Role of TNF-α in the Mechanisms Responsible for Preterm Delivery Induced by Stx2 in Rats

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Summary

BACKGROUND AND PURPOSE. Shiga toxin-producing *Escherichia coli* (STEC) infections could be one of the causes of fetal morbimortality in pregnant women. We have previously reported that Shiga toxin type 2 (Stx2) causes preterm delivery in pregnant rats. In this study, we evaluate the role of tumor necrosis factor alpha (TNF-α), prostaglandins (PGs), and nitric oxide (NO) in the Stx2-induced preterm delivery.

EXPERIMENTAL APPROACH. Pregnant rats were treated with 0.7 ng Stx2/g of body weight and killed at different times after treatment. Placenta and decidua were used to analyze NO synthase (NOS) activity by conversion L-[\(^{14}\)C]arginine into L-[\(^{14}\)C]citruline, PGE2 and PGF2α by radioimmunoassay, and cyclooxygenases (COX) protein by Western blot. TNF-α level was analyzed in serum by ELISA and by L929 cytotoxicity. Aminoguanidine (AG, inducible NOS inhibitor), meloxicam (Melo, COX-2 inhibitor) and etanercept (ETA, competitive inhibitor of TNF-α) were used alone or combined to inhibit NO, PGs and TNF-α production respectively, to prevent Stx2-induced preterm delivery.

KEY RESULTS. Stx2 increased placental PGE2 and decidual PGF2α levels as well as COX-2 expression in both tissues. AG and Melo delayed the preterm delivery time but did not prevent it. ETA blocked the TNF-α increase after Stx2 treatment and reduced the preterm delivery by approximately 30%. The combined action of AG and ETA prevented Stx2-induced preterm delivery by roughly 70%.

CONCLUSIONS AND IMPLICATIONS. Our results demonstrate for the first time that the increase of TNF-α and NO produced by Stx2 are mostly responsible for the preterm delivery in rats.

Key words: Preterm delivery, Stx2 treatment, Nitric oxide, Prostaglandins, Tumor necrosis factor-alpha, Pregnant rats, Cyclooxygenases, nitric oxide synthase

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List of abbreviations:

AG: aminoguanidine

ANOVA: analysis of variance

bwt: body weight

Cyclooxygenases: COX

ETA: etanercept

Gb3: globotriaosylceramide

gd: gestation day

HUS: hemolytic uremic syndrome

IL: interleukins

iNOS: inducible NO synthase

i.p.: intraperitoneal

LB: Luria Broth

LPS: lipopolysaccharide

Melo: meloxicam

NO: nitric oxide

NOS: NO synthase

PBS: phosphate buffer solution
PGs: prostaglandins

PGE2: PGE-2

PGF2α: PGF-2α

PVDF: polyvinylidene difluoride

SEM: Standard error of measurement

STEC: Shiga toxin-producing Escherichia coli

Stx: Shiga toxin

Stx2: Shiga toxin type 2

TNF-α: tumor necrosis factor alpha
Introduction
Gastrointestinal infection with Shiga toxin (Stx)-producing *E. coli* (STEC) strains causes diarrhea and hemorrhagic colitis and, in addition, it is the leading cause of hemolytic uremic syndrome (HUS) (Richardson *et al*., 1988). HUS is characterized by a triad of hemolytic anemia, thrombocytopenia, and acute renal failure (Gianantonio *et al*., 1964; Repetto, 1997).

HUS is most commonly seen in young children but it is occasionally present in adults (Scully *et al*., 1995; Repetto, 1997; Frank *et al*., 2011), including post-partum women. The most postpartum cases had preceded upper respiratory or gastrointestinal symptoms (Strauss *et al*., 1976; Steele *et al*., 1984) and at least in one case, HUS was detected after a preterm delivery at 32 weeks (Steele *et al*., 1984). However, to our knowledge, an increased risk of preterm delivery linked to STEC infection has not yet been evaluated.

HUS is a systemic complication attributed to the expression of Stx (Karmali *et al*., 1983). Two types of Stx may be produced by STEC strains: Stx1 and/or Stx2 with their variants (Paton and Paton, 1998). In Argentina, STEC strain O157:H7 producing only Stx2 is highly prevalent (Rivas *et al*., 2006). Stx is an AB5 holotoxin possessing a single A subunit in noncovalent association with five B subunits (Fraser *et al*., 1994). The B subunits form a pentameric ring and mediate toxin binding to the glycolipid receptor globotriaosylceramide (Gb3) expressed in vascular endothelial cells, podocytes, mesangial cells, and proximal tubule epithelial cells of the kidney (Lingwood *et al*., 1987; Lingwood, 1996). The A-subunit is an N-glycosidase, which removes an adenine of the 28S ribosomal RNA resulting in cell death (Endo *et al*., 1988).

We have previously reported that intraperitoneal (i.p.) administration of Stx2 in the late stage of pregnancy produces preterm delivery of dead fetuses (Burdet *et al*., 2009). An
overproduction of nitric oxide (NO) and damage in placenta prevented by aminoguanidine (AG), an inducible NO synthase (iNOS) inhibitor, demonstrated that NO plays an important role in placental toxicity and fetal mortality induced by Stx2 (Burdet et al., 2010).

During inflammation, prostaglandins (PGs) are released simultaneously with NO and their overproduction could be detrimental. PGs promote uterine contractions contributing to embryonic expulsion (Aisemberg et al., 2007). Stx may act in concert with bacterial lipopolysaccharide (LPS) and induce the production of tumor necrosis factor alpha (TNF-α) and interleukins (IL) as IL-1 and IL-6 by macrophages rendering the vascular endothelial cells more sensitive to the toxin (Tesh et al., 1994; Louise et al., 1991). Furthermore, high concentrations of many cytokines in the vaginal or cervical secretions including TNF-α and IL-6 in women with symptoms of preterm labor are associated with early preterm delivery (Inglis et al., 1994).

These observations led us to investigate whether PGs and TNF-α could be involved in Stx2-induced preterm delivery in rats.
Materials and Methods

Drugs and Chemicals

L-[\textsuperscript{14}C]Arginine (specific activity 360 mCi/mmol), [5,6,8,11,12,14,15-\textsuperscript{3}H(N)]-PGE\textsubscript{2} (specific activity 150 Ci/mmol), and [5,6,8,9,11,12,14,15-\textsuperscript{3}H(N)]-PGF\textsubscript{2\alpha} (specific activity 200 Ci/mmol) were from Perkin Elmer Life and Analytical Sciences, USA. ECL detection system, Biotrak rat TNF-\textalpha ELISA and actinomycin D were from GE Healthcare Life Sciences, USA. Meloxicam (Melo) was purchased from Boehringer Ingelheim, USA. AG, PGE-2 (PGE\textsubscript{2}) and PGF-2 alpha (PGF\textsubscript{2\alpha}) standards, monoclonal \textbeta-actin antibody, secondary antibodies, Luria-Bertani broth (LB), and ampicillin were from Sigma Chemical Corp. USA. ETA was from Wyeth Lab, UK. The Dowew AG50-X8 column (Na\textsuperscript{+} form), nitrocellulose membranes, and all other Western blot reagents were from Bio-Rad Lab, USA. Filters were from Millipore Corp, USA. Primary cyclooxygenase (COX) antibodies were from Cayman Chemical. The HEK-Blue LPS Detection Kit was from InvivoGen, USA. The BCA Protein Assay Kit was from Pierce Biotechnology Inc, USA. All other chemicals were analytical grade.

Animals

To obtain timed pregnant females, male and virgin female Sprague-Dawley rats between 250 and 300 g of body weight (bwt) were acquired from the animal facility of the School of Veterinary, University of Buenos Aires. Mating was performed placing the female rats in the cages of the male rats from the same strain for several days. Day 1 of gestation was determined when sperm was observed in the vaginal smear. The animals received food and water \textit{ad libitum} and were housed under controlled conditions of light (12-h light, 1-h dark) and temperature (23-25°C). This study was carried out in strict
accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved for the Committee for the Care and Use of Laboratory Animals of the University of Buenos Aires (CICUAL, Permit Number 1209–10).

Experimental Protocols

Pregnant rats on day 15 of gestation (gd) were randomly divided into groups of at least three rats each. The Stx2 injury was induced as previously described (Burdet et al., 2009). Briefly, Stx2-treated rats were injected (i.p.) with 0.5 ml of culture supernatant from recombinant *E. coli* containing 0.7 ng Stx2 and 50 pg LPS/g bwt. Control rats were inoculated with 0.5 ml of culture supernatant containing only LPS (50 pg LPS/g bwt). In separate experiments, control and Stx2-treated rats were injected (i.p.) with AG (100 µg/g bwt) 24 h before and 4 h after toxins injection; Melo (2 µg/g bwt) simultaneously to the toxins and 12, 24 or 36 h later; ETA (100 µg/g bwt) 6 h before toxins; a combination of AG and Melo; or AG and ETA according the protocol previously described for each one. Rats from the different experimental groups were observed daily to evaluate delivery time and fetal status. Some of them were anesthetized and killed by cervical dislocation at different times after treatment. Placenta and decidua tissues were removed to evaluate PGs synthesis, NO synthase (NOS) activity, COX-1 and COX-2 protein expression. Serum and amniotic fluid were obtained to measure TNF-α production.

Determination of Prostaglandins
Placenta and decidua were used to measure PGE$_2$ and PGF$_{2\alpha}$ levels as previously detailed (Ribeiro et al., 2005). Firstly, tissues were incubated for 1 h in Krebs-Ringer bicarbonate solution under a 5%-CO$_2$ atmosphere at 37°C. Afterwards, medium was acidified and PGs were extracted twice with ethyl acetate. PGs concentration was determined by radioimmunoassay. Sensitivity was 5–10 pg per tube and values are expressed as pg PGs per mg of protein.

**Determination of NOS activity**

NOS enzyme activity in placenta was quantified from the conversion of L-$^{14}$Carginine into L-$^{14}$Ccitrulline as described before (Burdet et al., 2010). In a few words, samples were weighed, homogenized and incubated with 10 µM L-$^{14}$C arginine (0.3 µCi). After 15 min, samples were centrifuged for 10 min at 10,000 g and applied to a Dowex AG50-X8 column. L-$^{14}$Ccitrulline was eluted and measured by liquid scintillation counting. Enzyme activity is reported as fmoles of L-$^{14}$Ccitrulline per mg of protein per 15 min.

**TNF-α bioassay and immunoassay**

TNF bioactivity in serum and amniotic fluid samples was assayed employing the TNF-sensitive cell line L929 (Shiau et al., 2001). In brief, $1 \times 10^4$ L929 cells per well were seeded into a 96well tissue culture plate and cultured in RPMI 1640 medium supplemented with 5 µg/ml streptomycin, 5 U/ml penicillin, and 10% fetal calf serum. The medium was replaced with 0.1 ml of medium alone, or with samples from Control or Stx2-treated rats. The L929 cells were grown in arrested-grown conditions (actinomycin D, 10 µg/ml). After 24 h incubation, the cells were washed with PBS and viability was
assayed by using the neutral red uptake technique (Goldstein et al., 2007). Bioactive TNF-α was expressed as percentage of L929 cell viability. Concentration of TNF-α was measured with the Biotrak rat TNF-α ELISA system according to the manufacturer’s instructions.

**Western Blot Analysis**

Tissues were processed according to the method explained before (Burdet et al., 2010). In short, placental and decidual tissues were fragmented and homogenized on ice in an appropriated buffer with protease inhibitors. Homogenates were pre-centrifuged at 2,500 g for 10 min at 4º C and the collected supernatants were additionally centrifuged at 7,800 g for 10 min at 8º C. The supernatants were collected and stored at -70º C until western blotting was performed. Protein concentration was determined with the BCA Protein Assay Kit. One hundred micrograms of protein were loaded in each line. Samples were separated on 10 % (w/v) sodium dodecyl sulphate-polyacrylamide gel by electrophoresis and transferred to a PVDF membrane. The blots were incubated 48 h at 4ºC with anti-COX-1 or anti-COX-2 rabbit polyclonal antibody diluted 1:250 in PBS. The membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit Ig G antibody (1:3000). Proteins were detected through ECL detection system. To determine the uniformity of loading, protein 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 β-actin bands.

**Statistics**
Statistical analysis was performed using the Graph Pad Prism Software (San Diego, CA, USA). Comparison between values of different groups was performed using one-way ANOVA. Significance was determined using Tukey’s multiple comparison test for unequal replicated or Student’s t-test. Fisher exact test was used to compare the term and preterm delivery under different treatments. Statistical significance was set at $p < 0.05$. 
RESULTS

Stx2 increases PGs levels in placenta and decidua

Taking into account that during inflammation PGs are released simultaneously with NO, we examined the PGE2 and PGF2α expression in placenta and decidua from rats treated with Stx2 alone or in the presence of AG. In placenta, PGE2 showed an increase 12 h after Stx2 injection (p < 0.01, n = 18) while PGF2α remained constant. The treatment with AG plus Stx2 caused a greater increase in PGE2 (p < 0.001, n = 18) than the increase detected only with Stx2 and also caused an increase in PGF2α (p < 0.01, n = 18) (Figure 1A). In decidua, PGE2 decreased and PGF2α increased (p < 0.05, n = 18) in Stx2-treated rats while both PGs were similar to the controls after AG plus Stx2 treatment (Figure 1B).

Stx2 stimulates the COX-2 expression in placenta and decidua

An increase in COX-2 protein expression was detected in placenta from rats treated with Stx2 alone or in the presence of AG (Figure 2A and C, p< 0.05, n= 6). The increase in COX-2 expression was also detected in decidua from Stx2-treated rats (p< 0.05, n= 6) although the expression was similar to the controls under AG treatment (Figure 2B and D). None of the applied treatments changed the expression level of COX-1 in placental and decidual tissues of Stx2-treated rats (data no shown). These results suggest that changes in the PGs protein levels caused by Stx2 are mediated by COX-2 and modulated by NO production.

AG and Melo delay the preterm delivery induced by Stx2
In order to determine whether the alterations in the PGs levels detected in placenta and decidua from Stx2-treated rats may be in part responsible for preterm delivery, Melo, a selective inhibitor of COX-2, was used. Table 1 shows that Melo did not prevent preterm delivery in Stx2-treated rats. One hundred percentage of the rats treated with Melo and Stx2 had premature delivery of dead fetuses while Control rats treated with Melo delivered normal live pups at term (Table 1, row 4 vs 3). Furthermore, we studied the ability of AG combined with Melo to prevent Stx2-induced preterm delivery. Previously, we have showed that AG administration did not prevent the premature delivery of dead fetuses (Table 1, row 6) (Burdet et al., 2010). Instead, co-administration of AG and Melo delayed preterm delivery around 2 days in 50% of the rats, 3 days in 17% of the rats and 4 days in 33% of the rats but did not prevent the preterm delivery of dead fetuses (Table 1, row 8). These results suggest that both NO and PGs are involved in the Stx2 action on pregnant rats, although their blockade was not enough to reverse the Stx2-induced preterm delivery.

**Stx2 induces deregulation of serum TNF-α production**

TNF-α is one of the main cytokines that mediates the inflammatory response to Stx2 and its deregulation, either systemic or locally, could be contributing to the changes observed during the development of pregnancy. To test this hypothesis, we determined the TNF-α levels in serum and amniotic fluid of Stx2-treated rats as well as its toxicity on TNF-sensitive cell line L929.

An increase in TNF-α protein level in serum of Stx2-treated rats was maximal at 2 h after Stx2 injection, after which levels returned to normal at 6 h and 12 h (Figure 3A). Consequently, the toxic activity of TNF-α showed a reduction in the viability of L929...
cells to approximately 47% at 2 h after Stx2 injection (Figure 3B). However, the TNF-α level did not change in the amniotic fluid of pregnant rats treated with Stx2 (data not shown).

The pretreatment with ETA was able to prevent the increase of TNF-α production in serum samples induced by Stx2 (Figure 4). ETA also prevented the significant increase in placental PGE₂ (p < 0.001, n = 18) and decidual PGF₂α (p < 0.001, n = 18) levels caused by Stx2 (Figure 5A and B). In contrast, no significant difference in placental NOS activity was found in rats treated with ETA + Stx2 compared with Stx2 alone. In both cases, a significant increase in NOS activity in placental tissues (p< 0.01, n = 6) was observed compared with the Control (Figure 6).

**ETA and AG prevent the preterm delivery induced by Stx2**

To determine whether the increase in TNF-α production and NOS activity triggers the Stx2 induced preterm delivery, we evaluated the action of ETA on the delivery time and fetal status of Stx2-treated rats. Administration of ETA 6 h before Stx2 injection prevented the preterm delivery by roughly 30% compared with their Controls (Table 1 row 10 vs 9). Furthermore, we evaluated the combined action of AG and ETA in Stx2-treated rats compared with Control rats. For the first time, we observed that the treatment with both inhibitors, AG plus ETA, significantly (p< 0.005, n = 10) prevented Stx2-induced preterm delivery by 70 % of the cases (Table 1, row 12). The pups of these rats were born alive and had normal development compared with their Controls (Table 1, row 11). These results suggest that the overproduction of TNF-α and NO is the major responsible of the preterm delivery of dead fetuses induced by Stx2.
Discussion

In this study, a significant increase in placental PGE$_2$ and decidual PGF$_{2\alpha}$ was found in Stx treated rats. Additionally, in both tissues COX-2 protein expression was stimulated by Stx2, although Melo, a specific inhibitor of COX-2, was unable to prevent placental toxicity and fetal mortality. Here, we found that production of PGs and expression of COX-2 protein were modulated by NOS activity in Stx2-treated rats. Thus, it is reasonable to infer that NO could elicit different effects on the progression of pregnancy, some directly related with its overproduction and others related with the regulation of PG production (Aisemberg et al., 2007). We reported before that AG prevented placental damages in Stx2-treated rats but did not prevent preterm delivery (Burdet et al., 2010). Here, the administration of AG together with Melo delayed preterm delivery but did not prevent it, which indicates that other factors are involved in the Stx2-induced preterm delivery. It is well known that Stx may act in concert with LPS to elicit cellular dysfunction (Louise and Obrig, 1992). Stx is able to bind to Gb3 receptors expressed on the membrane of monocytes/macrophages and leads to cellular activation and secretion of cytokines such as TNF-$\alpha$, IL-1$\beta$ and IL-8 that increase the endothelial susceptibility to Stx (Ibarra and Palermo, 2010). Many studies conducted in human and experimental animals have established that a correct balance of cytokines at the maternal-fetal interface is an essential requirement for proper placental development and therefore reproductive success (Peltier, 2003; Gravett et al., 2007). In pregnant rats, administration of TNF-$\alpha$ produced placental injury and fetal death similar to the effects observed after LPS exposure (Silen et al., 1989). In this study, we showed that a significant increase in serum TNF-$\alpha$ observed 2 h after Stx2 treatment may be responsible for preterm delivery. Pregnancy loss was triggered by abnormal
inflammation as reported by other researchers (Grigsby et al., 2003; Renaud et al., 2011). TNF-α in amniotic fluid was not detected suggesting that maternal more than fetal TNF-α exert its detrimental effects on pregnancy. Administration of ETA completely prevented the increase in serum TNF-α level caused by Stx2 as previously demonstrated in astrocytes treated with LPS and Stx1 (Landoni et al., 2010). Moreover, ETA prevented by 30% the preterm delivery caused by Stx2 and, although this is not significant, shows a tendency to the Stx2 effect prevention. Our results indicate that TNF-α may play a causal role in Stx2 pregnancy loss consistent with previous studies reported in human and animal models (Silver et al., 1994; Raghupathy et al., 2000). ETA also inhibited the induction of placental PGE₂ and decidual PGF₂α synthesis in Stx2-treated rats even in the presence of AG. These findings indicate that TNF-α stimulates PG production independent of NO production as it is described in the literature (Romero et al., 1988; Sato et al., 2003). ETA did not inhibit the increase of placental NOS activity caused by Stx2. Thus, it is reasonable to infer that Stx2-induced increase in NO production is not mediated by TNF-α. We assayed the combined action of AG and ETA on fetal status and delivery time in Stx2-treated rats. Co-administration of AG and ETA significantly prevented the preterm delivery caused by Stx2 by approximately 70% of the cases. The pups of these rats were similar in size and weight to those observed in the controls. These results indicate that the preterm delivery of dead fetuses induced by Stx2 is triggered by TNF-α and mediated by an increase in NOS production. Both placental PGE₂ and decidual PGF₂α increase secondarily to the increase in TNF-α and output of iNOS and contribute to the mechanisms that lead to the preterm delivery.
In conclusion, the model presented in this study is relevant because it shows that Stx2 increases TNF-α production, which renders the feto-maternal unit more susceptible to Stx2 through the stimulation of local PGs synthesis, which in turn may activate uterine contractions and cervical dilation. This proinflammatory environment could promote the contraction of the uterus and the expulsion of the fetuses. Additionally, Stx2 induces the overproduction of NO as a consequence of an increase in the levels of iNOS protein in placental tissues, which plays an important role in placental toxicity and fetal mortality.

It is important to notice the fact that the administration of AG and ETA partially prevented preterm delivery of dead fetuses induced by Stx2. This suggests that they could be used in the future as therapeutic agents in this pathological consequence of HUS.
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Conflicts of interest
None.
List of references


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Figure legends

Figure 1: Effects of Stx2 and AG on PGs production. Pregnant rats (gd 15) were treated with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 12 h post-treatment. Some animals were i.p. injected with AG (100 µg/g bwt) 24 h before and 4 h after toxin injection. PGE$_2$ and PGF$_{2α}$ levels were evaluated in placenta (A) and decidua (B) by RIA. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n = 18). *p < 0.05 vs Control, **p < 0.01 vs Control, ***p < 0.001 vs Control, ^p < 0.05 vs Stx2.

Figure 2: Effect of Stx2 and AG on COX-2 protein expression. Pregnant rats were injected with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 12 h post-treatment. Some animals were i.p. injected with AG (100 µg/g bwt) 24 h before and 4 h after toxin treatment. Placenta and deciduas were removed for Western blot analysis. To determine the uniformity of loading, blots were probed with a monoclonal anti-β-actin antibody. Representative gel for COX-2 protein expression in placenta (A) and decidua (B) and the corresponding densitometry of the bands (C and D, respectively) are showed. Values correspond to the mean of 2 different pools of 6 animals ± SEM. *p < 0.05 vs Control, ^p < 0.05 vs Stx2 + AG.

Figure 3: TNF-α production in pregnant rats. Pregnant rats were injected with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 0, 2, 6, 12 and 24 h post-injection. Serum samples were used to determine amount of TNF-α by ELISA assay (A) and TNF-α cytotoxic activity by viability assay in L929 cells (B). Data
corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n=6). *p < 0.05 vs Control.

Figure 4: Effects of ETA on TNF-α production. Pregnant rats were injected with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 2 h post-injection. Some animals were i.p. injected with ETA (100 µg/g bwt) 6 h before toxin treatment. Serum samples were used to determine amount of TNF-α by ELISA assay. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n=6). *p < 0.05 vs Control.

Figure 5: Effect of ETA and AG on PGs production. Pregnant rats (gd 15) were injected with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 12 h post-injection. Some animals were i.p. injected with ETA (100 µg/g) 6 h before toxin treatment. Others were treated with a combination of AG and ETA according the protocol described before for each one. PGE₂ and PGF₂α levels were evaluated in placenta (A) and decidua (B) by RIA. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n=18). a*p< 0.05 vs Control, b*p< 0.01 vs Control, c*p< 0.001 vs Control, d*p< 0.001 vs ETA control, ETA + Stx2 and AG + ETA + Stx2.

Figure 6: Effect of ETA on NOS activity. Pregnant rats (gd 15) were injected with 50 pg LPS/g bwt (Control) or with 0.7 ng Stx2 and 50 pg LPS/g bwt (Stx2) and killed 12 h post-injection. Some animals were treated with ETA (100 µg/g) 6 h before toxin treatment. NOS activity in placenta was quantified by measuring the conversion of L-[¹⁴C] arginine into L-[¹⁴C] citrulline. Data corresponding to at least two separate experiments are shown.
experiments are shown. Values are expressed as the mean ± SEM (n=6). **p< 0.01 vs Control.
Table 1: Effect of Melo, AG and ETA on delivery time and pups status

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<td>0</td>
<td>100</td>
<td>100/0</td>
<td></td>
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<tr>
<td>ETA + Stx2</td>
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<td>30</td>
<td>30</td>
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<td>10</td>
<td>0</td>
<td>30</td>
<td>30/70</td>
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<tr>
<td>AG + ETA Control</td>
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<tr>
<td>AG + ETA + Stx2</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>30</td>
<td>0</td>
<td>70</td>
<td>70/30</td>
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</table>

*Melo: 2 µg/g bwt, AG: 100 µg/g bwt, ETA: 100 µg/g bwt, *N= number of rats.

*<p<0.005 vs Control; †<p<0.005 vs Melo Control; ‡<p<0.005 vs AG Control; ††<p<0.005 vs AG + Melo Control; ‡‡<p<0.005 vs Stx2. *a,b,c,d,e Calculated by Fisher exact test.
Figure 1.tif
Figure 5.tif
Figure 6