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Effect of methotrexate on rat placenta development

Jing Sun [a], Akihiko Sugiyama [a,b,*], Syouta Inoue [b], Takashi Takeuchi [a,c], Takashi Takeuchi [a,b], Satoshi Furukawa [d]

[a] United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi, Yamaguchi 753-8515, Japan
[b] Courses of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4-101 Koyama-cho, Tottori, Tottori 680-8553, Japan
[c] Courses of Laboratory Animal Science, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4-101 Koyama-cho, Tottori, Tottori 680-8553, Japan
[d] Toxicology and Environmental Science Department, Biological Research Laboratories, Nissan Chemical Industries, Ltd., 1470 Shiraoka, Minamisaitama, Saitama 349-0294, Japan

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ABSTRACT

The sequential changes in the placenta from rats exposed to methotrexate were examined histopathologically. Methotrexate was intraperitoneally administered at 0.2 mg/kg/day during gestation days (GDs) 11–12 or GDs 13–14, and the placentas were sampled on GDs 13, 15, 17 and 21. The fetal mortality rates tended to increase throughout the experimental period, and fetal weights were significantly decreased on GD13 in the GD11,12-treated group. A significant reduction in placental weights was detected on GDs 13 and 15 in the GD11,12-treated group. Histopathologically, in the GD11,12-treated group, a significant thinning of the basal zone was detected throughout the experimental period, whereas the thickness of the labyrinth zone decreased significantly during GDs 13–17. The severity of thinning of the basal zone was higher than that of the labyrinth zone. In addition, a marked decrease in glycogen cell-islands in the basal zone was detected on GD 15. A significant decrease in Phospho-Histone H3-positive cells and a significant increase in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were detected on GD 13 in the basal zone of the GD11,12-treated group. In the GD13,14-treated group, there were no other significant changes in placentas and fetuses throughout the experimental period other than thinning of the labyrinth zone on GD 17. These results suggest that component cells of the basal and labyrinth zones during GDs 11–12 are more highly sensitive for methotrexate than those during GDs 13–14 and methotrexate affects the component cells of basal zone more strongly than those of the labyrinth zone.

1. Introduction

The placenta is an interface between the dams and the embryos/fetuses, and is a multifaceted organ that performs a number of essential and significant functions that are modified during gestation. These include anchoring the developing fetus to the uterine wall, mediating maternal immune tolerance, and maintaining O_2/CO_2 exchange and nutrient/metabolite requirements during embryonic development (Bauer et al., 1998; Furukawa et al., 2011). It also functions as a barrier protecting embryos/fetuses from xenobiotics, and releases a variety of steroids, hormones and cytokines (Furukawa et al., 2011). Thus, the growth and function of the placenta play important roles in the maintenance of pregnancy, and influences fetal development. The placenta is a target organ highly susceptible to drug- or chemical material-induced adverse effects (Furukawa et al., 2011). Drug- or chemical material-induced placental functional disorders result in developmental abnormality of the fetus (Furukawa et al., 2011). Thus, the placenta is an important organ for evaluating embryonic developmental toxicity and understanding its mechanism.

Folates function in various one-carbon transfer reactions, including purine and thymidylate biosynthesis, amino acid metabolism, and formate oxidation (Wagner, 1995). Purine and thymidylate biosynthesis is a fundamental requisite event underlying DNA and RNA synthesis (Tamura and Picciano, 2006). These folate-dependent reactions are essential for fetal development and maternal well-being (Tamura and Picciano, 2006). Folic acid may also have important roles in other physiological pathways needed for successful pregnancy, including angiogenesis (Sasaki et al., 2003; Williams et al., 2011), methylation of the homocysteine (Ciaccio et al., 2008), antioxidant effect (Joshi et al., 2001), and endothelial-dependent vascular relaxation (Griffith et al., 2005). These processes are essential for the establishment of

* Corresponding author at: Courses of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4-101 Koyama-cho, Tottori, Tottori 680-8553, Japan. Tel.: +81 857 31 5591; fax: +81 857 31 5591.
E-mail address: sugiyama@muses.tottori-u.ac.jp (A. Sugiyama).

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fetoplacental circulation. However, little is known about histopathological changes in placentas induced by folate metabolism impairment or folate deficiency.

Folate metabolism antagonist, methotrexate (MTX), a structural analog of folic acids, inhibits the reduction of dihydrofolate to tetrahydrofolate, resulting in the inhibition of DNA and RNA synthesis (Margolis et al., 1971; Jolivet et al., 1983). This results in MTX-mediated inhibition of cell proliferation, disruption of cell cycle and induction of apoptosis in susceptible cells (Genestier et al., 2000). MTX has been used in the treatment of neoplastic diseases, gestational trophoblast disease, and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, psoriasis, and psoriatic arthritis (Cuellar and Espinoza, 1997; DeLoia et al., 1998; Genestier et al., 2000). MTX is also used in the medical management of ectopic pregnancy and for medical termination of pregnancy (Creinin and Krohn, 1997; Lipscomb, 2007). In humans, maternal exposure to MTX during pregnancy induces miscarriages, neonatal malformations and developmental delays (Del Campo et al., 1999; Martinez Lopez et al., 2009). MTX is embryotoxic and teratogenic in rats, mice and rabbits (Skalko and Gold, 1974; Tsibangu et al., 1975; Jordan et al., 1977), and is abortifacient in rhesus monkeys (Wilson et al., 1979). However, there have been few reports on the detailed histopathological findings of the placenta exposed to MTX, and it remains unclear how placental pathology might affect fetal growth.

In the present study, we investigated the sequential histopathological changes in the placenta after exposure to MTX in pregnant rats to clarify the role of the placenta in the induction of fetal developmental disability induced by folate metabolism antagonist, MTX.

2. Materials and methods

2.1. Animals

All experiments were performed using female Wistar Imamichi rats, 9 weeks of age, 223.66 ± 1.69 g (mean ± SE) in weight and obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were reared in a room with the temperature controlled at 22 ± 2 °C, humidity at 50 ± 5%, with ventilation 11 times per hour and lighting set at 12:12-h light/dark cycle (light cycle, 7:00–19:00), and they were given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The present experiments were performed following the provisions approved by the Animal Research Committee of Tottori University.

2.2. Experimental design

Day 0 of gestation (GD 0) was designated as the day when the presence of a vaginal plug was identified. A total of 44 animals were divided into three different groups as follows: (1) saline-treated control rats ($n = 16$), (2) MTX-GDs 11 and 12-treated rats ($n = 16$), (3) MTX-GDs 13 and 14-treated rats ($n = 12$). MTX (Pfizer Japan Inc., Tokyo, Japan) was dissolved in saline and administered intraperitoneally. The rats received intraperitoneal injections (i.p.) with MTX (0.2 mg/kg body weight) or saline (the control) during GDs 11–12 or GDs 13–14. The dose level in the present study was previously reported to induce an impairment of the fetus in rats (Jordan et al., 1977; Wilson et al., 1979). The specific timing of MTX administration was selected because the glycogen cells, which constitute one of the important elements in the basal zone, are detected from GD 11 (Peel and Bulmer, 1977). Maternal body weight was recorded on GDs 0–21. Placenta and fetus samples were collected under pentobarbital anesthesia (100 mg/kg, i.p.) on GDs 13 (except for the GD13,14-treated group), 15, 17 and 21. All embryos/fetuses were removed from the placentas. Half of the placentas were separated between the basal zone and decidua basalis, removed from the uterine wall, and weighed. The embryos/fetuses and removed placentas were weighed. The fetuses on GD21 were macroscopically examined for external malformations. All procedures were conducted between 9 and 11 a.m.

2.3. Histopathological examination of rat placentas

All placentas were fixed in 10% neutral buffered formalin. These tissues were embedded in paraffin, sectioned 4 μm in thickness, and stained with hematoxylin and eosin (HE). The thickness of the labyrinth zone, basal zone, decidua basalis and metrial gland close to the central portion were measured in placentas from each dam with histomorphometric analysis software (Olympus Corporation, Tokyo, Japan).

2.4. Immunohistochemical examinations of rat placentas

For immunohistochemistry, mouse monoclonal antibodies for Phospho-Histone H3 (Epitomics, Inc., CA, USA) were used as the primary antibodies. All sections were dewaxed, rehydrated, rinsed with 0.05 M Tris-buffered saline (TBS; pH 7.6), treated with 3% hydrogen peroxide, and then rinsed again with TBS. Tissue sections for detection of both antigens were immersed in 0.01 M citrate buffer (pH 6.0/Dako, Glostrup, Denmark) and autoclaved for 15 min at 121 °C to retrieve the antigens. Slides were incubated with the primary antibody (1:300 dilution) for 30 min at room temperature, rinsed with TBS, and treated with Simple Stain MAX-PO (Nichirei, Tokyo, Japan) for 30 min at room temperature. They were then rinsed with TBS before being treated with a 3,3′-diaminobenzidine solution containing 0.01% hydrogen peroxide to facilitate a peroxidase color reaction. After a further wash with TBS, the slides were counterstained with Mayer's hematoxylin.

Apoptotic bodies in the placenta were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, USA). The criteria of apoptosis included TUNEL staining and the presence of pyknotic nuclei.

The number of TUNEL or Phospho-Histone H3-positive cells was counted in ten different fields in the labyrinth and the basal zones by light microscopy with a 20× objective lens, and with above-mentioned histomorphometric analysis software.

2.5. Statistical analysis

All data were expressed as means ± standard error (SE) in each group. The results in each group were compared by Student's *t*-test or Dunnett's multiple comparison test with statistical software (SSRI Co., Ltd., Tokyo, Japan). $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

3. Results

3.1. Effects on dams

Body weight gains of dams decreased significantly during GDs 13–21 in the GD11,12-treated group compared with the control group, whereas in the GD13,14-treated group, significant declining body weight gains of dams were not observed throughout the experimental period compared with the control group (Fig. 1). However, dams neither in the control nor the two MTX-treated groups showed any other clinical signs during the experimental period.