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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 (E_2) 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 E_2. These results indicate that ketoconazole-induced E_2 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 (E_2) retarded placental growth [5], and the reduction of blood E_2 concentrations following ovariectomy with exogenous hormonal replacement induced excessive placental hypertrophy [6-7]. Furthermore, treatment with the antibody to E_2 caused increases in placental weights [8]. These findings suggest that a deficiency of E_2 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

altidude 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 E_2 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

mL/kg.

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 E_2 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 E_2 levels decreased 3 h after dosing of KTZ [41].

2.5. Effects of treatment with KTZ alone or with E_2 on Days 12 to 14 of pregnancy on placentas

E_2 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+0E_2, KTZ+0.1E_2, or KTZ+1E_2 group, respectively). Controls received vehicle for KTZ and solvent for E_2 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 E_2 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+0E_2, KTZ+0.1E_2, and KTZ+1E_2 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+0E_2 and KTZ+1E_2 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+0E_2, and KTZ+1E_2 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+0E_2, and KTZ+1E_2 groups were used (n=5 in each group).

2.6. Hormone assay (E_2 measurement)

Plasma E_2 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-E_2 antibody with E_2, 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.

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179 *2.7. Histology*

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

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196 *2.8. Determination of the placental blood flow*

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198 The blood flow was evaluated according to the method of Hakkinen et al. [46].
199 Briefly, at 0, 4, 8 and 24 h after dosing KTZ or its vehicle on Day 14 of pregnancy, the
200 rats in the control (n=19), KTZ+0E_2 (n=18) and KTZ+1E_2 (n=19) groups were
201 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:

$$Qs = (As \cdot Qr)/Ar,$$

where Qs and Qr represent the flow in the sample tissue and in the reference blood, respectively, and As and Ar 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 couterstained 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 E_2 levels between the control and KTZ-treated groups and that of placental blood flow between the control and KTZ+$0E_2$ or KTZ+$1E_2$ 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+0E_2 group were significantly higher than those in the other groups. There was no significant difference in the value between controls and the KTZ+1E_2 group. The

placental weights in the KTZ+0.1E_2 and KTZ+1E_2 groups were significantly lower than those in the KTZ+0E_2 group and decreased in a dose-dependent manner of E_2.

3.4. Effect of treatment with KTZ alone or with E_2 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+0E_2 group (Fig. 2B). The expanded sinusoids were associated with thinning of the trophoblast cell. The labyrinth structure in the KTZ+0.1E_2 and KTZ+1E_2 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+0E_2 group was greater than that in the other groups. Although there was no difference in the sinusoid area between the KTZ+0.1E_2 and KTZ+1E_2 groups, the value in the KTZ+0.1E_2 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+0E_2 group (Fig. 3B). Histological characteristic in the KTZ+1E_2 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+0E_2 and KTZ+1E_2 groups. The ratio of fetal erythrocytes to maternal erythrocytes in the KTZ+0E_2 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+0E_2 group when compared to controls (Table 3). Supplementation of E_2 (KTZ+1E_2 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 E_2 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+0E_2 or KTZ+1E_2 group before the treatment with KTZ or its vehicle (0 h). Although values in the control and KTZ+1E_2 groups kept a constant level after the treatment, the value in the KTZ+0E_2 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+1E_2 groups. Thereafter, no differences in the placental blood flow were seen between the control and KTZ+0E_2 or KTZ+1E_2 groups.

3.6. Effect of treatment with KTZ alone or with E_2 on immunohistochemical staining for

pimonidazole in placentas

In the placentas of the KTZ+0E_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_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_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_2 in a dose-dependent manner, suggesting that the decrease in E_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_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 E_2 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 E_2 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 E_2 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 E_2. These results suggest that reduced estrogen production after KTZ

394 treatment induces decreased placental blood flow followed by placental hypoxia and
395 ~~causes~~ later placental changes. Because E_2 has been reported to induce vasodilatation
396 through an NO-mediated mechanism [56], reduction of placental blood flow by
397 estrogen deficiency may be related to a change in nitric oxide (NO), one of the
398 endothelium derived relaxing factors.

399 The results of this study indicate the involvement of hypoxia in the KTZ-induced
400 changes in placentas, which is consistent with the reports indicating that oxygen supply
401 or uteroplacental blood flow affects development of the placenta [16-26]. Placentation
402 has been shown to be dependent upon the hypoxia inducible factor signaling pathway
403 regulated by oxygen levels [57]. VEGF is a key regulator of vasculogenesis and
404 angiogenesis [58, 59], and its production is up regulated by hypoxia in human cell lines
405 [60] and in rat placental villous explants [61]. Since the treatment with KTZ on Days 12
406 to 14 of pregnancy has been reported to increase the number of mitotic cells in the
407 labyrinth zone on Day 15 of pregnancy in rats [32], it may be possible that a hypoxic
408 environment is related to the increased mitosis through VEGF regulation in the placenta
409 of the KTZ-treated rat. Dilatation of the sinusoids in the labyrinth zone accompanied by
410 thinning of the trophoblast cells seen in the histological examination also might be the
411 result of hypoxia in the placenta because a hypoxic environment inhibits the formation
412 of stress fibers, the cytoskeletal structures in the rat Rcho-1 trophoblast cell line [62, 63].
413 Therefore, the reduction of placental blood flow followed by a hypoxic environment
414 might have triggered a reduction in the cytoskeletal structure, and then dilatation of the
415 placental sinusoids occurred. Thinning of the barrier separating maternal and fetal
416 sinusoids could provide larger diffusion capacity for the oxygen supply. Although the
417 mechanism underlying the pathophysiology of the thinning of trophoblast cells remains

418 to be studied, placental ischemia could be a key factor. From these findings, it was
419 speculated that one of the causes of KTZ-induced placental weight gain is a hypoxic
420 condition followed by increased vasculogenesis and dilatation of labyrinthine sinusoids.
421 An adequate blood flow to the placenta is critical for normal placental growth. Although
422 changes in blood flow has not been examined, estrogen deficiency by the treatment with
423 epoxiconazole during pregnancy has been shown to induce placental degeneration
424 characterized by cystic dilatation of maternal sinuses in rats [64]. To the best of our
425 knowledge, no previous studies have established impaired placental blood flow caused
426 by estrogen deficiency and further studies are needed to clarify the morphological and
427 functional changes in the placentas related to placental blood flow. The possibility that
428 decreased placental blood flow and increased placental weights at late pregnancy may
429 be independent process remains to be elucidated.
430
431
432 **5. Conclusions**
433
434 This study showed that daily administration of KTZ (25 mg/kg/day) on specific days
435 (Days 12 to 14 of pregnancy) induced placental weight gain associated with increased
436 sinusoid area on Day 20 of pregnancy in rats. The administration decreased the blood E_2
437 concentration and placental area of maternal sinusoid and caused transient decrease of
438 placental blood flow associated with placental hypoxia on Day 14 of pregnancy. All of
439 the changes by the KTZ treatment were reversed by subcutaneous E_2 infusion. These
440 results indicate that KTZ-induced estrogen deficiency induces transient decrease in
441 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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References

[1] M.K. Bauer, J.E. Harding, N.S. Bassett, B.H. Breier, M.H. Oliver, B.H. Gallaher, P.C. Evans, S.M. Woodall, P.D. Gluckman, Fetal growth and placental function, Mol. Cell Endocrinol. 140 (1998) 115-120.

[2] D.W. Matt, G.J. MacDonald, *In vitro* progesterone and testosterone production by the rat placenta during pregnancy, Endocrinology 115 (1984) 741-747.

[3] H.O. Garland, J.C. Atherton, C. Baylis, M.R. Morgan, C.M. Milne, Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: correlation with renal studies, J. Endocrinol. 113 (1987) 435-444.

[4] N. Chernoff, R.W. Setzer, D.B. Miller, M.B. Rosen, J.M. Rogers, Effects of chemically induced maternal toxicity on prenatal development in the rat, Teratology 42 (1990) 651-658.

[5] R. K. Bartholomeusz, N.W. Bruce, A.-M. Lynch, Embryo survival, and fetal and placental growth following elevation of maternal estradiol blood concentrations in the rat. Biol. Reprod. 61 (1999) 46-50.

[6] A.I. Csapo, W.G. Wiest, Plasma steroid levels and ovariectomy-induced placental hypertrophy in rats, Endocrinology 93 (1973) 1173-1177.

[7] A.I. Csapo, E.F. Csapo, Ovariectomy induced placental hypertrophy, Prostaglandins 4 (1973) 189-200.

[8] A. Csapo, F. Dray, T. Erdos, Letter: Oestradiol 17β: inhibitor of placental growth, Lancet 2 (1974) 51-52.

[9] K.J. Ryan, Conversion of delta 5-androstene-3β, 16α, 17β-triol to estriol by human

placenta, Endocrinology 63 (1958) 392-394.

[10] B. Hoffmann, G. Schuler, The bovine placenta; a source and target of steroid hormones: observations during the second half of gestation, Domest. Anim. Endocrinol. 23 (2002) 309-320.

[11] J.A. Mondragon, R. Ocadiz-Delgado, C. Miranda, J. Valencia, A.M. Rosales, P. Gariglio, M.C. Romano, Expression of P450-aromatase in the goat placenta throughout pregnancy. Theriogenology 68 (2007) 646-653.

[12] K. Uchida, Production of estrogen in rat placenta studied by electron microscope autoradiography and biochemical analysis, Arch. Histol. Jpn. 36 (1973) 57-71.

[13] M.L. Warshaw, D.C. Johnson, I. Khan, B. Eckstein, G. Gibori, Placental secretion of androgens in the rat, Endocrinology 119 (1986) 2642-2648.

[14] S.G. Bassett, G.J. Pepe, Utilization of circulating androstenedione and testosterone for estradiol production during gestation in the rat, Biol. Reprod. 37 (1987) 606-611.

[15] M.O. Pulkkinen, E. Csapo, Placental hypertrophy in the rat induced by ovariectomy, J. Reprod. Fertil. 18 (1969) 125-127.

[16] N.W. Bruce, D.A. Cabral, Effects of maternal blood loss on embryonic and placental development in the rat, J. Reprod. Fertil. 45 (1975) 349-365.

[17] N.W. Bruce, The effect of ligating a uterine artery on fetal and placental development in the rat, Biol. Reprod. 14 (1976) 246-247.

[18] J.R. Wellstead, N.W. Bruce, A. Rahima, Effects of indomethacin on spacing of conceptuses within the uterine horn and on fetal and placental growth in the rat, Anat. Rec. 225 (1989) 101-105.

[19] J. Richichi, P. Vasilenko, The effects of nifedipine on pregnancy outcome and

501 morphology of the placenta, uterus, and cervix during late pregnancy in the rat, Am.
502 J. Obstet. Gynecol. 167 (1992) 797-803.
503 [20] K.M. Godfrey, C.W. Redman, D.J. Barker, C. Osmond, The effect of maternal
504 anaemia and iron deficiency on the ratio of fetal weight to placental weight, Br. J.
505 Obstet. Gynaecol. 98 (1991) 886-891.
506 [21] R.M. Lewis, C.B. Doherty, L.A. James, G.J. Burton, C.N. Hales, Effects of
507 maternal iron restriction on placental vascularization in the rat, Placenta 22 (2001)
508 534-539.
509 [22] G.X. Rosario, T. Konno, M.J. Soares, Maternal hypoxia activates endovascular
510 trophoblast cell invasion, Dev. Biol. 314 (2008) 362-375.
511 [23] A.M. Lynch, N.W. Bruce, Placental growth in rats exposed to carbon monoxide at
512 selected stages of pregnancy, Biol. Neonate 56 (1989) 151-157.
513 [24] H.G. Richter, E.J. Camm, B.N. Modi, F. Naeem, C.M. Cross, T. Cindrova-Davies,
514 O. Spasic-Boskovic, C. Dunster, I.S. Mudway, F.J. Kelly, G.J. Burton, L. Poston,
515 D.A. Giussani, Ascorbate prevents placental oxidative stress and enhances birth
516 weight in hypoxic pregnancy in rats, J. Physiol. 590 (2012) 1377-1387.
517 [25] S. Zamudio, The placenta at high altitude, High Alt Med Biol, 4 (2003) 171-191.
518 [26] N.A. Beischer, M. Holsman, W.H. Kitchen, Relation of various forms of anemia to
519 placental weight, Am. J. Obstet. Gynecol. 101 (1968) 801-809.
520 [27] R. Resnik, A.P. Killam, F.C. Battaglia, E.L. Makowski, G. Meschia, The
521 stimulation of uterine blood flow by various estrogens, Endocrinology 94 (1974)
522 1192-1196.
523 [28] A.P. Killam, C.R. Rosenfeld, F.C. Battaglia, E.L. Makowski, G. Meschia, Effect of
524 estrogens on the uterine blood flow of oophorectomized ewes, Am. J. Obstet.

525 Gynecol. 115 (1973) 1045-1052.
526 [29] S.M. Kalman, The effect of estrogens on uterine blood flow in the rat, J. Pharmacol.
527 Exp. Ther. 124 (1958) 179-181.
528 [30] H.S. Buttar, J.H. Moffat, C. Bura, Pregnancy outcome in ketoconazole-treated rats
529 and mice, Teratology 39 (1989) 444.
530 [31] A. Ichikawa, N. Seki, T. Sugimoto, Y. Ooshima, Effects of ketoconazole on
531 placental weight in rats, Congenit. Anom. 46 (2006) A23.
532 [32] S. Furukawa, S. Hayashi, K. Usuda, M. Abe, I. Ogawa, Histopathological effect of
533 ketoconazole on rat placenta, J. Vet. Med. Sci. 70 (2008) 1179-1184.
534 [33] M. Borgers, H. Van den Bossche, M. De Brabander, The mechanism of action of
535 the new antimycotic ketoconazole, Am. J. Med. 74 (1983) 2-8.
536 [34] H. Van den Bossche, G. Willemsens, W. Cools, F. Cornelissen, W.F. Lauwers, J.M.
537 van Cutsem, In vitro and in vivo effects of the antimycotic drug ketoconazole on
538 sterol synthesis, Antimicrob. Agents Chemother. 17 (1980) 922-928.
539 [35] M.A. Ghannoum, L.B. Rice, Antifungal agents: mode of action, mechanisms of
540 resistance, and correlation of these mechanisms with bacterial resistance, Clin.
541 Microbiol. Rev. 12 (1999) 501-517.
542 [36] M.M. Weber, A. Will, B. Adelmann, D. Engelhardt, Effect of ketoconazole on
543 human ovarian C17,20-desmolase and aromatase, J. Steroid. Biochem. Mol. Biol.
544 38 (1991) 213-218.
545 [37] D. Feldman, Ketoconazole and other imidazole derivatives as inhibitors of
546 steroidogenesis, Endocr. Rev. 7 (1986) 409-420.
547 [38] S. Haidar, P.B. Ehmer, S. Barassin, C. Batzl-Hartmann, R.W. Hartmann, Effects of
548 novel 17α-hydroxylase/C17, 20-lyase (P450 17, CYP 17) inhibitors on androgen

549 biosynthesis in vitro and in vivo, J. Steroid. Biochem. Mol. Biol. 84 (2003)
550 555-562.
551 [39] W. Wouters, R. De Coster, N. Goeminne, D. Beerens, J. van Dun, Aromatase
552 inhibition by the antifungal ketoconazole, J. Steroid. Biochem. 30 (1988) 387-389.
553 [40] S. Malozowski, V. Mericq, F. Cassorla, Effects of ketoconazole on rat ovarian
554 steroidogenic enzymatic activities, Res. Commun. Chem. Pathol. Pharmacol. 52
555 (1986) 403-406.
556 [41] H. Watanabe, J.A. Menzies, Depression of ovarian estradiol-17β following single
557 oral dose of ketoconazole, Res. Commun. Chem. Pathol. Pharmacol. 48 (1985)
558 141-144.
559 [42] F. Latrille, C. Charuel, A.M. Monro, J. Stadler, B.C. Sutter, Imidazole antifungal
560 agents reduce production of 17β-oestradiol by rat ovaries in vitro, Biochem.
561 Pharmacol. 36 (1987) 1863-1866.
562 [43] R.G. Dyer, S. Mansfield, Anaesthesia with alphaxalone plus alphadolone acetate
563 blocks the oestrogen-stimulated LH surge and impairs pulsatile LH secreation in
564 ovariectomized female rats, J. Endocrinol. 102 (1984) 27-31.
565 [44] M.J. Kilpatrick, B.T. Donovan, Effect of anesthesia on the negative feedback action
566 of gonadal steroids on gonadotrophin secretion in the ovariectomized guinea-gig, J.
567 Endocrinol. 89 (1981) 167-177.
568 [45] M.E. Cruz, A. Flores, M.T. Palafox, G. Melendez, J.O. Rodriguez, R. Chavira, R.
569 Dominguez, The role of the muscarinic system in regulating estradiol secretion
570 varies during the estrous cycle: the hemiovariectomized rat model, Reprod. Biol.
571 Endocrinol. 4 (2006) 43.
572 [46] J.P. Hakkinen, M.W. Miller, A.H. Smith, D.R. Knight, Measurement of organ blood

573 flow with coloured microspheres in the rat, Cardiovasc. Res. 29 (1995) 74-79.
574 [47] J.A. Raleigh, S.C. Chou, G.E. Arteel, M.R. Horsman, Comparisons among
575 pimonidazole binding, oxygen electrode measurements, and radiation response in
576 C3H mouse tumors, Radiat. Res. 151 (1999) 580-589.
577 [48] B. Varga, E.S. Patay, E. Horvath, G. Folly, Ovarian venous outflow, progesterone
578 and 17β-oestradiol secretion and peripheral blood level during pregnancy in the rat,
579 Acta. Physiol. Acad. Sci. Hung. 58 (1981) 141-146.
580 [49] M.D. Al-Bader, Estrogen receptors alpha and beta in rat placenta: detection by
581 RT-PCR, real time PCR and Western blotting, Reprod. Biol. Endocrinol. 4 (2006)
582 13.
583 [50] F. Dray, A. Csapo, T. Erdos, Effects of antibodies to estradiol-17β and to
584 progesterone on the placental weight and pregnancy in rats – a quantitative study, J.
585 Steroid. Biochem. 6 (1975) 547-548.
586 [51] S. Matsuura, A. Itakura, Y. Ohno, Y. Nakashima, Y. Murata, M. Takeuchi, M.
587 Kobayashi, S. Mizutani, Effects of estradiol administration on feto-placental growth
588 in rat, Early Hum. Dev. 77 (2004) 47-56.
589 [52] G.R. Ross, U. Yallampalli, P.R. Gangula, L. Reed, K. Sathishkumar, H. Gao, M.
590 Chauhan, C. Yallampalli, Adrenomedullin relaxes rat uterine artery: mechanisms
591 and influence of pregnancy and estradiol, Endocrinology 151 (2010) 4485-4493.
592 [53] R.R. Magness, T.M. Phernetton, J. Zheng, Systemic and uterine blood flow
593 distribution during prolonged infusion of 17β-estradiol, Am. J. Physiol. 275 (1998)
594 H731-743.
595 [54] J.M. Storment, M. Meyer, G. Osol, Estrogen augments the vasodilatory effects of
596 vascular endothelial growth factor in the uterine circulation of the rat, Am. J. Obstet.

Gynecol. 183 (2000) 449-453.

[55] M.M. Veras, N.R. Damaceno-Rodrigues, E.G. Caldini, A.A.C. Maciel Ribeiro, T.M. Mayhew, P.H.N. Saldiva, M. Dolhnikoff, Particulate urban air pollution affects the functional morphology of mouse placenta, Biol. Reprod. 79 (2008) 578-584.

[56] K.E. Vagnoni, C.E. Shaw, T.M. Phernetton, B.M. Meglin, I.M. Bird, R.R. Magness, Endothelial vasodilator production by uterine and systemic arteries. III. Ovarian and estrogen effects on NO synthase, Am. J. Physiol. 275 (1998) H1845-1856.

[57] D.M. Adelman, M. Gertsenstein, A. Nagy, M.C. Simon, E. Maltepe, Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses, Genes Dev. 14 (2000) 3191-3203.

[58] D.S. Charnock-Jones, A.M. Sharkey, C.A. Boocock, A. Ahmed, R. Plevin, N. Ferrara, S.K. Smith, Vascular endothelial growth factor receptor localization and activation in human trophoblast and choriocarcinoma cells, Biol. Reprod. 51 (1994) 524-530.

[59] P. Evans, T. Wheeler, F. Anthony, C. Osmond, Maternal serum vascular endothelial growth factor during early pregnancy, Clin. Sci. (Lond.) 92 (1997) 567-571.

[60] C.M. Taylor, H. Stevens, F.W. Anthony, T. Wheeler, Influence of hypoxia on vascular endothelial growth factor and chorionic gonadotrophin production in the trophoblast-derived cell lines: JEG, JAr and BeWo, Placenta 18 (1997) 451-458.

[61] E.M. George, K. Cockrell, T.H. Adair, J.P. Granger, Regulation of sFlt-1 and VEGF secretion by adenosine under hypoxic conditions in rat placental villous explants, Am. J. Physiol. Regul. Integr. Comp. Physiol. 299 (2010) R1629-1633.

[62] A.D. Gultice, K.L. Selesniemi, T.L. Brown, Hypoxia inhibits differentiation of lineage-specific Rcho-1 trophoblast giant cells, Biol. Reprod. 74 (2006) 1041-1050.

621 [63] A.D. Gultice, K. Kulkarni-Datar, T.L. Brown, Hypoxia-inducible factor 1alpha
622 (HIF1A) mediates distinct steps of rat trophoblast differentiation in gradient oxygen,
623 Biol. Reprod. 80 (2009) 184-193.
624 [64] M.C. Rey Moreno, K.C. Fussell, S. Groters, S. Schneider, V. Strauss, S.
625 Stinchcombe, I. Fegert, M. Veras, B. van Ravenzwaay, Epoxiconazole-induced
626 degeneration in rat placenta and the effects of estradiol supplementation, Birth
627 Defects Res. B Dev. Reprod. Toxicol. 98 (2013) 208-221.