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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 12–14 of pregnancy induced reduction of plasma estradiol-17β (E_2) concentration, transient decrease in placental blood flow and increased intensity of a hypoxia-related marker in the placenta 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.

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1. Introduction

The placenta is a pivotal organ that synthesizes several hormones and other mediators 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-17ß (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. The placenta produces estrogen 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 in rats, estrogen is produced mainly in the ovary from androgen, which is generated in the placenta [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 weight gain 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

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biosynthesis pathway that metabolizes lanosterol to ergosterol in fungi [35]. Certain cytochrome P450 enzymes such as C17, 20-lyase and 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 11–12 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 h) for at least 1 week before use. Virgin females (13–18 weeks old) were mated overnight with males (14–25 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, g-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 miniosmotic 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 5–7 animals. KTZ was administered orally by gavage at a dose of 25 mg/kg/day on Days 9–11, 12–14, or 15–17 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 live fetuses and their placentas 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 12–14 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 18,500g for 1 min 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 12–14 of pregnancy on placentas

E_2 was administered into the dorsal subcutis using a miniosmotic 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 12–14 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.

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

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