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 Stx2induced 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

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 preceeded 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-[^{14}C]Arginine (specific activity 360 mCi/mmol), [5,6,8,11,12,14,15-^{3}H(N)]-PGE_2 (specific activity 150 Ci/mmol), and [5,6,8,9,11,12,14,15-^{3}H(N)]- $PGF_{2\alpha}$ (specific activity 200 Ci/mmol) were from Perkin Elmer Life and Analytical Sciences, USA. ECL detection system, Biotrak rat TNF-α ELISA and actinomycin D were from GE Healthcare Life Sciences, USA. Meloxicam (Melo) was purchased from Boehringer Ingelheim, USA. AG, PGE-2 (PGE_2) and PGF-2 alpha ($PGF_{2\alpha}$) standards, monoclonal β-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^+ form), nitrocellulose membranes, and all other Western blot reagents were from Bio-Rad Lab, USA. Filters were from Millipore Corp, USA. Primary cyclooxigenase (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 *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 with 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 measures 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%-CO2 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}C]arginine into L[^{14}C]citrulline 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}C]citrulline was eluted and measured by liquid scintillation counting. Enzyme activity is reported as fmoles of L-[^{14}C]citrulline 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 × 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.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, PGE_2 showed an increase 12 h after Stx2 injection ($p < 0.01$, $n = 18$) while $PGF_{2\alpha}$ remained constant. The treatment with AG plus Stx2 caused a greater increase in PGE_2 ($p < 0.001$, $n = 18$) than the increase detected only with Stx2 and also caused an increase in $PGF_{2\alpha}$ ($p < 0.01$, $n = 18$) (Figure 1A). In decidua, PGE_2 decreased and $PGF_{2\alpha}$ 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 no 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 PGE_2 ($p < 0.001$, $n = 18$) and decidual $PGF_{2\alpha}$ ($p < 0.001$, $n = 18$) 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-α, IL-1β 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-α 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-α 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 PGE_2 and decidual $PGF_{2\alpha}$ 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 PGE_2 and decidual $PGF_{2\alpha}$ 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

Aisemberg J, Vercelli C, Billi S, Ribeiro ML, Ogando D, Meiss R *et al*. (2007) Nitric oxide mediates prostaglandins' deleterious effect on lipopolysaccharide-triggered murine fetal resorption. Proc Natl Acad Sci USA 104: 7534–7539.

Burdet J, Zotta E, Cella M, Franchi AM, Ibarra C (2010). Role of Nitric Oxide in Shiga toxin-2induced premature delivery of dead fetuses in rats. PLoS One 5: e15127.

Burdet J, Zotta E, Franchi AM, Ibarra C (2009). Intraperitoneal administration of Shiga toxin type 2 in rats in the late stage of pregnancy produces premature delivery of dead fetuses. Placenta 30: 491–496.

Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K (1988). Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem 171: 45–50.

Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M *et al*. (2011). Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. N Engl J Med 365: 1771–1780.

Fraser ME, Chernaia MM, Kozlov YV, James MNG (1994). Crystal structure of the holotoxin from Shigella dysenteriae at 2.5 Å resolution. Nat Struct Biol 1: 59–64.

Gianantonio C, Vitacco M, Mendizharzu F, Rutty A (1964). The hemlytic-uremic syndrome. J. Pediatr 64: 478–491.

Goldstein J, Loidl CF, Pistone Creydt V, Boccoli J, Ibarra C (2007). Intracerebroventricular administration of Shiga toxin type 2 induces striatal neuronal death and glial alterations: An ultrastructural study. Brain Res 1161:106–115.

Gravett MG, Adams KM, Sadowsky DW, Grosvenor AR, Witkin SS, Axthelm MK *et al.* (2007). Immunomodulators plus antibiotics delay preterm delivery after experimental intraamniotic infection in a nonhuman primate model. Am J Obstet Gynecol 197: 518.e1–8.

Grigsby, PL, Hirst JJ, Scheerlinck JP, Phillips DJ, Jenkin G (2003). Fetal responses to maternal and intra-amniotic lipopolysaccharide administration in sheep. Biol Reprod 68: 1695–1702.

Ibarra C, Palermo M (2010). Host responses to pathogenic *Escherichia coli*. Cap 9 pp 122-141. In Pathogenic *Escherichia coli* in Latin America. 1st Edition. Betham Publ. Texas, USA. http://www.bentham.org/ebooks/9781608051922/index.htm.

Inglis SR, Jeremias J, Kuno K, Lescale K, Peeper Q, Chervenak FA *et al.* Detection of tumor necrosis factor-alpha, interleukin-6, and fetal fibronectin in the lower genital tract during pregnancy: relation to outcome. Am J Obstet Gynecol 1994;171:5-10

Karmali MA, Steele BT, Petric M, Lim C (1983). Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. Lancet 1: 619–620.

Landoni VI, de Campos-Nebel M, Schierloh P, Calatayud C, Fernandez GC, Lapponi ML, *et al.* (2010) Shiga toxin 1-induced inflammatory response in lipopolysaccharide-sensitized astrocytes is mediated by endogenous tumor necrosis factor alpha. Infect Immun 78: 1193–1201.

Lingwood CA (1996). Role of verotoxin receptors in pathogenesis. Trends Microbiol 4: 147– 153.

Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S, *et al.* (1987). Glycolipid binding of purified and recombinant *Escherichia coli*-produced verotoxin *in vitro*. J Biol Chem 262: 8834–8839.

Louise CB, Obrig TG (1991). Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1 beta, and tumor necrosis factor alpha on human vascular endothelial cells *in vitro*. Infect Immun 59: 4173–4179.

Louise CB, Obrig TG (1992). Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells *in vitro*. Infect Immun 60: 1536–1543.

Paton JC, Paton AW (1998). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 11: 450–479.

Peltier MR (2003). Immunology of term and preterm labor. Reprod Biol Endocrinol 1:122.

Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R (2000). Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod 15: 713–718.

Renaud SJ, Cotechini T, Quirt JS, Macdonald-Goodfellow M, Othman M, Graham CH (2011). Spontaneous pregnancy loss mediated by abnormal maternal inflammation in rats is linked to deficiente uteroplacental perfusion. J Immunol 186: 1799-1808.

Repetto HA (1997). Epidemic hemolytic-uremic syndrome in children. Nephrology Forum. Kidney Int 52: 1708–1719.

Ribeiro ML, Aisemberg J, Billi S, Farina MG, Meiss R, McCann S, *et al*. (2005). Epidermal growth factor prevents prepartum luteolysis in the rat. Proc Natl Acad Sci USA. 102: 8048– 8053.

Richardson SE, Karmali MA, Becker LE, Smith CR (1988). The histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing Escherichia coli. Human Pathology, 19: 1102-1110.

Rivas M, Miliwebsky E, Chinen I, Deza N, Leotta G (2006). The epidemiology of hemolytic uremic syndrome in Argentina. Diagnosis of the etiologic agent, reservoirs and routes of transmission. Medicina (B Aires) 66: 27–32.

Romero R, Mazor M, Wu YK, Sirtori M, Oyarzum E, Mitchell MD, *et al.* (1988). Infection in the pathogenesis of preterm labor. Semin Perinatol 12: 262–279.

Sato TA, Keelan, JA, Mitchell MD (2003). Critical Paracrine Interactions Between TNF-α and IL-10 regulate pipopolysaccharide-stimulated human choriodecidual cytokine and prostaglandin E2 production. *J Immunol* 170:158–166.

Scully RE, Mark EJ, McNeely WF, Ebeling SH, Phillips LD (1995). Case records of the Massachusetts General Hospital. N Engl J Med 336: 1587–1594.

Shiau MY, Chiou HL, Lee YL, Kuo TM, Chang YH (2001). Establishment of a consistent L929 bioassay system for TNF-alpha quantitation to evaluate the effect of lipopolysaccharide, phytomitogens and cytodifferentiation agents on cytotoxicity of TNF-alpha secreted by adherent human mononuclear cells. Mediators Inflamm 10: 199–208.

Silen ML, Firpo A, Morgello S, Lowry SF, Francus T (1989). Interleukin-1 alpha and tumor necrosis factor alpha cause placental injury in the rat. Am J Pathol 135: 239-244.

Silver RM, Lohner WS, Daynes RA, Mitchell MD, Branch DW (1994). Lipopolysaccharide-induced fetal death: the role of tumor-necrosis factor alpha. Biol Reprod 50: 1108–1112.

Steele BT, Goldie J, Alexopoluou I, Shimizu A (1984). Post-partum haemolytic uraemicsyndrome and verotoxin-producing *Escherichia coli*. Lancet 1: 511.

Strauss RC, Alexander RW (1976). Post-partum hemolytic uremic syndrome. Obst Gynecol 47: 169–173.

Tesh VL, Ramegowda B, Samuel JE (1994). Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. Infect Immun 62: 5085–5094.

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 $PGF_{2\alpha}$ 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, ^{a}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-ß-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= 4). *p< 0.05 *vs* Control, $^{\#}$p< 0.05 vs ETA + Stx2.

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 killed12 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. PGE_2 and $PGF_{2\alpha}$ 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 killed12 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-[^{14}C] arginine into L-[^{14}C] citrulline. Data corresponding to at least two separate

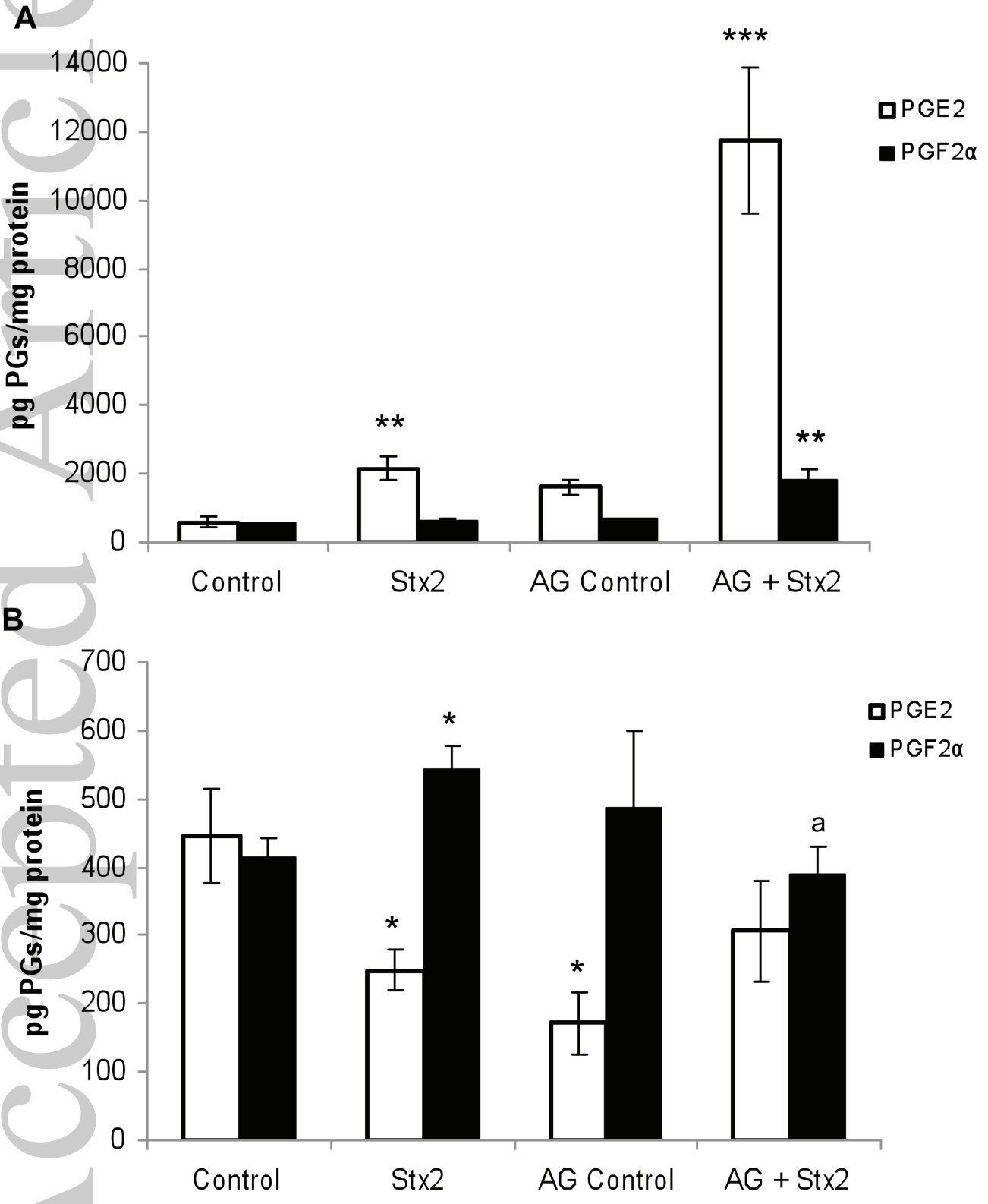
experiments are shown. Values are expressed as the mean ± SEM (n=6). **p< 0.01 *vs* Control.

Table 1: Effect of Melo, AG and ETA on delivery time and pups status

		Preterm delivery (%)						Term delivery (%)		Live/dead pups (%)
Treatment	***N**	**gd 16-17**	**gd 18**	**gd 19**	**gd 20**	**gd 21**		**gd 22**	**gd 23**	
Control	6	0	0	0	0	0		0	100	100/0
Stx2	8	100	0	0	0	0		0	0[a]	0/100
[†]Melo Control	6	0	0	0	0	0		0	100	100/0
[†]Melo + Stx2	8	100	0	0	0	0		0	0[b]	0/100
[#]AG Control	6	0	0	0	0	0		0	100	100/0
[#]AG + Stx2	6	100	0	0	0	0		0	0[c]	0/100
[#]AG+ [†]Melo Control	6	0	0	0	0	0		0	100	100/0
[#]AG+ [†]Melo + Stx2	6	0	0	50	17	33		0	0[d]	0/100
[¥]ETA Control	3	0	0	0	0	0		0	100	100/0
[¥]ETA + Stx2	10	30	30	0	0	10		0	30	30/70
[#]AG + [¥]ETA Control	3	0	0	0	0	0		0	100	100/0
[#]AG + [¥]ETA + Stx2	10	0	0	0	0	30		0	70[e]	70/30

[†]Melo: 2 µg/g bwt, [#]AG: 100 µg/g bwt, [¥]ETA: 100 µg/g bwt, *N= number of rats.

[a]p<0.005 *vs* Control; [b]p<0.005 *vs* Melo Control; [c]p<0.005 *vs* AG Control; [d]p<0.005 *vs* AG + Melo Control; [e]p<0.005 *vs* Stx2. [a,b,c,d,e]Calculated by Fisher exact test.



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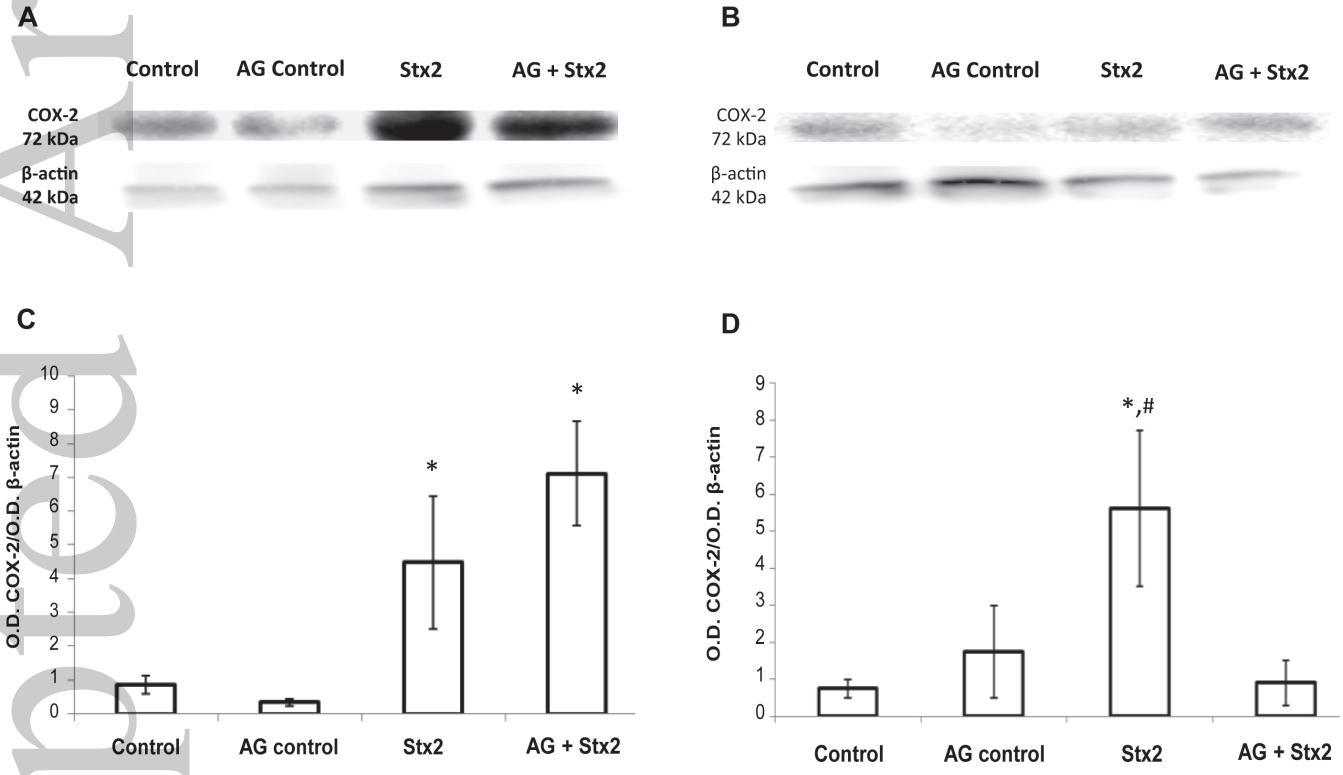

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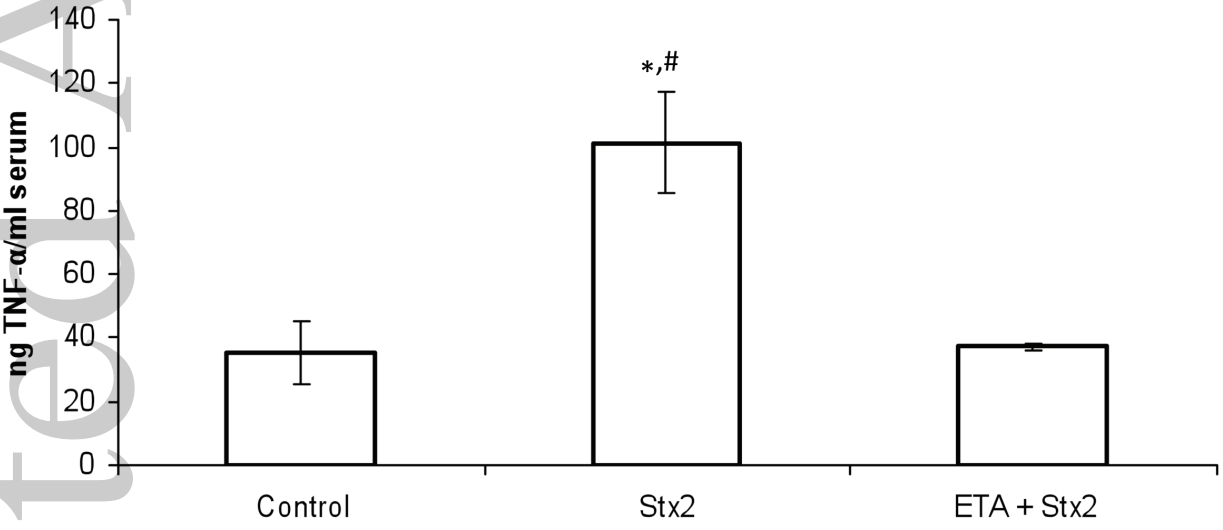

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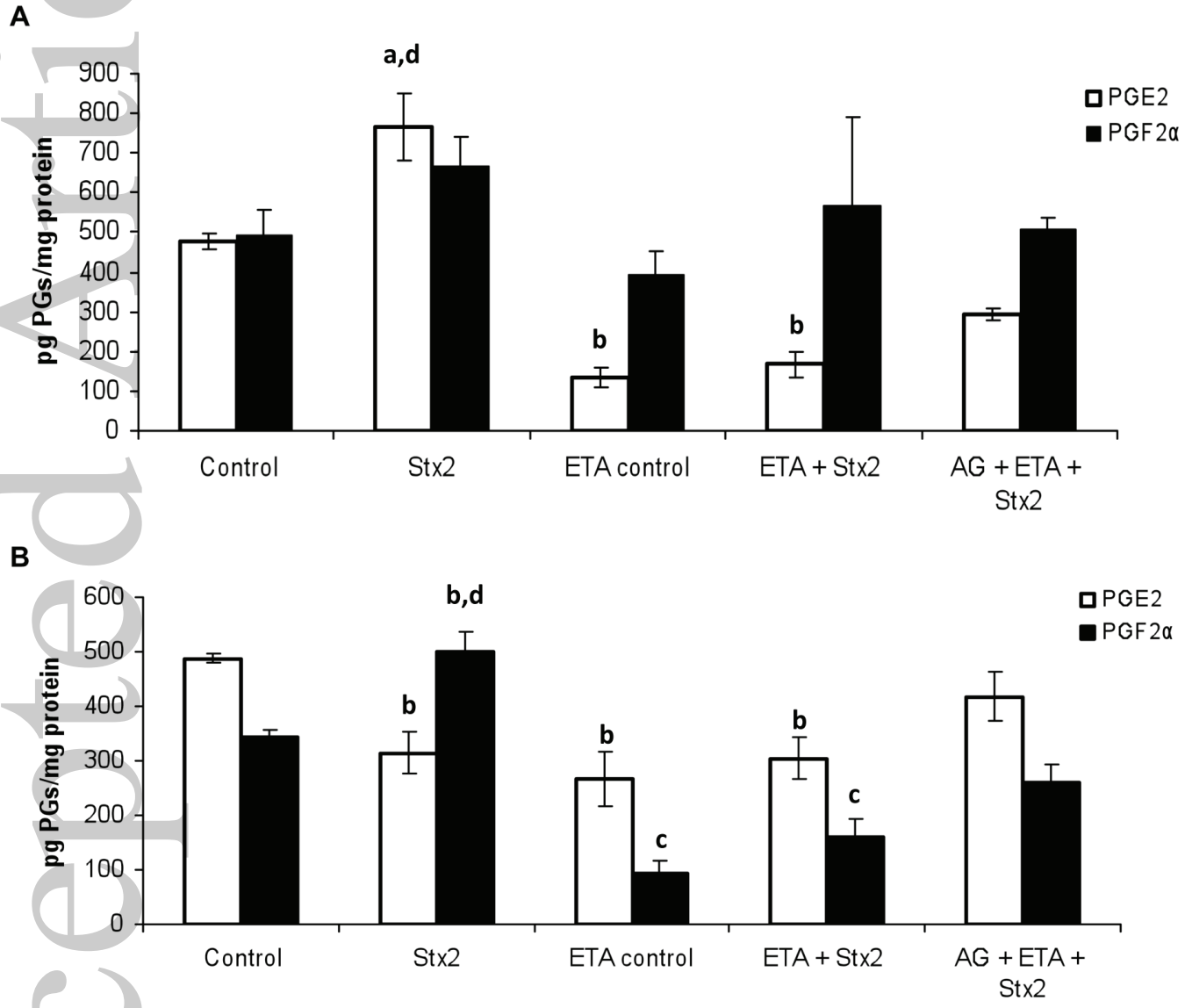

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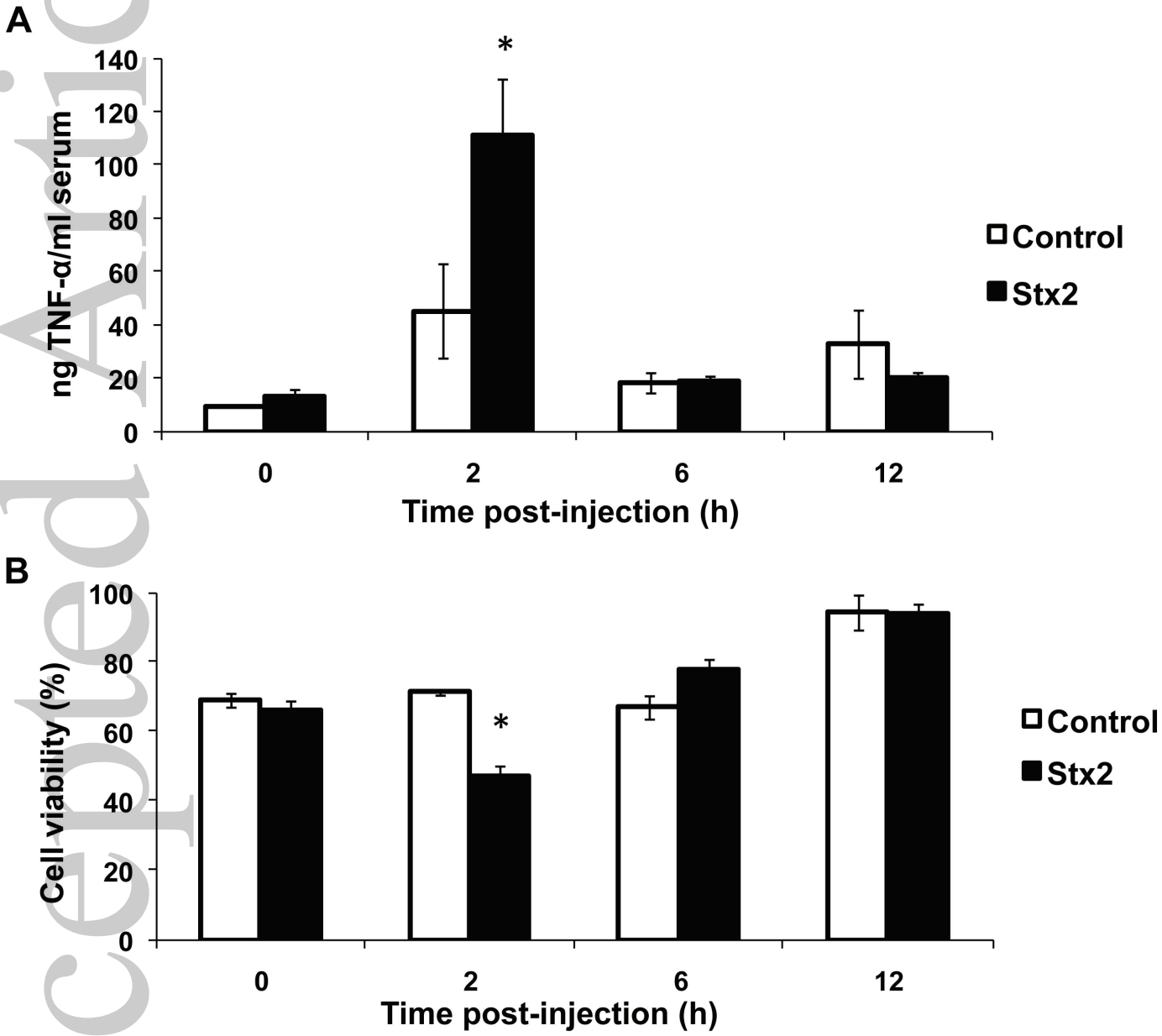

Figure_3.jpg

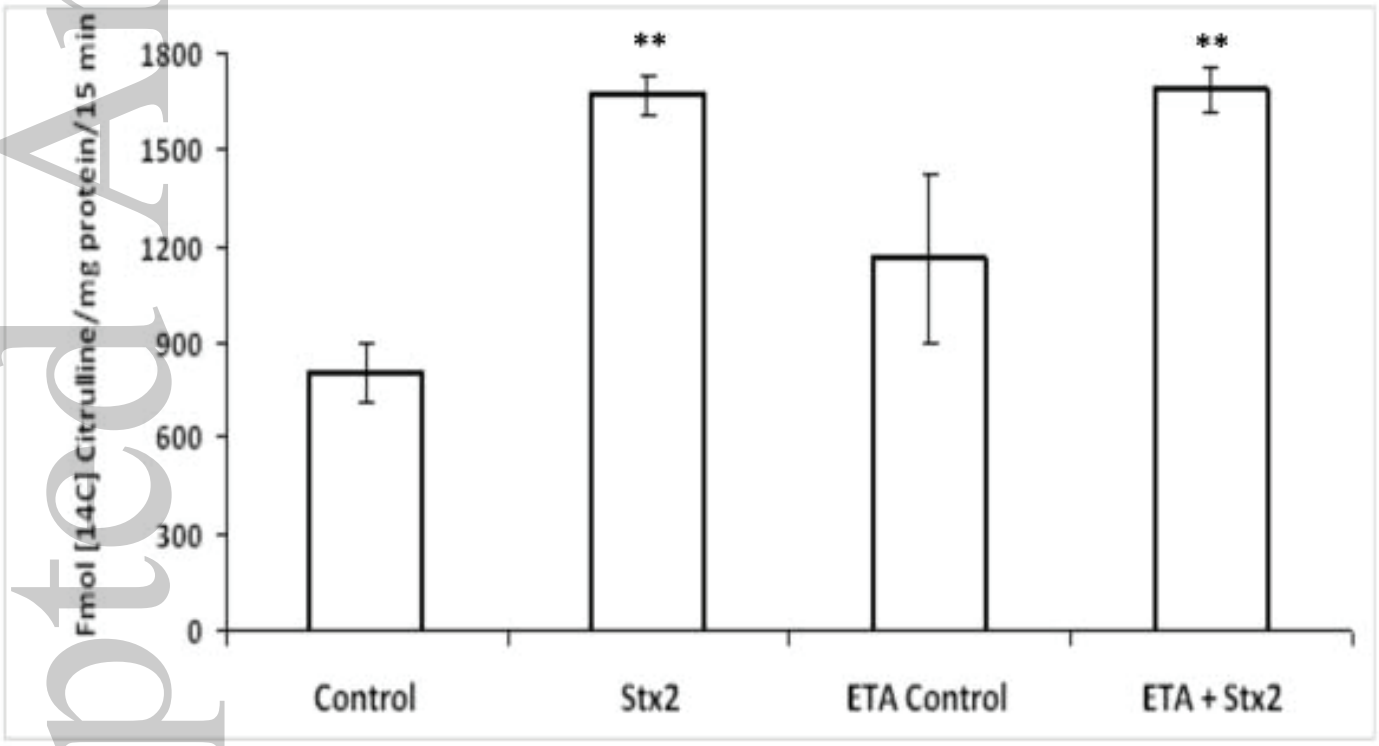

Figure_6.jpg