supply or uteroplacental blood flow plays an important role in the development of the placenta. Although estrogen affects uterine blood flow [27-29], the relationship between placental growth and changes in the uteroplacental blood flow by estrogen deficiency has not been evaluated.

Daily administration of ketoconazole (KTZ) from Day 6 through late pregnancy induces intrauterine growth retardation, delayed parturition, and abnormal postnatal development in mice and rats [30], and administration of KTZ for a few days during pregnancy induces placental hypertrophy in rats [31, 32]. KTZ is a synthetic antifungal agent that interferes with the fungal synthesis of ergosterol, the main constituent of cell membranes [33, 34]. KTZ primarily inhibits cytochrome P450, an enzyme involved in the steroid biosynthesis pathway that metabolizes lanosterol to ergosterol in fungi [35]. Certain cytochrome P450 enzymes such as C17, 20-lyase, or aromatase are responsible for androgen or estrogen biosynthesis in mammals [36-38]. KTZ, both in vivo and in vitro, reduces ovarian E₂ levels dose dependently in rats [39-42]. In order to examine the etiology of KTZ-induced placental weight increase, this study investigated the relationship among estrogen, placental blood flow, and placental weight gain in KTZ-treated rats.

2. Materials and methods

2.1. Animals and housing

Female Crl:CD (SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan)
were obtained at 11 to 12 weeks of age. The rats were acclimated in the laboratory at 23±3°C and with a 12-h light and 12-h dark cycle (light: 0700-1900 hour) for at least 1 week before use. Virgin females (13 to 18 weeks old) were mated overnight with males (14 to 25 weeks old) of the same strain at proestrus on a one to one basis. The day when a copulation plug was found was designated Day 0 of pregnancy. The animals were individually housed in metal cages with wire mesh bottoms and provided with tap water and a laboratory animal diet (CR-LPF, γ-ray irradiated, Oriental Yeast, Co. Ltd., Tokyo, Japan) ad libitum. Animals were euthanized by exsanguination under ether anesthesia except when otherwise noted. All procedures were performed in accordance with the institutional guidelines for animal care at Takeda Pharmaceutical Company Limited in conformity to the National Institutes of Health guide for the care and use of Laboratory Animals.

2.2. Chemicals and preparation for treatments

Methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) was dissolved in injection-grade distilled water to make a 0.5% (w/v) solution. KTZ (Wako Pure Chemical Industries, Tokyo, Japan) was weighed and mixed with the solution using a defoaming conditioning mixer (MX-201, THINKY Corporation, Tokyo, Japan) to make a 0.5% (w/v) suspension of KTZ. Batches of the dosing suspensions sufficient for several days of dosing (maximum 5 days) were prepared and were stored in a refrigerator (set at 4°C) until use. Prior to dose administration, the dosing suspension was allowed to warm to room temperature. The dose volume for each animal was 5
E$_2$ was purchased from CALBIOCHEM (La Jolla, CA) and mini-osmotic pumps (model 1003D; 1.0 μL/h delivery rate, 3 days, Alzet®, DURECT Corporation, Cupertino, CA) were used to infuse E$_2$. The pumps were filled with approximately 90 μL of E$_2$ solution at a concentration of 0, 0.42 or 42 μg/mL in a mixture of 0.5% ethanol and 99.5% propylene glycol.

Pimonidazole hydrochloride was purchased from HPI (Hypoxyprobe Plus kit, Burlington, MA), dissolved in physiological saline to give a 60 mg/mL solution and filter sterilized prior to intraperitoneal injection.

2.3. Effect of KTZ treatment during different periods of pregnancy on placental weight

Pregnant rats were allocated to 4 groups, each containing 5 to 7 animals. KTZ was administered orally by gavage at a dose of 25 mg/kg/day on Days 9 to 11, 12 to 14, or 15 to 17 of pregnancy (the dams were dosed daily between 09:00 and 11:00). The dose of KTZ was based on the report that a single oral dose of 20 mg/kg KTZ depressed ovarian concentrations of E$_2$ [41]. Control animals received vehicle only. On Day 20 of pregnancy, the dams were euthanized and the placentas and live fetuses were weighed using an electric balance.

2.4. Effect of KTZ treatment on plasma E$_2$ concentration
Maternal plasma E2 concentration on Day 14 of pregnancy was measured in the group treated with KTZ (25 mg/kg/day) on Days 12 to 14 of pregnancy (n=6) and in the controls (n=5). Approximately 0.8 mL blood samples were collected from the jugular vein using heparinized syringe without anesthesia on Day 14 of pregnancy at 4 h after the KTZ treatment. The blood samples were centrifuged at 18,500 × g for 1 minute to obtain plasma, and the plasma samples were kept frozen (below -20°C) until the hormone assay. The sampling time was based on reports that showed peripheral E2 levels decreased 3 h after dosing of KTZ [41].

2.5. Effects of treatment with KTZ alone or with E2 on Days 12 to 14 of pregnancy on placentas

E2 was administered into the dorsal subcutis using a mini-osmotic pump at the rate of 0, 0.1, or 1 μg/rat/day in combination with the oral administration of 25 mg/kg/day of KTZ for 3 days from Days 12 of pregnancy (abbreviated as KTZ+0E2, KTZ+0.1E2, or KTZ+1E2 group, respectively). Controls received vehicle for KTZ and solvent for E2 in the same manner. Under ether anesthesia, the pumps were implanted and removed 3 days after the implantation. Although some anesthetics modify secretion of luteinizing hormone which stimulates steroidogenesis [43, 44], ether anesthesia does not affect serum E2 concentration in rats [45]. Therefore, ether was used with carefully monitoring animals during and after anesthesia.

On Day 20 of pregnancy, the rats in the control, KTZ+0E2, KTZ+0.1E2, and KTZ+1E2 groups (n=12 in each group) were euthanized and the placentas were weighed.
Among these placentas, 2 from 3 rats in each group were fixed in 10% neutral buffered formalin for histological examination.

On Days 14 of pregnancy, the rats in the control, KTZ+0E2 and KTZ+1E2 groups (n=3 in each group) were euthanized 4 h after the treatment with KTZ or its vehicle, and 2 placentas from each rat were fixed in 10% neutral buffered formalin for histological examination.

The placental blood flow on Day 14 of pregnancy at 0, 4, 8, and 24 h after the treatment with KTZ or its vehicle was evaluated by the microspheres technique in the control, KTZ+0E2, and KTZ+1E2 groups. Four to 5 rats per group were used for each sampling point, and 56 animals were euthanized for this evaluation.

For immunohistochemical staining for pimonidazole on Day 14 of pregnancy, the rats in the control, KTZ+0E2, and KTZ+1E2 groups were used (n=5 in each group).

2.6. Hormone assay (E2 measurement)

Plasma E2 levels were measured by a double-antibody radioimmunoassay (RIA) with a commercially available kit (Diagnostic Products Corporation, LA). According to the manufacturer, cross-reactivities of the anti-E2 antibody with E2, estrone, estriol, testosterone, androstenedione, and progesterone were 100%, 10.0%, 0.32%, 0.001%, <0.001% and <0.001%, respectively. All of the samples were quantified within a single assay. The intra-assay coefficient of variation and the lower limit of sensitivity were 5.0% and 5 pg/mL, respectively.
2.7. Histology

Formalin-fixed, paraffin-embedded placentas were sectioned at 4-µm thickness, stained with hematoxylin and eosin (HE), and examined under a light microscope. Six images obtained from 6 placentas from 3 dams, which showed representative histological characteristics in each placenta, were examined for each group. Quantitative analysis of erythrocyte counts and size of labyrinthine sinusoids on the photomicrographic images were performed on a Microsoft computer using digital image analysis software (MicroAnalyzer® Nihon Poladigital, KK, Tokyo, Japan). On Day 14 of pregnancy the number of maternal and fetal erythrocytes, which are located in the maternal and fetal sinusoids, respectively, in an enclosed area of 400 square micrometers were counted. The area of the labyrinthine sinusoids on Days 14 (maternal and fetal sinusoids, respectively) and 20 of pregnancy (overall sinusoids) was measured by counting the number of pixels on the image within the enclosed area of 400 square micrometers.

2.8. Determination of the placental blood flow

The blood flow was evaluated according to the method of Hakkinen et al. [46]. Briefly, at 0, 4, 8 and 24 h after dosing KTZ or its vehicle on Day 14 of pregnancy, the rats in the control (n=19), KTZ+0E2 (n=18) and KTZ+1E2 (n=19) groups were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) for the
implantation of two catheters that were filled with saline into the femoral artery and left ventricle. A PE-10 catheter was positioned into the abdominal aorta through the femoral artery for the direct measurement of the arterial pressure and collection of ‘reference blood’. The second catheter was inserted into the left ventricle through the right carotid artery for the infusion of colored microspheres. A 1 mL solution of 300,000 yellow microspheres was infused at the rate of 1 mL/min, and at the same time 1 mL of ‘reference blood’ was collected at the rate of 1 mL/min. The animals were euthanized and their placentas were removed and weighed. The sample tissue and the reference blood were properly treated to isolate the microspheres. The absorption spectrum peak for the yellow microspheres was obtained at 440 nm.

For each infusion, the tissue flow rates were calculated according to the following formula:

\[ Q_s = \frac{(A_s \cdot Q_r)}{A_r} \]

where \( Q_s \) and \( Q_r \) represent the flow in the sample tissue and in the reference blood, respectively, and \( A_s \) and \( A_r \) represent the peak absorption of the tissue sample and of the reference blood, respectively. The blood flow rates were divided by the tissue weights to yield mL/min/g. The catheter position was confirmed by cardiotomy during necropsy.

2.9. Immunohistochemistry

Immunohistochemical staining for pimonidazole was performed to examine the hypoxic state of placentas. Pimonidazole is water soluble and rapidly distributes to all tissues after peritoneal injection. It forms adducts with proteins in cells having an
oxygen concentration less than 14 micromolar [47]. The rats received an intraperitoneal injection of pimonidazole hydrochloride solution (60 mg/kg) 5 h after the treatment with KTZ or its vehicle on Day 14 of pregnancy. Ninety minutes after the injection, the rats were anesthetized and the uteri including fetuses and placentas were excised and fixed in 10% neutral buffered formalin solution. Two placentas were randomly taken from each dam and embedded in paraffin. Sagittal sections were made for each placenta. The sections were deparaffinized and rehydrated, and stained for the presence of the pimonidazole adduct (hypoxia marker) based on the manufacturer’s instructions (HPI, Burlington, MA). Briefly, the rehydrated sections were treated with trypsin (Difco, NJ) in TRIS-buffered saline (TBS) for antigen retrieval and then incubated with mouse monoclonal antibodies (Cayman Chemical Company, MI) at 1:2500 dilution. Antibody binding was detected after incubation with a secondary biotinylated horse anti-mouse antibody (Lab Vision, Fremount, CA) and reagents in the Vectastatin immunohistochemical staining kit (Vector Laboratories, Burlingame, CA). Immunostained sections were lightly counterstained with hematoxylin.

2.10. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Evaluation of the number of live fetuses and erythrocytes was performed by Bartlett’s test for homogeneity of variance followed by an analysis of variance (ANOVA). Weights of the placentas and fetuses were analyzed by two-way analysis of variance, with the variance being partitioned between (groups)- and within (gender of fetuses)-animal bases,
followed by multiple comparison using the Tukey-Kramer method. Comparison of plasma E₂ levels between the control and KTZ-treated groups and that of placental blood flow between the control and KTZ+0E₂ or KTZ+1E₂ groups at each sampling time were performed by the F test for homogeneity of variance followed by Student’s t test (when the variances were homogeneous) or the Welch’s t test (when the variances were heterogeneous). The Bonferroni correction was used to determine if the t tests were significant after multiple testing for the values of placental blood flow. The percent of sinusoid area and ratio of fetal erythrocytes were subjected to arcsine transformation before Bartlett’s test for homogeneity of variance followed by ANOVA and the Tukey-Kramer method. The significance level was set at \(p<0.05\). The analyses were done using Statcel (the add-in forms on Excel, 3rd ed.; OMS Ltd., Tokorozawa, Japan).

3. Results

3.1. Effect of KTZ treatment during different periods of pregnancy on placental weights

Table 1 shows the placental and fetal weights on Day 20 of pregnancy when KTZ was given to pregnant rats during various periods. The number of live fetuses was not different among the groups. In the analysis of fetal weight by 2-way ANOVA, there was no effect of either gender or treatment. Regarding analysis of placental weights by 2-way ANOVA, although effects of neither gender nor interaction between treatment and gender were observed, the effect of treatment was significant. Regardless of fetal gender, placental weights were significantly greater in the group treated with KTZ on
Days 12 to 14 of pregnancy than in the other groups, and the values were not different between controls and the group treated with KTZ on Days 9 to 11 or 15 to 17 of pregnancy. These results indicate that the time at which placental growth is most responsive to KTZ treatment is approximately Days 12 to 14 of pregnancy. Therefore, the time of KTZ treatments were settled at these critical periods in the following experiments.

3.2. Effect of KTZ treatment on plasma $E_2$ concentrations

At 4 h after the treatment with KTZ or its vehicle on Day 14 of pregnancy, maternal plasma $E_2$ concentration (mean ± SEM) was significantly lower ($p<0.05$) in the group treated with KTZ on Days 12 to 14 of pregnancy (17.2 ± 6.6 pg/mL, n=6) than in the controls (34.9 ± 7.6 pg/mL, n=5).

3.3. Effect of treatment with $E_2$ on placental weight in the KTZ-treated rat

Placental weights on Day 20 of pregnancy in the groups treated with KTZ and $E_2$ on Days 12 to 14 of pregnancy are shown in Fig. 1. In the 2-way ANOVA, although there were no effects of gender or interaction between gender and treatment, the effect of treatment was significant. Regardless of the fetal gender, placental weights in the KTZ+0$E_2$ group were significantly higher than those in the other groups. There was no significant difference in the value between controls and the KTZ+1$E_2$ group. The
placental weights in the KTZ+0.1E2 and KTZ+1E2 groups were significantly lower than those in the KTZ+0E2 group and decreased in a dose-dependent manner of E2.

3.4. Effect of treatment with KTZ alone or with E2 on placental histology

On day 20 of pregnancy, when compared to controls (Fig. 2A), markedly dilated labyrinthine sinusoids filled with erythrocytes were observed in the KTZ+0E2 group (Fig. 2B). The expanded sinusoids were associated with thinning of the trophoblast cell. The labyrinth structure in the KTZ+0.1E2 and KTZ+1E2 groups (Fig. 2C and 2D) were comparable to that in controls. Because differentiation between fetal and maternal erythrocytes was difficult, the areas of fetal and maternal sinusoids were combined for quantitative measurement. Table 2 shows that the area of the sinusoid per unit area of labyrinth zone in the KTZ+0E2 group was greater than that in the other groups. Although there was no difference in the sinusoid area between the KTZ+0.1E2 and KTZ+1E2 groups, the value in the KTZ+0.1E2 group was greater than that in the controls.

On Day 14 of pregnancy, fetal erythrocytes with nuclei were clearly distinguished from maternal erythrocytes, which have no nuclei. When compared to controls (Fig. 3A), the number of maternal erythrocytes was markedly decreased in the labyrinth zone and fetal erythrocytes were increased in widely expanded sinusoids in the KTZ+0E2 group (Fig. 3B). Histological characteristic in the KTZ+1E2 group (Fig. 3C) was similar to that in the controls. Table 3 shows quantitative analyses of erythrocytes and sinusoid area of labyrinth zone of placentas among groups. The total
number of erythrocytes was not different among the control, KTZ+0E₂ and KTZ+1E₂
groups. The ratio of fetal erythrocytes to maternal erythrocytes in the KTZ+0E₂ group
was significantly higher than those in the other groups. Analysis of the area of
labyrinthine sinusoids per unit area shows that the ratio of maternal sinusoids was
lower and that of fetal sinusoids was higher in the KTZ+0E₂ group when compared to
controls (Table 3). Supplementation of E₂ (KTZ+1E₂ group) increased the ratio of
maternal sinusoids when compared to controls and restored the ratio of fetal sinusoids
to the control level.

3.5. Effect of treatment with KTZ alone or with E₂ on placental blood flow

Fig. 4 shows placental blood flow after treatment with KTZ or its vehicle on Day
14 of pregnancy. The values were not different between the control and KTZ+0E₂ or
KTZ+1E₂ group before the treatment with KTZ or its vehicle (0 h). Although values
in the control and KTZ+1E₂ groups kept a constant level after the treatment, the value
in the KTZ+0E₂ group remarkably decreased 4 h after KTZ treatment and were
significantly lower than that in the control group. At this time there was no difference
in the value between the control and KTZ+1E₂ groups. Thereafter, no differences in
the placental blood flow were seen between the control and KTZ+0E₂ or KTZ+1E₂
groups.

3.6. Effect of treatment with KTZ alone or with E₂ on immunohistochemical staining for
pimonidazole in placentas

In the placentas of the KTZ+0E\textsubscript{2} group (Fig. 5B), stronger intensity of immunostaining for pimonidazole hydrochloride was observed when compared to controls (Fig. 5A). Slight staining for pimonidazole was seen in the placentas of the control and KTZ+1E\textsubscript{2} group (Fig. 5C).

4. Discussion

In this study, the window of sensitivity for KTZ treatment to increase placental weight was found to be Days 12 to 14 of pregnancy, and the effect was valid in the placenta of both male and female fetuses. KTZ decreased plasma E\textsubscript{2} concentrations to a half at 4 h after the treatment when compared to that of controls, and the increase in placental weights by the KTZ treatment was negated by a continuous infusion of E\textsubscript{2} in a dose-dependent manner, suggesting that the decrease in E\textsubscript{2} levels could be a cause of the KTZ-induced placental weight increase. Although the reason is unclear as to why the sensitivity to KTZ for increasing placental weights is limited to such a short period of pregnancy, dramatic changes in placental morphology during gestation may be involved. The labyrinth zone appears and the maternal E\textsubscript{2} concentrations tend to increase around Day 12 of pregnancy [48]. The KTZ treatment during Days 9 to 11 may not affect placental growth because the placental labyrinth, which is a major constituent of placental growth, is absent at this stage. The reduced sensitivity to KTZ treatment after Day 15 of pregnancy may be related to the number of placental estrogen receptors (ER)
because the ER in the rat placenta decreases during late pregnancy [49].

Although accumulating evidence suggests that E2 inhibits placental growth in rats [5-8, 15, 50, 51], the mechanism by which estrogen deficiency induces placental weight gain is not known. It has been reported that E2 increases blood flow in the uterus [27-29, 52-54], and reduction of the oxygen supply by anemia or blood loss induces placental weight increase [16, 20]. This study examined the relationship among estrogen, placental blood flow and placental weight gain in the KTZ-treated rats. The treatment with KTZ on Days 12 to 14 of pregnancy, which decreased blood E2 concentration, caused a transient decrease in placental blood flow after the treatment and placental weight gain on Day 20. Histological observation also showed that the area and number of blood cells in maternal sinusoids markedly decreased at 4 h after KTZ treatment on Day 14 of pregnancy, and the total area of maternal and fetal sinusoids increased on Day 20. Since the decrease of maternal blood space in the placenta has been suggested to be harmful for fetal growth [55], regulation of the sinusoid areas could be important for the maintenance of pregnancy and fetal development. Furukawa et al. [32] also observed in the KTZ-treated rats a multiple cystic dilatation of maternal sinusoids in some placentas on Days 15, 17, and 21 of pregnancy; however, quantitative analysis was not performed. Furthermore, the treatment with KTZ increased immunoreactivity for pimonidazole, a hypoxia marker, in the placenta after KTZ treatment on Day 14. Expansion of fetal sinusoids observed on Day 14 in the KTZ-treated group may be a response of the fetal blood vessels in the placenta to a hypoxic condition of the fetuses. The KTZ-induced blood flow reduction, histological changes, hypoxia, and later weight gain with increased sinusoid area in the placenta were all reversed by concomitant subcutaneous infusion with E2. These results suggest that reduced estrogen production after KTZ
treatment induces decreased placental blood flow followed by placental hypoxia and causes later placental changes. Because E\textsubscript{2} has been reported to induce vasodilatation through an NO-mediated mechanism [56], reduction of placental blood flow by estrogen deficiency may be related to a change in nitric oxide (NO), one of the endothelium derived relaxing factors.

The results of this study indicate the involvement of hypoxia in the KTZ-induced changes in placentas, which is consistent with the reports indicating that oxygen supply or uteroplacental blood flow affects development of the placenta [16-26]. Placentation has been shown to be dependent upon the hypoxia inducible factor signaling pathway regulated by oxygen levels [57]. VEGF is a key regulator of vasculogenesis and angiogenesis [58, 59], and its production is up regulated by hypoxia in human cell lines [60] and in rat placental villous explants [61]. Since the treatment with KTZ on Days 12 to 14 of pregnancy has been reported to increase the number of mitotic cells in the labyrinth zone on Day 15 of pregnancy in rats [32], it may be possible that a hypoxic environment is related to the increased mitosis through VEGF regulation in the placenta of the KTZ-treated rat. Dilatation of the sinusoids in the labyrinth zone accompanied by thinning of the trophoblast cells seen in the histological examination also might be the result of hypoxia in the placenta because a hypoxic environment inhibits the formation of stress fibers, the cytoskeletal structures in the rat Rcho-1 trophoblast cell line [62, 63]. Therefore, the reduction of placental blood flow followed by a hypoxic environment might have triggered a reduction in the cytoskeletal structure, and then dilatation of the placental sinusoids occurred. Thinning of the barrier separating maternal and fetal sinusoids could provide larger diffusion capacity for the oxygen supply. Although the mechanism underlying the pathophysiology of the thinning of trophoblast cells remains
to be studied, placental ischemia could be a key factor. From these findings, it was speculated that one of the causes of KTZ-induced placental weight gain is a hypoxic condition followed by increased vasculogenesis and dilatation of labyrinthine sinusoids. An adequate blood flow to the placenta is critical for normal placental growth. Although changes in blood flow has not been examined, estrogen deficiency by the treatment with epoxiconazole during pregnancy has been shown to induce placental degeneration characterized by cystic dilatation of maternal sinuses in rats [64]. To the best of our knowledge, no previous studies have established impaired placental blood flow caused by estrogen deficiency and further studies are needed to clarify the morphological and functional changes in the placentas related to placental blood flow. The possibility that decreased placental blood flow and increased placental weights at late pregnancy may be independent process remains to be elucidated.

5. Conclusions

This study showed that daily administration of KTZ (25 mg/kg/day) on specific days (Days 12 to 14 of pregnancy) induced placental weight gain associated with increased sinusoid area on Day 20 of pregnancy in rats. The administration decreased the blood E₂ concentration and placental area of maternal sinusoid and caused transient decrease of placental blood flow associated with placental hypoxia on Day 14 of pregnancy. All of the changes by the KTZ treatment were reversed by subcutaneous E₂ infusion. These results indicate that KTZ-induced estrogen deficiency induces transient decrease in placental blood flow and later placental weight gain. Placental hypoxia due to decreased
placental blood flow may be related to later placental changes.

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