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Involvement of hypoxia and inflammation in early pregnancy loss mediated by Shiga toxin type 2

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ABSTRACT

Introduction: Symptomatic or asymptomatic Shiga toxin producing *Escherichia coli* (STEC) infections during early pregnancy may cause maternal or fetal damage mediated by Shiga toxin type 2 (Stx2). The aim of this study is to elucidate the mechanisms responsible for early pregnancy loss in rats treated with Stx2.
Methods: Sprague Dawley pregnant rats were intraperitoneally injected at day 8 of gestation with a sublethal dose (0.5 ng of Stx2/g of total body weight, 250 μl) of purified Stx2. Control rats were injected with the same volume of PBS. The expression of globotriaosylceramide (Gb3) glycosphingolipid receptor for Stx2 was evaluated by thin-layer chromatography (TLC). Regions of hypoxia in decidual tissue were determined by pimonidazole immunohistochemistry and vascular endothelial growth factor (VEGF) expression by Western blot and immunohistochemistry. Tumor necrosis factor-alpha (TNF-α) levels in serum and decidual tissue were evaluated by ELISA. Serum progesterone levels were determined by RIA.
Results: Decidual tissue from both, control and Stx2-treated rats showed similar expression of Gb3 receptor. Intrauterine growth restriction was observed in Stx2-treated rats, associated with hypoxia and an increase of decidual TNF-α levels. Decrease of serum progesterone levels and decidual VEGF expression were also demonstrated.
Discussion: Our findings indicate that Stx2 reaches the uteroplacental unit, binds Gb3 and triggers damage in decidual tissue. Poor oxygen supply accompanied with damage in the uteroplacental unit and inflammation could be responsible for the early pregnancy loss. Decrease in the pregnancy protective factors, serum progesterone and local VEGF, may contribute to the pregnancy loss.

1. Introduction

Spontaneous abortion occurs in 15–20% of all recognized pregnancies and is the most common adverse pregnancy outcome in humans [1]. Maternal infections during pregnancy have been associated with higher incidence of spontaneous abortion [2]. However, the precise cause of fetal loss in most instances is unknown. Previous reports support the hypothesis that symptomatic or asymptomatic Shiga toxin producing *Escherichia coli* (STEC) infections during pregnancy may cause maternal or fetal damage mediated by Shiga toxin type 2 (Stx2) [3–5].

Stx2 is a member of the AB_5 family of bacterial toxins. The A subunit (StxA) exerts N-glycosidase activity on the 28S rRNA of 60S ribosomes in the cytosol, resulting in inhibition of protein synthesis in eukaryotic cells and activation of proinflammatory signaling cascade referred as the ribotoxic stress response [6]. The five B subunits form a pentamer that binds to globotriaosylceramide (Gb3) glycosphingolipid receptor for Stx2 on the cell membrane of target cells, particularly endothelial cells present in kidneys, brain and other organs [7]. STEC express two types of Stx proteins (Stx1 and Stx2) and their variants, being Stx2 more virulent and epidemiologically more relevant than Stx1 [8]. Stx2 is responsible for the most serious consequence of STEC infection known as Hemolytic

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Uremic Syndrome (HUS) characterized by hemolytic anemia, thrombocytopenia and acute renal failure [9].

STEC infection is mostly seen in young children including neonates [10,11] although the outbreak in 2011 in central Europe caused by Stx2-producing STEC affected more adults than children, and women were overrepresented [12–14]. To our knowledge, there are no reports of Stx2 effects during human pregnancy or described complications in the early pregnancy associated to STEC infection. However, several cases of Hemolytic Uremic Syndrome (HUS) after STEC infections in post partum women and neonate have been reported [10,15,16].

We have previously demonstrated that Stx2 intraperitoneally (i.p.) injected in rats in the early stage of pregnancy after the event of implantation, causes spontaneous abortion by a direct cytotoxic effect of Stx2 in the highly perfused uteroplacental unit [5]. The purpose of this study was to elucidate the mechanisms responsible for early pregnancy loss mediated by Stx2.

2. Materials and methods

2.1. Drugs and chemicals

Purified Stx2 was purchased from Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA and it was checked for lipopolysaccharide (LPS) contamination by *Limulus amoebocyte* lysate assay (Biowhittaker Inc. Maryland, USA). Toxin was diluted with sterile PBS before injection. Stx2 contained <10 pg LPS/ng of pure Stx2. Gb3 standard was purchase from Matreya (Pleasant Gap, PA, USA). Hypoxia marker, pimonidazole hydrochloride and mouse antibody anti-pimonidazole (Hypoxiprobe-1) were supplied by Natural Pharmacia International, Inc. (Burlington, MA, USA). Reagents for thin-layer chromatograph (TLC), immunohistochemistry and Western blot were provided by Sigma–Aldrich Co (St Louis. MI, USA). Progesterone antibody for RIA was provided by G.D. Nishwender (Colorado State University, Fort Colling, CO, USA).

2.2. Animals

Timed pregnant rats were obtained as described previously [5]. Briefly mating was performed placing female rats (Sprague–Dawley; 200–280 g; 2–3 months of age, acquired from the animal facility of the School of Pharmacy and Biochemistry) in the cages of the male rats for several days. Day 1 of gestation was determined when sperm was observed in the vaginal smear. Animals received food and water *ad libitum* and were housed under controlled conditions of light (12-h light, 12-h dark) and temperature (23–25 °C).

2.3. Experimental protocol

Sprague Dawley pregnant rats were intraperitoneally (i.p.) injected on gestation day 8 (gd 8) with a sublethal dose (0.5 ng of Stx2/g of total body weight (bwt), 250 μl) of purified Stx2. Control rats were injected with the same volume of phosphate-buffered saline (PBS). The rats were individually housed under controlled conditions of illumination, humidity, and temperature, with food and water being available *ad libitum*. Different groups of experimental and control rats were euthanized after treatment to obtain blood and uteroplacental units (flow chart Fig. 1). Two or three uteroplacental units were processed from each rat of control or Stx2-treated group when appropriate. The experiments were repeated at least twice.

2.4. Ethic statement

The experimental procedures reported here were carried out in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved for the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine, University of Buenos Aires (CICUAL, Permit Number 1494/2013).

2.5. Thin-layer chromatography

Decidual tissue (100 mg) from Stx2 and control rats 4 days after treatment, were homogenized on ice in an appropriate buffer and mixed with chloroform/methanol (2:1 ratio), incubated on ice for 15 min and centrifuged at 3000 rpm for 5 min to separate phases. The aqueous phase was discarded and the organic phase with extracted lipids was allowed to air-dry. One ml of methanol and 0.1 ml of 1.0 M NaOH were added to the dried residue and incubated 16 h at 37 °C. After the addition of 2 ml of chloroform and 0.5 ml water and separation of the phases, the upper phase was removed. The lower phase, corresponding to glycolipid extract, was brought to dryness. Fractionated lipids were subjected to TLC with a silica gel 60 aluminum plate previously activated by incubation 15 min at 100 °C, in a glass tank with a mixture of chloroform/methanol/water (65:35:8). A purified Gb3 standard was added to the plate for comparison. After the solvent front reached the top of the plate, the silica plate was air dried and treated with a solution of orcinol (Acros Organics, Morrys Plains, NJ, USA) to visualize the separated neutral glycolipid components.

2.6. Hypoxia marker and its administration

To assess hypoxia in the uteroplacental unit, immunohistochemical staining for pimonidazole was performed. Pimonidazole is a compound that selectively binds thiol groups in proteins of cells exposed to hypoxia (pO_2 < 10 mm Hg). Protein adducts of reductively activated pimonidazole are stable and can be detected immunohistochemically in paraffin sections.

Control and experimental rats at 2 days post Stx2 treatment (gd 10) were i.p. injected with pimonidazole hydrochloride (60 mg/kg) 90 min before euthanasia. Uteroplacental units were then extracted from the mothers, fixed with formalin 10% in PBS at room temperature, dehydrated, longitudinally dissected and embedded in paraffin for immunohistochemistry.

2.7. Utero-fetoplacental measurement

Uteri from Stx2 treated rats at 4 days post injection were extracted and photographed. Images were analyzed by Image-Pro Plus 5.0 software to estimate and compare uteroplacental unit sizes. Longitudinal and transversal size measurements of every uteroplacental unit were determined. Volume was estimated considering an ellipsoid ($4/3 \cdot \pi \cdot R_1 \cdot R_2^2$) where R_1 is the longitudinal size and R_2 is the transversal size. The mean volume of utero-placental units from Stx2-treated rats and control rats were compared. The experiment was repeated three times.

2.8. Immunohistochemistry

For hypoxia pimonidazole adducts, tissue sections, from Hypoxiprobe treated rats, of 5 μm thick were dewaxed and rehydrated in graded ethanol washes. Antigenic sites were retrieved by immersing slides in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 min at 95 °C. Slides were allowed to cool for 20 min at room temperature. Sections were then processed for immunohistochemistry with antibodies anti-pimonidazole adducts (Hyproxyprobe-1; 1:50). For Vascular endothelial growth factor (VEGF) detection, tissues from rats at 2 days after treatment were incubated with anti-VEGF (Abcam VG-1; 1:200). Primary antibody

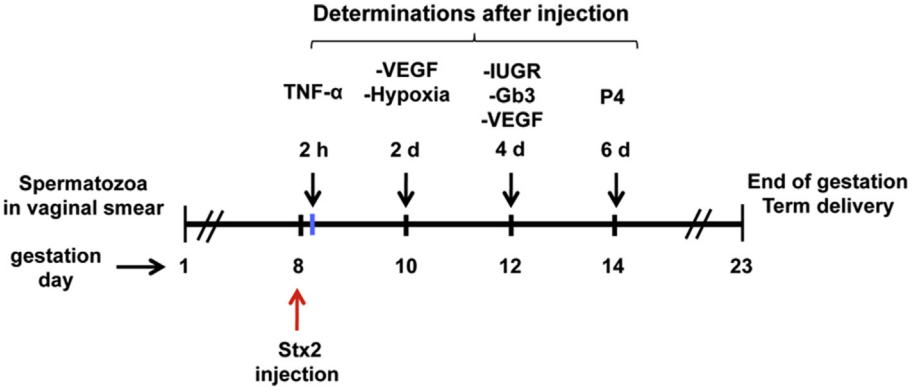

Fig. 1. Timeline indicating Stx2 treatment and sampling during pregnancy. The figure shows the timeline of pregnancy progression where is indicated when the experimental studies were performed.

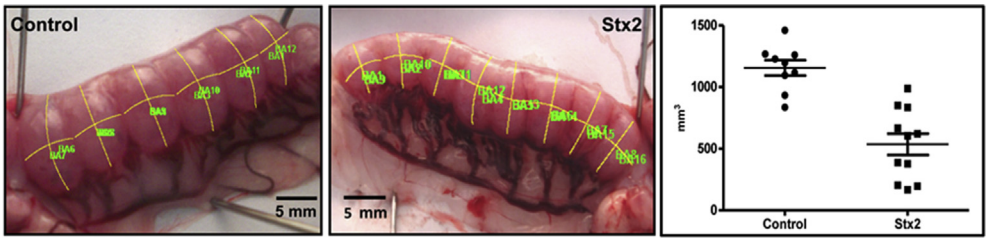

Fig. 2. Intrauterine growth restriction in Stx2 treated rats. Pregnant rats were intraperitoneally injected on gd 8 with 0.5 ng Stx2/g bwt (n = 4) or Control (n = 3) and sacrificed 4 days after treatment. Uteri were extracted and the volume of every uteroplacental unit was analyzed using Image-Pro Plus software. The experiment was repeated three times and analyzed 140 and 136 uteroplacental units for Stx2 and Control respectively that correspond to 11 rats for Stx2 and 9 rats for Control. Volume was estimated considering an ellipsoid $(4/3\pi \cdot R_1 \cdot R_2^2)$ where R_1 is the longitudinal size and R_2 is the transversal size. The uteroplacental unit volumes within a litter were averaged. The median volume from Stx2-treated rats was significantly smaller than controls (Mann Whitney test, $P < 0.0001$).

incubation was followed by addition of biotin-conjugated goat anti-mouse IgG antibody (GE Whole Ab Mouse IgG, RPN1177, 1:100). Staining was developed using streptavidin-conjugated peroxidase (Dako P0397 1:300) followed by diaminobenzidine hydrochloride (DAB, Sigma–Aldrich). Sections were lightly counterstained with hematoxylin and eosin and observed by light microscopy (Nikon Eclipse 200, NY, USA).

In the case of immunohistochemical detection (brown staining) of pimonidazole adducts, at least 3 images from the area of glycogenic wings of every slide (20×) were analyzed and then a global score was assigned per slide. Glycogenic wings are the regions, between the lateral sinusoids and antimesometrial decidua that contain cells with high glycogenic content and is commonly known as the lateral decidua [17]. Every image was blinded scored by two independent observers. A scale from 0 to 4 was used being 0 = no DAB staining; 1 = 0–25% of the image stained, 2 = 25–50% of the image stained, 3 = 50–75% of the image stained and 4 = more than 75% of the image stained.

2.9. Western blot analysis

Decidual tissues were dissected and homogenized on ice in an appropriate buffer with protease inhibitors and centrifuged at 7500 rpm for 10 min. Supernatants were separated and stored at −70 °C until western blotting was performed. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce Biotech. Inc, USA). Equal amounts of protein (100 μg) were loaded in each line. Samples were separated on 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel. Proteins were transferred to a PVDF membrane were incubated 18 h at 4 °C with anti-VEGF antibody (Abcam VG-1, 1:250). The blots were then incubated 1 h at room temperature with HRP-conjugated goat anti-mouse (Biotrak, 1:3000). Proteins were detected through ECL detection system. Specific bands were revealed with luminol staining. To determine the uniformity of loading, blots were probed with a monoclonal anti-β-actin antibody (1:4000). Band intensities were measured using the Quantity One densitometry software package (Bio-Rad Lab, USA). Protein bands were normalized to their respective b-actin bands.

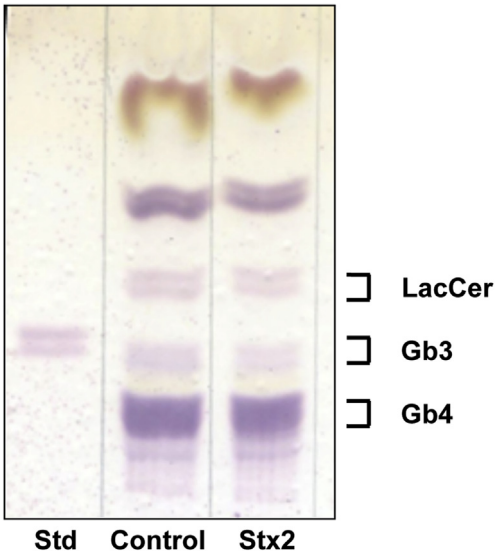

Fig. 3. Gb3 expression in decidua from Stx2 treated rats is similar to controls. Decidual tissues (3–4 deciduas, aprox. 100 mg) from Control and Stx2-treated rats (Stx2) at 4 days post injection were subjected to lipid extraction followed by TLC. Two bands located at the same distance of Gb3 standard (Std) revealed the expression of Gb3 in both control and Stx2-treated rats. In addition, LacCer, the precursor of Gb3 and Gb4, the immediate precursor of Gb3, were also similar. The experiment was repeated twice.

2.10. Determination of TNF-α level by enzyme immunoassay

Maternal serum and decidual tissue were collected from at least three dams at 2 h after treatment from rats non-treated (control) or treated with 0.5 g Stx2/g bwt. For TNF-α tissue determination, 100 mg of decidua were homogenized in 120 ul of PBS buffer containing 10% fetal bovine serum, and supplemented with 20 ul of a cocktail of protease inhibitors. Samples were cleared by centrifugation for 20 min at 13,000 rpm. TNF-α levels were estimated with enzyme linked immunosorbent assay (ELISA) kit (TNF [Mono/Mono] Set of BD Biosciences Pharmingen (San Diego, CA, USA), according to the manufacturer's instructions. All measurements were performed in duplicate. Protein concentration was determined with the BCA Protein Assay Kit (Pierce Biotechnology Inc, USA). Levels of systemic TNF-α were measured with the Biotrak rat TNF-α ELISA system according to the manufacturer's instructions.

2.11. Determination of progesterone levels by RIA

Progesterone (P4) levels were measured in serum extracted from rats treated with PBS (controls) or with 0.5 g Stx2/g bwt at 6 days after treatment. Blood was allowed to clot and was centrifuged at 655 g for 10 min. Progesterone levels were determined by radioimmunoassay (RIA) using specific antibodies as previously described [18].

2.12. Statistical analysis

Statistical analysis was performed using the Graph Pad Prism Software 5.0 (San Diego, CA, USA). Differences between Stx2-treated and control rats were analyzed by Mann Whitney-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Intrauterine growth restriction in Stx2 treated rats

Pregnant rats were intraperitoneally injected on gd 8 with 0.5 ng Stx2/g of total body weight (Stx2) or PBS (Control) and sacrificed 4 days post injection. Uteroplacental units from Stx2-treated rats were significantly smaller than those from controls (Fig. 2).

3.2. Gb3 expression in decidual cells is not affected by Stx2 treatment

We have previously demonstrated histological damage in decidual tissues at 4 days after Stx2 treatment [5] and since Gb3 was demonstrated to be upregulated by Stx2 [19], we assess its expression by TLC. Two bands located at the same distance of Gb3 standard revealed the expression of Gb3 in decidual tissue from Stx2-treated rats (Fig. 3). Based on glycolipid mobility [20], Gb3 and the other neutral glycolipid comprising lactosylceramide (LacCer,

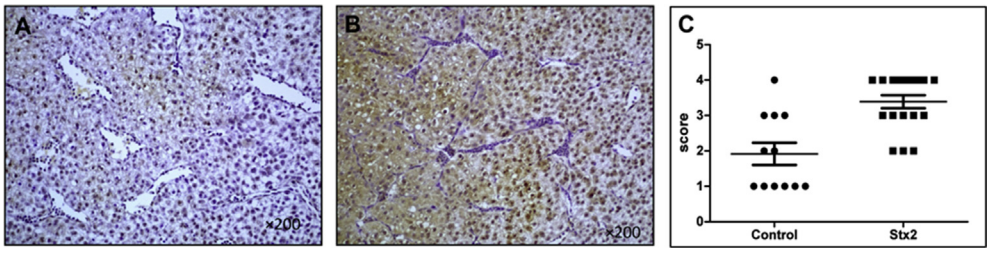

Fig. 4. Stx2 causes hypoxia in the uteroplacental units of pregnant rats. Pregnant rats were intraperitoneally injected on gd 8 with 0.5 ng Stx2/g bwt (n = 4) or PBS (n = 4) 2 days before sacrifice. Ninety minutes before euthanasia, all rats were intraperitoneally injected with pimonidazole (60 mg/kg), a compound used to detect regions of hypoxia in tissues. Immunohistochemical detection (brown staining) of pimonidazole adducts in decidual tissue, particularly in the glycogenic wings of the implantation sites in Control (A) and Stx2 (B) was observed. Blinding scoring of hypoxia regions (C) was performed with a scale from 0 to 4 being 0 = no DAB staining and 4 more than 75% of the image stained. The experiment was repeated twice and analyzed 12 and 16 uteroplacental units for Stx2 and Control respectively. Significance ($P < 0.005$) between Control and Stx2 was determined by Mann Whitney test.

the precursor of Gb3) and globotetraosylceramide (Gb4, to which Gb3 is the immediate precursor) in decidual tissue isolated from control and Stx2-treated rats were similar expression (Fig. 3).

3.3. Stx2 causes hypoxia in the implantation sites of pregnant rats

Pimonidazole immunostaining was used to detect hypoxia in the uteroplacental unit from Stx2-treated rats at 2 days after treatment. Areas of uteroplacental unit, particularly in the glycogenic wings of the implantation sites exhibited a stronger staining on decidual tissue isolated from Stx2-treated rats (Fig. 4B) compared with deciduas from control rats (Fig. 4A). The immunopathology score from the area of glycogenic wings, showed a significant difference in hypoxia levels from Stx2-treated rats compared with the controls (Fig. 4C).

3.4. VEGF expression in decidual tissue decreases after maternal Stx2 exposure

VEGF immunoreactivity was detected in decidual tissues from control (Fig. 5A) and Stx2-treated rats at 2 days after treatment (Fig. 5B). Because VEGF could be increased as a protective factor, after vascular injury caused by Stx2, Western blot analysis were performed (Fig. 6). Two distinct bands of 24 kDa and 45 kDa in decidua tissues from controls and Stx2-treated rats were revealed after 2 days (A) and 4 days (B) after treatment. Although the relative optical densities of the bands showed a decrease after 2 days of Stx2 treatment (C), the decline in VEGF optical density only reached statistical significance after 4 days of treatment (D).

3.5. TNF-α level decreases in decidual tissue after maternal Stx2 exposure

TNF-α is one of the main cytokines that mediates the inflammatory response to Stx2 and its deregulation, either systemic or locally, could contribute to the changes observed during the development of pregnancy. To test this hypothesis, TNF-α levels in serum samples and decidual tissue from Stx2-treated rats were determined by ELISA assay. Results showed a significant increase in TNF-α levels in deciduas isolated from Stx2-treated rats 2 h after treatment compared with deciduas isolated from controls (Fig. 7A). No significant differences with the controls were detected in serum TNF-α levels in pregnant rats 2 h after Stx2 treatment (Fig. 7B).

3.6. Maternal progesterone level decreases in Stx2-treated rats

Previous results showed that Stx2 administered to pregnant rats on gd 8 produces 100% of fetal resorption 6 days after treatment [5]. Considering that there is a close relationship between the amount of circulating P4 and ongoing pregnancy, we found a significant decrease in serum P4 levels 6 days after Stx2 treatment compared with the controls (58 ± 4 *vs* 120 ± 15 ng/ml serum, $P < 0.05$, $n = 4$).

4. Discussion

In previous studies, we showed significant morphological and histological damages in the uteroplacental unit as well as feto maternal resorptions in rats treated with sublethal Stx2 doses in the early stage of pregnancy. In addition, we found Stx2 binding to the microvasculature and decidual cells [5]. It is well known that Stx2 binds Gb3 receptors on the endothelial cells, internalizes

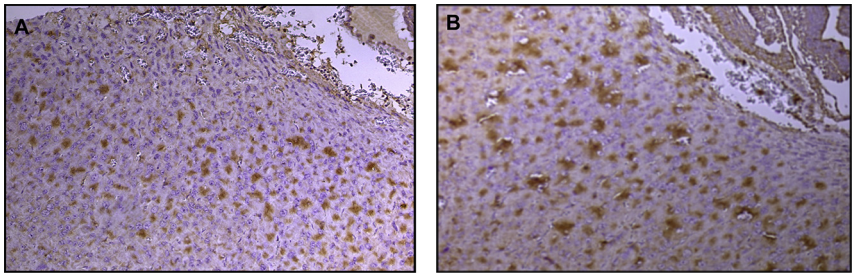

Fig. 5. Vascular endothelial growth factor (VEGF) expression in decidual tissue. Pregnant rats were intraperitoneally injected on gd 8 with PBS (A, n = 4) or 0.5 ng Stx2/g bwt (B, n = 4) and sacrificed at 2 days post injection. VEGF expression was detected in decidual tissue by immunohistochemistry. Two uteroplacental units were processed from each control and experimental rat. This assay was repeated twice. Original magnification: 200×.

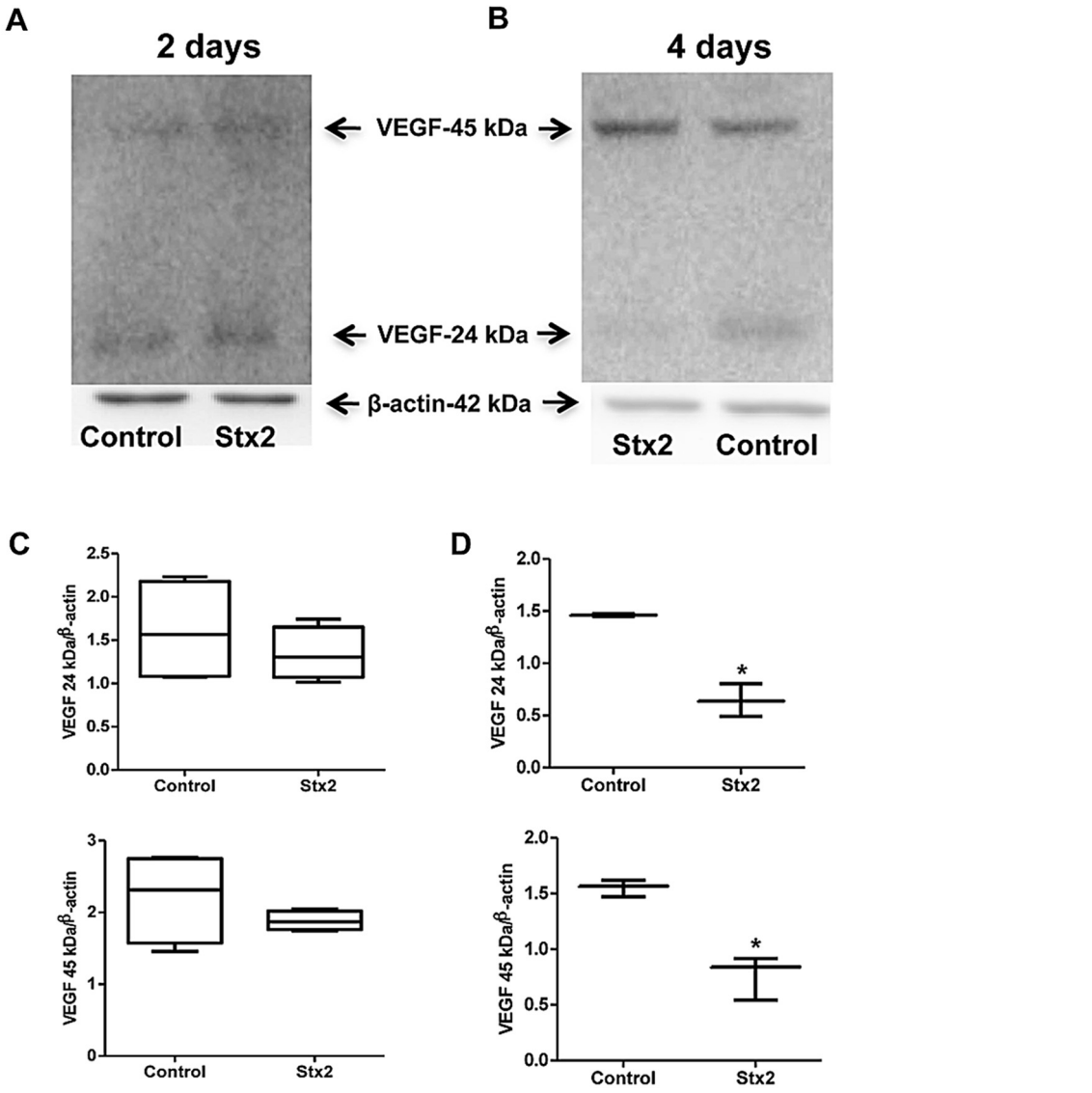

Fig. 6. Vascular endothelial growth factor (VEGF) expression in decidual tissue decreases after maternal Stx2 exposure. Pregnant rats were injected on gd 8 with 0.5 ng of Stx2/g bwt (n = 4) or PBS (n = 4) and killed 2 days (A) and 4 days (B) after injection. Decidual tissues were used to analyze VEGF by Western blots. Two or three uteroplacental units were processed from Stx2 and Control groups. The experiment was repeated twice. Two sizes of VEGF products (24 and 45 kDa) were detected in both experimental conditions. Relative intensity of VEGF bands (C and D) shows a significantly lower expression of VEGF at 4 days after Stx2 treatment compared to Control (D). Data are shown as median and range (min. to max. *P < 0.05).

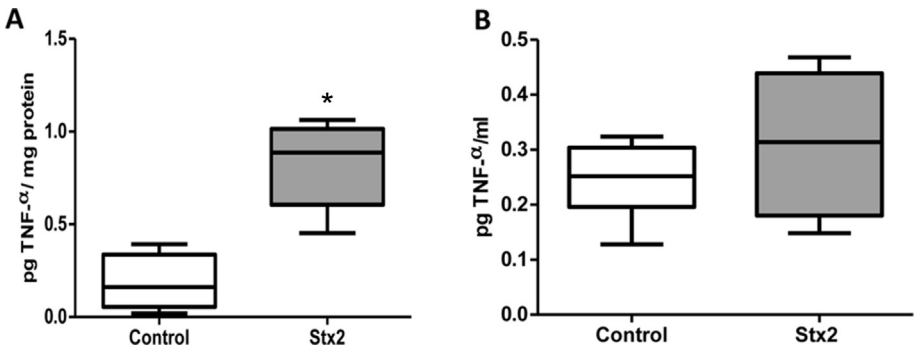

Fig. 7. TNF-α levels in serum and decidual tissue of pregnant rats. Pregnant rats were injected on gd 8 with 0.5 ng of Stx2/g bwt (n = 3) or PBS (n = 2) and killed 2 h post-injection. Decidual tissue (A) and serum samples (B) were used to determine amount of TNF-α by ELISA assay. For tissue analysis two or three uteroplacental units were processed from each of control and Stx2 treated group. Data are shown as median and range (min. to max.) from at least three independent experiments performed in triplicate (*P < 0.05).

following a retrograde transport to the Golgi apparatus and induces cell death by inhibition of protein synthesis and induction of cellular apoptosis [21]. In this study, we detected a similar glycolipid profile in decidual tissue of Stx2-treated rats compared with the controls. The expression of Gb3 in the uteroplacental unit from Stx2-treated rats indicates that the cell injury caused by Stx2 could be a Gb3-dependent process. Consistently, Stx2 can alter the maternal microvasculature and reduce blood flow and oxygen delivery to the fetus. In this study, we observed hypoxia in the glycogenic wings of the implantation sites and intrauterine growth restriction in Stx2-treated rats 4 days after treatment.

Several studies have revealed VEGF increased after vascular injury and a period of hypoxia support the hypothesis that VEGF has a role in repair endothelial damage [22]. However, in the present study, the amount of VEGF was significant reduced in decidual tissue after 4 days of Stx2 administration. The amount of VEGF was also reduced in kidneys from D + HUS patients [23] where endothelial damage in the renal microvasculature by Stx2 is considered a hallmark of the pathogenesis of this illness [24,25]. Much information is now available including microarray data, that show up or down-regulation of renal genes by Stx2 [26]. Therefore, it may be possible that Stx2 down-regulates the amount of VEGF in decidual tissue 4 days after Stx2 treatment. It may be also possible that Stx2 up-regulates TNF-α expression in the uteroplacental unit as occur in other target organs [27]. In this study, a significant increase of TNF-α levels in decidual tissue but not in maternal circulation was observed 2 h after Stx2 administration. We previously observed leukocyte infiltration in decidual tissue 4 days after Stx2 treatment, which could be the result of transmigration from the blood microvasculature to decidual tissue due to the endothelial damage caused by Stx2 [5]. Several authors have reported the importance of the inflammatory responses in the development of HUS induced by Stx2 [28,29] and we have previously reported an increase of maternal systemic TNF-α levels 2 h after Stx2 treatment in rats in the late stage of pregnancy suggesting that inflammation is mostly responsible for the preterm delivery in rats [30]. In this study, we consider that the direct cytotoxic effect of Stx2 in the uteroplacental unit is responsible for abortion. The local inflammatory response enhanced the cytotoxic action of Stx2 as occurs in other target organs [31] contributing to pregnancy loss. In this regard, Stx2 is able to cause the detrimental damage in the uteroplacental unit by necrosis and apoptosis and acts in concert with other bacterial endotoxins such as LPS to induce the production of an inflammation state rendering the cells more sensitive to the toxin [32,33].

Concerning P4, we found a significant decrease in serum P4 at 6 days after Stx2 treatment compared with control rats. In rodents, ovarian steroidogenesis is required to sustain pregnancy and recent studies suggest that, during early phases of pregnancy, local P4 synthesis in decidual tissue is important for successful implantation and/or maintenance of pregnancy [34]. Therefore, the detrimental effects of Stx2 on the uteroplacental unit may explain the fall of progesterone concomitant with pregnancy loss, although it should not be ruled out a possible effect of the toxin on the ovaries. The abortifacient effect of the Stx2 might also be due to sensitization of the uterus by inflammatory products such as nitric oxide or TNF-α followed by increasing uterine contractile activity as P4 levels fall. In this regard, it is notable that serum P4 falls to very low levels newly 6 days after toxin exposure. Whether this is a direct result of the inflammatory process (e.g., prostaglandin-induced luteolysis [35] or a secondary outcome related to a failing pregnancy is not known, but our results seem to support the conclusion that toxin exposure activate different pathways to miscarriage and P4 withdrawal could be a consequence of them.

In summary, our findings indicate that Stx2 reaches the uteroplacental unit where Gb3 is present and triggers damage in decidual tissue, hypoxia, intrauterine growth restriction and an increase of local TNF-α levels. Poor oxygen supply accompanied with damages in the uteroplacental tissue and inflammation could be responsible for the early pregnancy loss. Finally, the decrease in the pregnancy protective factors, serum progesterone and local VEGF, may contribute to the pregnancy loss.

Conflict of interest

There is not conflict of interest.

Acknowledgments

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