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Letter

Inhibitory effects of L-NAME, a nitric oxide synthase inhibitor, on decidual cell proliferation in mid-to-late pregnant rats

Wataru Ishikawa, Shugo Kazama, Takehito Suzuki, Rei Yamana, Yoko Miyazaki, Kazuaki Tanaka, Makoto Usami and Tatsuya Takizawa

Graduate School of Veterinary Medicine, Azabu University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252–5201, Japan

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ABSTRACT — The effects of *N*G-Nitro-L-arginine-methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase, on decidual cell proliferation were examined to investigate possible NO depletion-mediated mechanisms of reproductive toxicity. Mid-to-late pregnant rats were injected intraperitoneally with L-NAME (100 mg/kg body weight) and 5-bromo-2′-deoxyuridine (BrdU, 100 mg/kg body weight) at 12 hr and 1 hr before sacrifice, respectively, on day 13.5, 17.5, or 21.5, and the BrdU-positive proliferating decidual cells were detected. In control rats, proliferating decidual cells were observed sporadically on days 13.5 and 17.5; however, fewer such cells were observed at day 21.5. L-NAME reduced the number of proliferating decidual cells by approximately half at days 13.5 and 17.5 but not at day 21.5. These results suggested that in mid-to-late pregnant rats, decidual cell proliferation was maintained by a mechanism involving NO signaling, and NO depletion reduced it only to a limited extent. The absent effect at day 21.5 may be due to the low incidence of proliferating decidual cells or the changing role of NO near the term of pregnancy.

Key words: BrdU, Cell proliferation, Decidua, Nitric oxide, Rat

INTRODUCTION

*N*G-Nitro-L-arginine-methyl ester (L-NAME) is an L-arginine analog that inhibits nitric oxide synthase (NOS) in a competitive manner. In mammals, there are three major isoforms of NOS: neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelial (eNOS or NOS3); although originally named according to their tissue expression and inducibility, they are all constitutively expressed in various tissues. As nitric oxide (NO) is very short-lived (Palmer *et al.*, 1987), the function of endogenous NO has often been investigated using NOS inhibitors, such as L-NAME that inhibits all NOS isoforms, as pharmacological tools (Alderton *et al.*, 2001).

NO functions as a signaling molecule in many biological processes, including reproduction (Dixit and Parvizi, 2001). For example, the decidua, a uterine tissue that differentiates from stromal cells after implantation for maintaining pregnancy (Renaud and Soares, 2011), appears to be maintained by NO signaling through the regulation of cell death. iNOS is the major isoform in rat decidua (Suzuki *et al.*, 2009), and its gene knockdown reduces decidual cell density and litter size in mice (Burnett *et al.*, 2002). The inhibition of endogenous NO synthesis by L-NAME induces decidual apoptosis in late pregnant rats when NO is abundant in the decidua (Suzuki *et al.*, 2010). The formation of the decidua (decidualization) requires the synergistic effect of NO and progesterone, as

Correspondence: Tatsuya Takizawa (E-mail: takizawa@azabu-u.ac.jp)

shown in a study using NOS inhibitors and an anti-progesterone agent in rats (Chwalisz *et al.*, 1999).

In this study, we examined the effects of L-NAME on decidual cell proliferation to investigate possible NO depletion-mediated mechanisms of reproductive toxicity. Mid-to-late pregnant rats were injected intraperitoneally with L-NAME and 5-bromo-2′-deoxyuridine (BrdU), and the incidence of proliferating decidual cells, detected as BrdU-positive cells, was determined.

MATERIALS AND METHODS

Animals

Wistar rats (Crlj: WI; Charles River Japan, Kanagawa, Japan) aged 10-15 weeks were used in the experiments. They were provided a commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and tap water *ad libitum* in an animal room maintained at a temperature of 22 ± 3°C, with a relative humidity of 55% ± 10% and a constant light/dark schedule (12L:12D). Three female rats were placed with a male rat overnight for mating (plug day = day 0.5 of pregnancy). All experimental procedures were performed according to the guidelines of the Committee for Animal Experimentation at Azabu University, Japan.

Chemicals and experimental treatment

Pregnant rats were treated with 100 mg/kg body weight of L-NAME (Sigma-Aldrich, St Louis, MO, USA) and 100 mg/kg body weight of BrdU (Sigma-Aldrich), each dissolved in physiological saline, via intraperitoneal injection at 12 hr and 1 hr before sacrifice, respectively. It has been shown that subcutaneous injection of L-NAME at the same dose depleted NO at and after 1 hr in the uterus of late pregnant rats (Suzuki *et al.*, 2009). The animals were sacrificed at day 13.5, 17.5, or 21.5 of pregnancy. Animals in the control groups received physiological saline in the same manner.

Detection of proliferating cells

Proliferating cells in the decidua of uterine samples were detected as BrdU-positive cells via immunohistochemistry. The whole uterus was fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned into 4-µm-thick slices. A primary antibody against BrdU (Calbiochem, San Diego, CA, USA) was used with the VECTASTAIN Elite ABC Mouse IgG Kit (Vector Laboratories, Inc., Burlingame, CA, USA) and the Peroxidase Stain DAB Kit (Brown Stain, Nacalai Tesque, Tokyo, Japan) according to the manufacturers' instructions. The sections were counterstained with Mayer's hematoxylin.

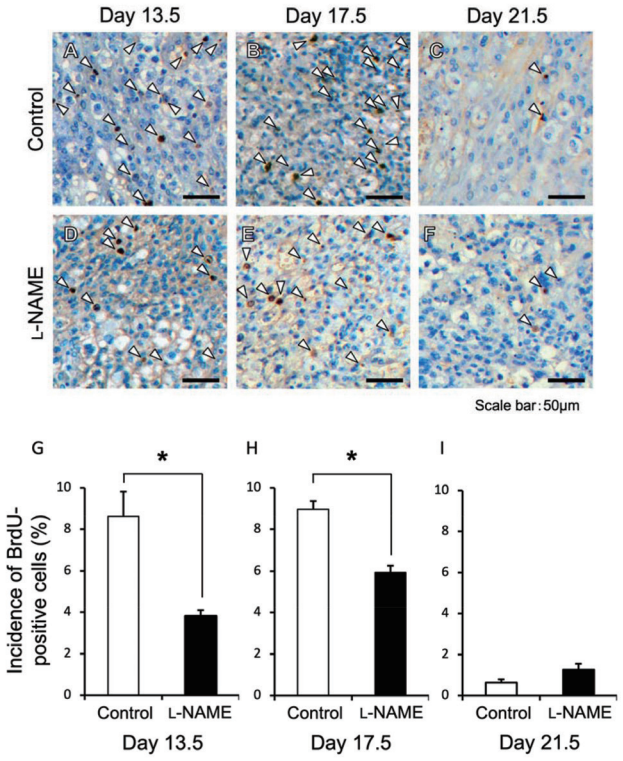

Fig. 1. Effects of N^G-Nitro-L-arginine-methyl ester (L-NAME) treatment on cell proliferation in rat decidua. Pregnant rats were treated with L-NAME (100 mg/kg body weight) and 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg body weight) via intraperitoneal injection at 12 hr and 1 hr before sacrifice, respectively. (A-F) Detection of proliferating cells as BrdU-positive cells in the decidua. Arrowheads indicate BrdU-positive cells stained brown. Scale bar, 50 µm. (G-I) Incidence of BrdU-positive cells in the decidua. The incidence in 1,500 or more cells counted randomly is indicated. The mean ± standard error of the mean for four or five animals is shown. An asterisk indicates statistical significance at a probability level of 0.05.

Statistical analysis

Statistical significance of differences between the control and L-NAME groups were examined using Welch's *t*-test at a probability level of 0.05.

RESULTS AND DISCUSSION

In the decidua of control rats, proliferating cells, detected as BrdU-positive cells, were observed sporadically on each day of pregnancy examined (Fig. 1A-C). Quantitative analysis showed that the incidence of pro-

liferating decidual cells was similar, at approximately 9.0%, between days 13.5 and 17.5 but was lower at day 21.5, indicating that decidual cell proliferation decreased near the term of pregnancy (Fig. 1G-I). These findings are intriguing because during pregnancy, decidual tissue degenerates owing to apoptosis starting from the anti-mesometrial region, leading to decidual shedding from the uterus in the afterbirth (Griffith *et al.*, 2017; Gu *et al.*, 1994). It is thus inferred that cell proliferation and apoptosis occur simultaneously to dynamically maintain decidual cells suitable for mid-to-late pregnancy toward parturition, when decidual cells decrease drastically, thus finishing their roles.

L-NAME injection caused no apparent changes in the incidence of proliferating decidual cells on each day of pregnancy examined (Fig. 1D-F). However, quantitative analysis indicated that L-NAME injection significantly reduced proliferating decidual cells by approximately half both at days 13.5 and 17.5 (Fig. 1G-I). There was no effect of L-NAME injection on the incidence of proliferating decidual cells at day 21.5, when proliferating decidual cells were fewer than those at days 13.5 and 17.5 (Fig. 1G-I).

These results suggest that in mid-to-late pregnant rats, decidual cell proliferation is maintained by a mechanism involving NO signaling, and accordingly, NO depletion reduces it only to a limited extent. In addition, the absent effect of L-NAME at day 21.5 may be due to the low incidence of proliferating decidual cells as described above or due to changing role of NO near the term of pregnancy (Maul *et al*., 2003).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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