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# DEXAMETHASONE PREVENTS MOTOR DEFICITS AND NEUROVASCULAR DAMAGE PRODUCED BY SHIGA TOXIN 2 AND LIPOPOLYSACCHARIDE IN THE MOUSE STRIATUM

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**Abstract—Shiga toxin 2 (Stx2) from enterohemorrhagic *Escherichia coli* (EHEC) causes bloody diarrhea and Hemolytic Uremic Syndrome (HUS) that may derive to fatal neurological outcomes. Neurological abnormalities in the striatum are frequently observed in affected patients and in studies with animal models while motor disorders are usually associated with pyramidal and extra pyramidal systems. A translational murine model of encephalopathy was employed to demonstrate that systemic administration of a sublethal dose of Stx2 damaged the striatal microvasculature and astrocytes, increase the blood brain barrier permeability and caused neuronal degeneration. All these events were aggravated by lipopolysaccharide (LPS). The injury observed in the striatum coincided with locomotor behavioral alterations. The anti-inflammatory Dexamethasone resulted to prevent the observed neurologic and clinical signs, proving to be an effective drug. Therefore, the present work demonstrates that: (i) systemic sub-lethal Stx2 damages the striatal neurovascular unit as it succeeds to pass through the blood brain barrier. (ii) This damage is aggravated by the contribution of LPS which is also produced and secreted by EHEC, and (iii) the observed neurological alterations may be prevented by an anti-inflammatory treatment. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: neuronal damage, inflammation, reactive astrocytes, Blood–Brain Barrier, microvasculature, encephalopathy, Hemolytic Uremic Syndrome.**

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*Abbreviations:* CNS, central nervous system; EHEC, enterohemorrhagic *Escherichia coli*; GFAP, Glial fibrillary acidic protein; HUS, Hemolytic Uremic Syndrome; IOD, integral optical density; LPS, lipopolysaccharide; PBS, phosphate-buffered solution; STEC, Stx2-producing *Escherichia coli*; Stx2, Shiga toxin 2; VEGF, vascular endothelial growth factor.

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## INTRODUCTION

Shiga toxin from enterohemorrhagic *Escherichia coli* (EHEC) causes hemorrhagic colitis and Hemolytic Uremic Syndrome (HUS) (Karmali, 2004), a triad of events that includes thrombocytopenia, microangiopathic hemolytic anemia and acute renal failure (Proulx et al., 2001).

Outbreaks by EHEC intoxication frequently occur worldwide in north and south hemispheres (Rivas et al., 2003; Grisaru et al., 2011). In 2011 an infrequent outbreak of foodborne hemorrhagic colitis took place in Germany and spread in Europe. About 3816 patients were intoxicated with Shiga toxin 2 (Stx2)-producing *Escherichia coli* (STEC) O104:H4 (Frank et al., 2011). Among them 845 developed HUS and 54 died (Bielaszewska et al., 2011; Frank et al., 2011; Scheutz et al., 2011). It is noteworthy that Argentina possesses the highest occurrence of HUS in the planet, with approximately 420 new cases reported every year and an incidence of 17/100.000 under the age of five (Rivas et al., 2003).

It has been reported that approximately 42% of the patients with HUS progress to any central nervous system (CNS) dysfunction (Gianantonio et al., 1973; Bale et al., 1980; Karmali et al., 1985). This turns to be significant because mortality rate derived from HUS ranges between 0–5% of the cases, and 7–40% when the CNS is involved (Upadhyaya et al., 1980; Sheth et al., 1986; Hahn et al., 1989). Moreover, CNS dysfunctions without or concomitantly with HUS symptoms have been reported on 9–15% of the affected patients (Brasher and Siegler, 1981; Karmali et al., 1985). It can be inferred from this a more prevailing local deleterious action of the toxin into the CNS than a collateral injury from affected organs like the kidney. To test this, Stx2 has been locally administered in the brain and consequently neuronal damaged was observed (Goldstein et al., 2007), probably through a Gb3 neuronal receptor for Stx2. Our group has localized the neuronal Gb3 receptor in different brain areas, including the striatum, and found that the expression of this receptor increased following Stx2 treatment (Tironi-Farinati et al., 2010). The striatum has been identified as one of the most vulnerable regions in the intoxication with STEC in patients (Obata, 2010).

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Neurological symptoms in individuals include decerebrate posture, hemiparesis, ataxia, cranial nerve palsy, ophthalmological dysfunctions, hallucinations, seizures and changes in level of consciousness (from lethargy to coma) (Gianantonio et al., 1973; Cimolai et al., 1992; Hamano et al., 1993; Tapper et al., 1995). Similar symptoms were observed in mice that included lethargy, shivering, abnormal gait, hind limb paralysis, spasm-like seizure, reduced spontaneous motor activity, abnormal gait and pelvic elevation (Tironi-Farinati et al., 2013). Some of these symptoms are consistent with motor damage related to the pyramidal and extra pyramidal systems.

As it is known, EHEC not only secrete Stx2, but it also releases the outer membrane component lipopolysaccharide (LPS), an endotoxin that induces tissular production of a variety of inflammatory mediators when secreted in the gut. The LPS lipid A is the bioactive component of LPS, and its structure is highly conserved along *E. coli* strains. Moreover, the *E. coli* lipid A is a powerful activator of the innate immune system, and it contains two phosphate groups and six acyl chains composed by 12 or 14 carbons which is related with the infectivity of bacteria. In contrast, the lipid A of other bacteria out of *E. coli* contains four acyl chains and it does not activate the immune system (Alexander and Rietschel, 2001).

Accordingly, the injurious effects of these products have been reported *in vitro* and in various organs in individuals and in animal models (Zhang et al., 1997). However, few reports have investigated the deleterious contribution of LPS in EHEC-derived encephalopathies, a condition not fully determined in animal models nor considered in patients with HUS. In the present work an integrative study of the neuropathogenicity triggered by peripheral administration of sub-lethal Stx2 is carry out by immunofluorescence, physiological and behavioral means in the striatum of mice.

Therefore, the objective of this work was: (a) to study the contribution of LPS to pathogenicity in the neurovascular unit in striatal brain mice following systemic administration of a sub-lethal dose of Stx2, (b) to find a behavioral correlation respect the observed damage in the neurovascular unit, and (c) to determine whether these neurological alterations may be prevented by an anti-inflammatory treatment.

## EXPERIMENTAL PROCEDURES

### $LD_{50}$ determination

Purified Stx2 was purchased at Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA. The canonical Stx2 used was obtained from phage 933 W, named Stx2a (Plunkett et al., 1999). Stx2 was checked for LPS contamination by the limulus amoebocyte lysate assay. It contained $< 10$ pg LPS/ng of pure Stx2. The LPS used was from *E. coli* (L2880, Sigma, St. Louis, MO, USA). The amount of LPS used in this work was obtained from a measurement of Stx2 and LPS produced in the supernatant culture medium incubated with E. coli, and the ratio between Stx2: LPS yielded 1:800 (Goldstein et al., 2007).

The lethal effects of the Stx2 were characterized in mice ($n = 4$ per dose). As previously described (Tironi-Farinati et al., 2013), different amounts of Stx2 (5–0.44 ng per animal) or vehicle were administered intravenously (i.v.) to mice weighing about 20 g. Survival time was established when 100% of the animals survived at least 8 days after administration of Stx2. It was determined that the $LD_{50}$ was 1.6 ng of toxin per animal, and with 1 ng (approximately 60% if this dose), mice survived even for more than 10 days (time when all survived animals were sacrificed). This amount was thus considered sub-lethal and it was selected for use in this study.

### Dexamethasone survival assay

Six groups of mice ($n = 4$) were use to determine the protective effect of Dexamethasone against lethality by Stx2. Three groups of mice were endovenously treated with two lethal doses (LD) of Stx2 (3.2 ηg/mice), and the other three with vehicle saline solution (control). Two of these groups (one with two $LD_{50}$ of Stx2 and the other with saline solution) were treated with two intraperitoneal (i.p.) administrations per day of saline solution (100 µl per injection), another two groups were treated with i.p. 7.5 mg/kg of Dexamethasone per day (3.75 mg per injection, data not shown) and the last two with i.p. 15 mg/kg of Dexamethasone per day (7.5 mg per injection). The assay lasted ten days. The number of animal's death per group and the day of the deaths were monitored.

### Neurovascular toxicity and protection assay

Adult female Swiss mice (20 g) were housed in an air conditioned and light-controlled (lights on between 06:00 am and 06:00 pm) animal facility. Food and water were provided *ad libitum*. They were divided into four groups ($n = 8$) and subjected to the following i.v. treatments: LPS (800 ng); Stx2 (1 ng); LPS + Stx2 (1 ng Stx2 + 800 ng LPS); vehicle infusion (saline solution). Each animal received one i.v. dose in the lateral tail vein with 100 µl of solution. One half of the animals from each group ($n = 4$) were treated with 7.5 mg/kg i.p. Dexamethasone (100 µl per dose), twice a day for four days, while the other half of each group ($n = 4$) received 100 µl of i.p. saline solution, also twice a day for four days. All i.p. treatments (Dexamethasone or saline) started when they received their respective i.v. treatments (vehicle, LPS, Stx2 or Stx2 + LPS), and this day was referenced as day 0. All the animals were perfused on the fifth day (day of the first injection counted as day 1) as described in the following sentence.

Mice were anesthetized with pentobarbital (100 mg/kg) and perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS) [fixative per animal weight (ml/g)] after 4 days of the respective treatment. Brains were removed from the skull and post-fixed with the same fixative solution for 2 h, and cryoprotected through a daily sequenced passage of increasingly concentrated of sucrose solutions (10%, 20% and 30%). Brain coronal sections (20- µm-thick) were cut with a

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