



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

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13

14 *Key Words:*

15 Ketoconazole

16 Placenta

17 Estradiol-17 β

18 Blood flow

19 Rat

20

21 **ABSTRACT**

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

33 **1. Introduction**

34

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

40 Estrogen is known as one of the factors involved in the development of the placenta.
41 In pregnant rats, injection of estradiol-17 β (E₂) retarded placental growth [5], and the
42 reduction of blood E₂ concentrations following ovariectomy with exogenous hormonal
43 replacement induced excessive placental hypertrophy [6-7]. Furthermore, treatment with
44 the antibody to E₂ caused increases in placental weights [8]. These findings suggest that
45 a deficiency of E₂ could be involved in the hypertrophic responses of the rat placenta
46 during pregnancy. The placenta produces estrogen during pregnancy in some
47 mammalian species [9-11] , while slight or negligible production of estrogen was
48 detected in rat placentas [2, 12]. During the second half of pregnancy estrogen is
49 produced mainly in the ovary from androgen, which is generated in the placenta in rats
50 [13, 14]. It has been assumed that placental hypertrophy by estrogen deficiency may be
51 a compensatory response related to an effective ‘luteo-placental shift’ by steroid
52 production in the support of the maintenance of pregnancy [7, 15].

53 Concerning the other factors regulating placental growth, hemorrhage [16], uterine
54 vessel ligation [17], or treatment with indomethacin [18] or nifedipine [19], which
55 reduces placental blood flow, has been reported to increase placental weights. A
56 reduction of oxygen transport as a result of maternal anemia, iron deficiency or high

57 altitude also causes increased placental weights [20-26]. From these findings, it has
58 been assumed that oxygen supply or uteroplacental blood flow plays an important role
59 in the development of the placenta. Although estrogen affects uterine blood flow
60 [27-29], the relationship between placental growth and changes in the uteroplacental
61 blood flow by estrogen deficiency has not been evaluated.

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

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77 **2. Materials and methods**

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79 *2.1. Animals and housing*

80

81 Female Crl:CD (SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan)

82 were obtained at 11 to 12 weeks of age. The rats were acclimated in the laboratory at
83 $23\pm 3^{\circ}\text{C}$ and with a 12-h light and 12-h dark cycle (light: 0700-1900 hour) for at least 1
84 week before use. Virgin females (13 to 18 weeks old) were mated overnight with males
85 (14 to 25 weeks old) of the same strain at proestrus on a one to one basis. The day when
86 a copulation plug was found was designated Day 0 of pregnancy. The animals were
87 individually housed in metal cages with wire mesh bottoms and provided with tap water
88 and a laboratory animal diet (CR-LPF, γ -ray irradiated, Oriental Yeast, Co. Ltd., Tokyo,
89 Japan) ad libitum. Animals were euthanized by exsanguination under ether anesthesia
90 except when otherwise noted. All procedures were performed in accordance with the
91 institutional guidelines for animal care at Takeda Pharmaceutical Company Limited in
92 conformity to the National Institutes of Health guide for the care and use of Laboratory
93 Animals.

94

95

96 *2.2. Chemicals and preparation for treatments*

97

98 Methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) was dissolved in
99 injection-grade distilled water to make a 0.5% (w/v) solution. KTZ (Wako Pure
100 Chemical Industries, Tokyo, Japan) was weighed and mixed with the solution using a
101 defoaming conditioning mixer (MX-201, THINKY Corporation, Tokyo, Japan) to make
102 a 0.5% (w/v) suspension of KTZ. Batches of the dosing suspensions sufficient for
103 several days of dosing (maximum 5 days) were prepared and were stored in a
104 refrigerator (set at 4°C) until use. Prior to dose administration, the dosing suspension
105 was allowed to warm to room temperature. The dose volume for each animal was 5

106 mL/kg.

107 E₂ was purchased from CALBIOCHEM (La Jolla, CA) and mini-osmotic pumps
108 (model 1003D; 1.0 µL/h delivery rate, 3 days, Alzet[®], DURECT Corporation, Cupertino,
109 CA) were used to infuse E₂. The pumps were filled with approximately 90 µL of E₂
110 solution at a concentration of 0, 0.42 or 42 µg/mL in a mixture of 0.5% ethanol and
111 99.5% propylene glycol.

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

115

116

117 *2.3. Effect of KTZ treatment during different periods of pregnancy on placental weight*

118

119 Pregnant rats were allocated to 4 groups, each containing 5 to 7 animals. KTZ was
120 administered orally by gavage at a dose of 25 mg/kg/day on Days 9 to 11, 12 to 14, or
121 15 to 17 of pregnancy (the dams were dosed daily between 09:00 and 11:00). The dose
122 of KTZ was based on the report that a single oral dose of 20 mg/kg KTZ depressed
123 ovarian concentrations of E₂ [41]. Control animals received vehicle only. On Day 20 of
124 pregnancy, the dams were euthanized and the placentas and live fetuses were weighed
125 using an electric balance.

126

127

128 *2.4. Effect of KTZ treatment on plasma E₂ concentration*

129

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

138

139

140 *2.5. Effects of treatment with KTZ alone or with E₂ on Days 12 to 14 of pregnancy on*
141 *placentas*

142

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

152

153 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.

154 Among these placentas, 2 from 3 rats in each group were fixed in 10% neutral buffered
155 formalin for histological examination.

156 On Days 14 of pregnancy, the rats in the control, KTZ+0E₂ and KTZ+1E₂ groups
157 (n=3 in each group) were euthanized 4 h after the treatment with KTZ or its vehicle, and
158 2 placentas from each rat were fixed in 10% neutral buffered formalin for histological
159 examination.

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

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

166

167

168 2.6. Hormone assay (E₂ measurement)

169

170 Plasma E₂ levels were measured by a double-antibody radioimmunoassay (RIA) with
171 a commercially available kit (Diagnostic Products Corporation, LA). According to the
172 manufacturer, cross-reactivities of the anti-E₂ antibody with E₂, estrone, estriol,
173 testosterone, androstenedione, and progesterone were 100%, 10.0%, 0.32%, 0.001%,
174 <0.001% and <0.001%, respectively. All of the samples were quantified within a single
175 assay. The intra-assay coefficient of variation and the lower limit of sensitivity were
176 5.0% and 5 pg/mL, respectively.

177

178

179 *2.7. Histology*

180

181 Formalin-fixed, paraffin-embedded placentas were sectioned at 4- μ m 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.

194

195

196 *2.8. Determination of the placental blood flow*

197

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+0E₂ (n=18) and KTZ+1E₂ (n=19) groups were
201 anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) for the

202 implantation of two catheters that were filled with saline into the femoral artery and left
203 ventricle. A PE-10 catheter was positioned into the abdominal aorta through the femoral
204 artery for the direct measurement of the arterial pressure and collection of ‘reference
205 blood’. The second catheter was inserted into the left ventricle through the right carotid
206 artery for the infusion of colored microspheres. A 1 mL solution of 300,000 yellow
207 microspheres was infused at the rate of 1 mL/min, and at the same time 1 mL of
208 ‘reference blood’ was collected at the rate of 1 mL/min. The animals were euthanized
209 and their placentas were removed and weighed. The sample tissue and the reference
210 blood were properly treated to isolate the microspheres. The absorption spectrum peak
211 for the yellow microspheres was obtained at 440 nm.

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

$$214 \quad Q_s = (A_s \cdot Q_r) / A_r,$$

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

219

220

221 *2.9. Immunohistochemistry*

222

223 Immunohistochemical staining for pimonidazole was performed to examine the
224 hypoxic state of placentas. Pimonidazole is water soluble and rapidly distributes to all
225 tissues after peritoneal injection. It forms adducts with proteins in cells having an

226 oxygen concentration less than 14 micromolar [47]. The rats received an intraperitoneal
227 injection of pimonidazole hydrochloride solution (60 mg/kg) 5 h after the treatment
228 with KTZ or its vehicle on Day 14 of pregnancy. Ninety minutes after the injection, the
229 rats were anesthetized and the uteri including fetuses and placentas were excised and
230 fixed in 10% neutral buffered formalin solution. Two placentas were randomly taken
231 from each dam and embedded in paraffin. Sagittal sections were made for each placenta.
232 The sections were deparaffinized and rehydrated, and stained for the presence of the
233 pimonidazole adduct (hypoxia marker) based on the manufacturer's instructions (HPI,
234 Burlington, MA). Briefly, the rehydrated sections were treated with trypsin (Difco, NJ)
235 in TRIS-buffered saline (TBS) for antigen retrieval and then incubated with mouse
236 monoclonal antibodies (Cayman Chemical Company, MI) at 1:2500 dilution. Antibody
237 binding was detected after incubation with a secondary biotinylated horse anti-mouse
238 antibody (Lab Vision, Fremont, CA) and reagents in the Vectastatin
239 immunohistochemical staining kit (Vector Laboratories, Burlingame, CA).
240 Immunostained sections were lightly counterstained with hematoxylin.

241

242

243 *2.10. Statistical analysis*

244

245 Data are expressed as mean \pm standard error of the mean (SEM). Evaluation of the
246 number of live fetuses and erythrocytes was performed by Bartlett's test for
247 homogeneity of variance followed by an analysis of variance (ANOVA). Weights of the
248 placentas and fetuses were analyzed by two-way analysis of variance, with the variance
249 being partitioned between (groups)- and within (gender of fetuses)-animal bases,

250 followed by multiple comparison using the Tukey-Kramer method. Comparison of
251 plasma E₂ levels between the control and KTZ-treated groups and that of placental
252 blood flow between the control and KTZ+0E₂ or KTZ+1E₂ groups at each sampling
253 time were performed by the F test for homogeneity of variance followed by Student's t
254 test (when the variances were homogeneous) or the Welch's t test (when the variances
255 were heterogeneous). The Bonferroni correction was used to determine if the t tests
256 were significant after multiple testing for the values of placental blood flow. The percent
257 of sinusoid area and ratio of fetal erythrocytes were subjected to arcsine transformation
258 before Bartlett's test for homogeneity of variance followed by ANOVA and the
259 Tukey-Kramer method. The significance level was set at $p < 0.05$. The analyses were
260 done using Statcel (the add-in forms on Excel, 3rd ed.; OMS Ltd., Tokorozawa, Japan).

261

262

263 **3. Results**

264

265 *3.1. Effect of KTZ treatment during different periods of pregnancy on placental weights*

266

267 Table 1 shows the placental and fetal weights on Day 20 of pregnancy when KTZ
268 was given to pregnant rats during various periods. The number of live fetuses was not
269 different among the groups. In the analysis of fetal weight by 2-way ANOVA, there was
270 no effect of either gender or treatment. Regarding analysis of placental weights by
271 2-way ANOVA, although effects of neither gender nor interaction between treatment
272 and gender were observed, the effect of treatment was significant. Regardless of fetal
273 gender, placental weights were significantly greater in the group treated with KTZ on

274 Days 12 to 14 of pregnancy than in the other groups, and the values were not different
275 between controls and the group treated with KTZ on Days 9 to 11 or 15 to 17 of
276 pregnancy. These results indicate that the time at which placental growth is most
277 responsive to KTZ treatment is approximately Days 12 to 14 of pregnancy. Therefore,
278 the time of KTZ treatments were settled at these critical periods in the following
279 experiments.

280

281

282 *3.2. Effect of KTZ treatment on plasma E₂ concentrations*

283

284 At 4 h after the treatment with KTZ or its vehicle on Day 14 of pregnancy, maternal
285 plasma E₂ concentration (mean ± SEM) was significantly lower (p<0.05) in the group
286 treated with KTZ on Days 12 to 14 of pregnancy (17.2 ± 6.6 pg/mL, n=6) than in the
287 controls (34.9 ± 7.6 pg/mL, n=5).

288

289

290 *3.3. Effect of treatment with E₂ on placental weight in the KTZ-treated rat*

291

292 Placental weights on Day 20 of pregnancy in the groups treated with KTZ and E₂
293 on Days 12 to 14 of pregnancy are shown in Fig. 1. In the 2-way ANOVA, although
294 there were no effects of gender or interaction between gender and treatment, the effect
295 of treatment was significant. Regardless of the fetal gender, placental weights in the
296 KTZ+0E₂ group were significantly higher than those in the other groups. There was
297 no significant difference in the value between controls and the KTZ+1E₂ group. The

298 placental weights in the KTZ+0.1E₂ and KTZ+1E₂ groups were significantly lower
299 than those in the KTZ+0E₂ group and decreased in a dose-dependent manner of E₂.

300

301

302 *3.4. Effect of treatment with KTZ alone or with E₂ on placental histology*

303

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

315 On Day 14 of pregnancy, fetal erythrocytes with nuclei were clearly
316 distinguished from maternal erythrocytes, which have no nuclei. When compared to
317 controls (Fig. 3A), the number of maternal erythrocytes was markedly decreased in
318 the labyrinth zone and fetal erythrocytes were increased in widely expanded sinusoids
319 in the KTZ+0E₂ group (Fig. 3B). Histological characteristic in the KTZ+1E₂ group
320 (Fig. 3C) was similar to that in the controls. Table 3 shows quantitative analyses of
321 erythrocytes and sinusoid area of labyrinth zone of placentas among groups. The total

322 number of erythrocytes was not different among the control, KTZ+0E₂ and KTZ+1E₂
323 groups. The ratio of fetal erythrocytes to maternal erythrocytes in the KTZ+0E₂ group
324 was significantly higher than those in the other groups. Analysis of the area of
325 labyrinthine sinusoids per unit area shows that the ratio of maternal sinusoids was
326 lower and that of fetal sinusoids was higher in the KTZ+0E₂ group when compared to
327 controls (Table 3). Supplementation of E₂ (KTZ+1E₂ group) increased the ratio of
328 maternal sinusoids when compared to controls and restored the ratio of fetal sinusoids
329 to the control level.

330

331

332 *3.5. Effect of treatment with KTZ alone or with E₂ on placental blood flow*

333

334 Fig. 4 shows placental blood flow after treatment with KTZ or its vehicle on Day
335 14 of pregnancy. The values were not different between the control and KTZ+0E₂ or
336 KTZ+1E₂ group before the treatment with KTZ or its vehicle (0 h). Although values
337 in the control and KTZ+1E₂ groups kept a constant level after the treatment, the value
338 in the KTZ+0E₂ group remarkably decreased 4 h after KTZ treatment and were
339 significantly lower than that in the control group. At this time there was no difference
340 in the value between the control and KTZ+1E₂ groups. Thereafter, no differences in
341 the placental blood flow were seen between the control and KTZ+0E₂ or KTZ+1E₂
342 groups.

343

344

345 *3.6. Effect of treatment with KTZ alone or with E₂ on immunohistochemical staining for*

346 *pimonidazole in placentas*

347

348 In the placentas of the KTZ+0E₂ group (Fig. 5B), stronger intensity of
349 immunostaining for pimonidazole hydrochloride was observed when compared to
350 controls (Fig. 5A). Slight staining for pimonidazole was seen in the placentas of the
351 control and KTZ+1E₂ group (Fig. 5C).

352

353

354 **4. Discussion**

355

356 In this study, the window of sensitivity for KTZ treatment to increase placental
357 weight was found to be Days 12 to 14 of pregnancy, and the effect was valid in the
358 placenta of both male and female fetuses. KTZ decreased plasma E₂ concentrations to a
359 half at 4 h after the treatment when compared to that of controls, and the increase in
360 placental weights by the KTZ treatment was negated by a continuous infusion of E₂ in a
361 dose-dependent manner, suggesting that the decrease in E₂ levels could be a cause of the
362 KTZ-induced placental weight increase. Although the reason is unclear as to why the
363 sensitivity to KTZ for increasing placental weights is limited to such a short period of
364 pregnancy, dramatic changes in placental morphology during gestation may be involved.
365 The labyrinth zone appears and the maternal E₂ concentrations tend to increase around
366 Day 12 of pregnancy [48]. The KTZ treatment during Days 9 to 11 may not affect
367 placental growth because the placental labyrinth, which is a major constituent of
368 placental growth, is absent at this stage. The reduced sensitivity to KTZ treatment after
369 Day 15 of pregnancy may be related to the number of placental estrogen receptors (ER)

370 because the ER in the rat placenta decreases during late pregnancy [49].

371 Although accumulating evidence suggests that E₂ inhibits placental growth in rats
372 [5-8, 15, 50, 51], the mechanism by which estrogen deficiency induces placental weight
373 gain is not known. It has been reported that E₂ increases blood flow in the uterus [27-29,
374 52-54], and reduction of the oxygen supply by anemia or blood loss induces placental
375 weight increase [16, 20]. This study examined the relationship among estrogen,
376 placental blood flow and placental weight gain in the KTZ-treated rats. The treatment
377 with KTZ on Days 12 to 14 of pregnancy, which decreased blood E₂ concentration,
378 caused a transient decrease in placental blood flow after the treatment and placental
379 weight gain on Day 20. Histological observation also showed that the area and number
380 of blood cells in maternal sinusoids markedly decreased at 4 h after KTZ treatment on
381 Day 14 of pregnancy, and the total area of maternal and fetal sinusoids increased on Day
382 20. Since the decrease of maternal blood space in the placenta has been suggested to be
383 harmful for fetal growth [55], regulation of the sinusoid areas could be important for the
384 maintenance of pregnancy and fetal development. Furukawa et al. [32] also observed in
385 the KTZ-treated rats a multiple cystic dilatation of maternal sinusoids in some placentas
386 on Days 15, 17, and 21 of pregnancy; however, quantitative analysis was not performed.
387 Furthermore, the treatment with KTZ increased immunoreactivity for pimonidazole, a
388 hypoxia marker, in the placenta after KTZ treatment on Day 14. Expansion of fetal
389 sinusoids observed on Day 14 in the KTZ-treated group may be a response of the fetal
390 blood vessels in the placenta to a hypoxic condition of the fetuses. The KTZ-induced
391 blood flow reduction, histological changes, hypoxia, and later weight gain with
392 increased sinusoid area in the placenta were all reversed by concomitant subcutaneous
393 infusion with E₂. 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 E₂ 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 E₂
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 E₂ 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

442 placental blood flow may be related to later placental changes.

443

444

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446

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451

452

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