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REVIEW ARTICLE

Development of a Mouse Model of Shiga Toxin 2 (Stx2) Intoxication for Testing Therapeutic Agents Against Hemolytic Uremic Syndrome (HUS)

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ARTICLE HISTORY

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Abstract: ***Background*:** Hemolytic Uremic Syndrome (HUS) caused by infections with Shiga toxin (Stx)-producing *E. coli* is a life-threatening complication characterized by acute renal failure, thrombocytopenia and hemolytic anemia. Stx is the main pathogenic factor. Therefore, the mouse model by intravenous administration of a single lethal dose of Stx is often used to explore its pathogenic mechanisms.

***Objective*:** The aim of this work was to develop an alternative mouse model of Stx type 2 (Stx2) intoxication to evaluate new therapeutic strategies.

***Methods and Results*:** One lethal dose of Stx2 was divided in four daily doses. We observed a dose-dependent toxicity characterized by neutrophilia, leukocytopenia and renal damage. Most importantly, we demonstrated that the polyclonal anti-Stx2 serum was able to protect mice from fatal evolution even when administered together the third dose of Stx2.

***Conclusion*:** This model would provide an advantage for evaluation of therapeutic strategies. Furthermore, the results presented herein suggest that appropriate treatment with anti-Stx2 agents following the appearance of initial clinical signs may block the ongoing outcome or may alleviate disease in patients who have just been diagnosed with HUS. However, the delay in the onset of therapy would be unsafe.

Maria Pilar Mejias

Keywords: Shiga toxin, mouse model, neutralizing antibodies, HUS, STEC, therapy.

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infections can cause illness with a wide spectrum of severity, from watery diarrhea and hemorrhagic colitis to hemolytic uremic syndrome (HUS), a life-threatening complication [1, 2]. Stx family members have an AB_5 structure [3]. The A subunit is the toxin's active component, and the five identical B monomers are the binding subunits to the specific receptor globotriaosylceramide (Gb3) on the host cells [4, 5]. Among the Stx family, Stx type 2 (Stx2) is the variant most related to HUS development [6].

Many animal models have been used to study STEC pathogenesis *in vivo*. These include the use of small animals, such as mice [7-10], rats [11] and rabbits [12], and in some cases, larger animals such as pigs [13], dogs [14], baboons and macaques [15, 16]. Models are divided mainly into two categories, those which evaluate the effects of Stx (in the absence of bacteria) and those that evaluate STEC infection. While each model can be used to study one or more components of STEC pathogenesis, no model that mimics the full spectrum of STEC-mediated disease in humans has been described up to date. Systemic distribution of Stx during STEC infection is the key and necessary event for the progression to HUS. Thus, mice intravenously injected with a high single dose of Stx develop systemic alterations that are characteristic of HUS patients, such as platelet activation, thrombocytopenia, neutrophilia, and acute epithelial and endothelial renal damage [17, 18]. This damage is irreversible and mice die between 72-96 hours post-intoxication. This model has also been used for testing new anti-Stx therapies, in spite of the low possibility to extrapolate conclusions to the human situation, in which Stx probably enters circulation gradually during several days.

The aim of the present study was to develop a mouse model that closer resembles the human situation, in order to test new therapeutic agents. Considering that sustained amounts of Stx would probably reach circulation in a period of several days during STEC infection, multiple sublethal and increasing doses of Stx2 were i.v. administered to mice for four consecutive days. The cumulative effect of the four doses induced death and the pathologic signs associated to Stx2 toxicity in mice. Most importantly, this model allowed demonstrating the protective capacity of an immune serum obtained from mice immunized with a recently developed immunogen [19, 20]. However, the efficacy of the immune serum was dependent on the timing of administration

MATERIALS AND METHODS

Mice

BALB/c mice were bred in the animal facilities of the Instituto de Medicina Experimental (IMEX), Buenos Aires. Experiments performed herein were approved by the IMEX Animal Care Committee in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals [21]. Mice were housed in standard transparent polypropylene cages under environmentally controlled conditions (temperature, 24 ± 2°C; humidity, 50% ±

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10%) with a 12-h light-dark cycle. Throughout these studies, the health and behavior of the mice were assessed three times a day. Any mice that became moribund were humanely euthanized in CO_2 chamber. IMEX Animal Care Committee guidelines were used to define humane endpoints.

Recombinant Stx2 (rStx2)

The plasmid pGEM-Stx2 for expression of rStx2 (corresponding to the Stx2a variant according the new nomenclature [22]) was generated previously [23, 24]. The culture of *E. coli* JM109 strain transformed with the recombinant plasmid pGEM-Stx2, was obtained by overnight incubation in Luria–Bertani broth supplemented with ampicillin. Bacterial cells were centrifuged, and the resultant pellet was resuspended in PBS with 1 mM PMSF and lysed by sonication. To obtain the crude preparation of rStx2, the lysate from JM109/pGEM-Stx2 was centrifuged (14,000 rpm, 20 min 4°C), and the supernatant was precipitated with ammonium sulfate solution (75%). The pellet was resuspended in PBS, dialyzed against the same buffer for 24 h, and stored at 0°C until use. rStx2 concentration was determined with RIDASCREEN Verotoxin kit (R-BIOPHARM, Darmstadt, Germany) [19].

Immune Serum

~~BLS-Stx2B~~ immune serum was obtained by immunization of BALB/c adult mice with 20 µg Stx2B/mice of BLS-Stx2B. Mice were immunized by intraperitoneal (i.p.) injection 3 times on day 0, 15 and 30. The first dose was administrated with Freund's complete adjuvant, the second with Freund's incomplete and the third without adjuvant. Sera were obtained 45 days after the last immunization.

Stx2-neutralizing activity in sera was determined by the Vero cell's cytotoxicity assay as previously described [19, 20]. One Neutralizing Unit (NU) was determined as the reciprocal value of the highest dilution that blocked 50% of Stx2 toxicity on Vero cells.

Experimental Design

One lethal dose of rStx2 (3 ng/mouse) was divided in four doses that were administrated intravenously (i.v.) once a day for 4 consecutive days. The first two doses were lower (0.5 ng/mouse) and the last two doses were higher (1 ng/mouse). Mice were monitored daily and those becoming moribund were humanely euthanized by CO_2 inhalation in a closed chamber. For evaluation of long-term damage, mice treated with the four doses of Stx2 and 4NU of immune serum at day 1, 2 or 3 were euthanized three months after intoxication and their kidneys were excised. Non Stx2-intoxicated control mice, age and sex paired, were evaluated in parallel.

Clinical Assessments

Blood samples were obtained for laboratory analyses that included total and differential blood cell count in Neubauer chamber, because it is reported that leukocytopenia and high neutrophilia are indicators of poor prognosis in both, HUS patients and experimental mouse models of HUS [17]. Blood urea nitrogen (BUN) was determined with a commercial kit (Wierner Lab, Argentina), as a good indicator of renal damage in the mouse model

Histopathology

Euthanized mice were perfused with 4% paraformaldehyde. Kidneys were excised and placed in 5 ml of fixing solution containing formol/PBS 10%, processed routinely, embedded in paraffin and sectioned at 4 µm. Samples were stained with hematoxylin-eosin or with Masson trichrome stain. Sections of paraffin-embedded tissue were examined by light microscopy (Eclipse E-200 Nikon). Tissue samples of all the animals were evaluated in a blinded fashion and separately by two researchers regarding knowledge of treatments.

Statistical Analysis

Data are presented as the mean±SEM of each group of mice. Statistical differences were determined using one-way Multiple Comparison analysis of variance by ANOVA followed by Newman Keuls Test, and $P<0.05$ was considered significant. The Log-rank test was used to compare survival curves. For ANOVA-Newman Keuls tests, the software used for analyzing data (GraphPad Prism®) did not provide a specific P value for each pair. Therefore, statistic results in these cases were presented as $P<0.05$ or $P<0.001$.

RESULTS

Dose-Dependent Mortality in the Incremental Split-Dose Model

A dose-dependent mortality was observed in mice treated with the incremental split dose model of Stx2 intoxication (Fig. **1A**). Thus, mice receiving the first two doses showed the lowest mortality rates (5.6%), mice receiving three doses (two low and one high) showed almost a lethal effect (91%) and only the group of mice receiving the complete protocol with the four doses of rStx2 showed 100% mortality.

Figure 1A shows that mice injected with the four doses, died between 5 and 6 days after the first inoculation. We analyzed clinical parameters associated with Stx2 toxicity. Particularly, we assayed the total number of leukocytes, the percentage of circulating polymorphonuclear (PMN) cells and the levels of BUN. Mice showed leukocytopenia and neutrophilia (Fig. **1B**) and an increase in BUN values (Fig. **1C**) from day 4 until death.

Finally, histopathological studies showed glomerular and tubular damage (Fig. **2A-D**). Altogether, these results show that mice injected with the split-dose model reproduced the pathologic parameters associated with Stx2 toxicity.

Evaluation of BLS-Stx2 Immune Sera in the Split-Dose Model

We recently reported the development of a new immunogen which was able to induce high protective neutralizing antibodies against Stx2. In order to determine if it is possible to counteract the Stx2 lethal effect when a therapeutic agent is administered after Stx2 reached circulation, a high single dose of immune serum (15 NU) was administrated i.v. on day 1, 2, 3 or 4 of the Stx2 split-dose model. Fig. **3A** shows that the immune serum was still able to fully protect mice even when administered on day 3. However, serum was not effective when administered together with the last dose at day 4.

These results determined a therapeutic window during which the cumulative Stx2 doses are not enough to induce a fatal damage. Thus, our next objective was to analyze the minimal dose of immune serum that was able to protect mice. Mice were injected with the split-dose model and with 4 NU or 2 NU of immune serum on day 3. Fig. **3B** shows that mice receiving 4 NU were completely protected against death. However, mice receiving 2 NU were only partially protected.

In order to assay a more sensitive indicator for Stx2-damage than lethality, we evaluated the same clinical parameters as described above in mice treated with the split-dose model and the immune serum at day three. Mice receiving 4NU of serum showed a slight and transitory increase in the %PMN cells and in BUN at day 5 but these values returned to normal by day 8 (Figs. **4A** and **4C**). These mice also showed lower glomerular and tubular histopathological damage in kidney (Fig. **5A** and **B**). Mice receiving 2NU of serum also showed an increase in the %PMN cells and in BUN concentration. While BUN values returned to normal in the only mouse that survived at day 8, the percentage of PMN cells in this mouse was still increased (Figs. **4A** and **4C**). In both cases, mice treated with 4NU or 2 NU, ~~mice~~ were not protected against leukocytopenia (Fig. **4B**).

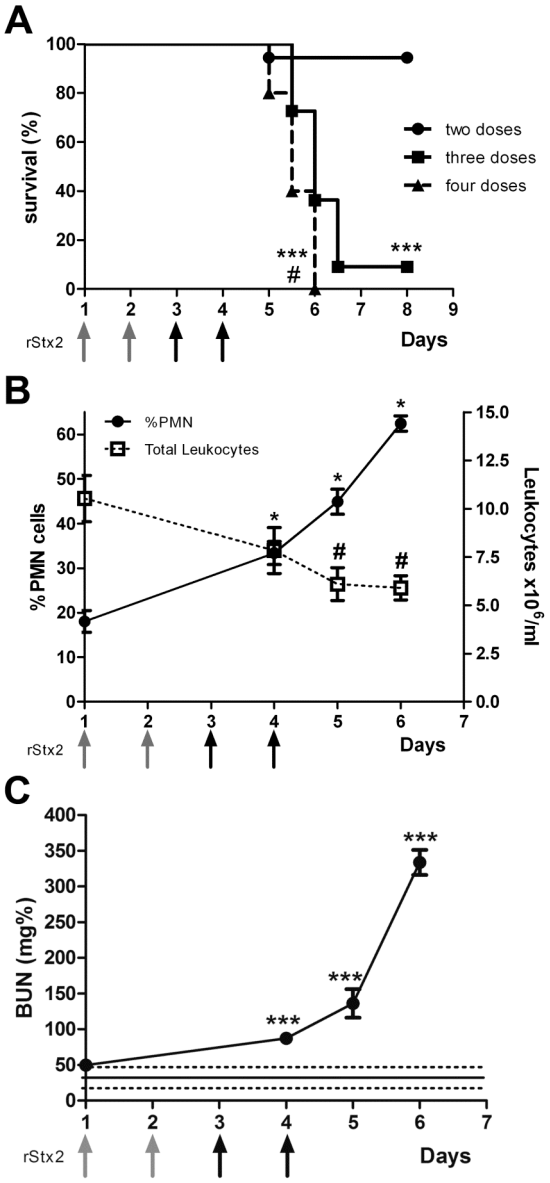

Fig. (1). Development of the split-dose model

Survival rates in response to rStx2 challenge in the split dose model. Mice were i.v. injected with 2 (0.5 ng each; n=18), 3 (two doses of 0.5 ng and one of 1 ng; n=11) or 4 (two doses of 0.5 ng and two of 1 ng; n=10) doses of rStx2, once a day. Mice were observed daily and mortality was evaluated. Grey arrows indicate a dose of 0.5 ng/mouse and black arrows indicate a dose of 1 ng/mouse of rStx2. ***P=0.0009 vs two doses. # P=0.0223 vs three doses. Log-Rank test.

Systemic signs of Stx2-associated toxicity. Mice were injected with the complete split-dose model (4 doses of rStx2) and bled at different times. Absolute numbers of total leukocytes and relative numbers of polymorphonuclear (%PMN) cells were analyzed. Each time point represents the mean±SEM for 6 mice. * and #, significantly different from % PMN and total leukocytes, respectively, in mice previous to challenge (P<0.05). ANOVA

Renal Stx2-induced toxicity. Mice were injected with the complete split-dose model (4 doses of rStx2) and bled at different times. BUN at different time points was measured as a biochemical parameter of renal damage. Solid and dashed horizontal lines represent the mean±2 SD of BUN values in control mice. Each time point represents the mean±SEM for 6 mice. ***, significantly different from control mice (P<0.001). ANOVA

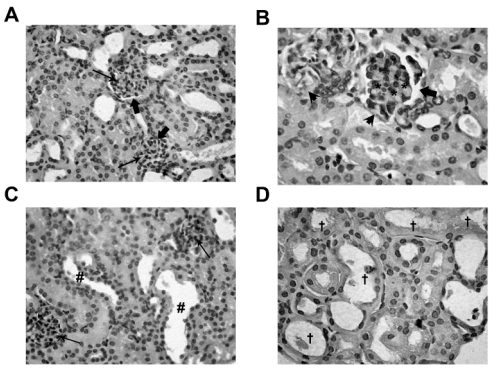

Fig. (2). Histological studies of kidneys. Mice were injected with the complete split-dose model (4 doses of rStx2) and euthanized at day 6. Tissues were fixed and stained with hematoxylin and eosin. Images were acquired using a C. Zeiss III photomicroscope (Oberkochen, Germany).

A) and B) Glomerular damage. Figures show glomeruli (thin arrow), with irregular sizes and shapes, retracted with relative increase in mesangial cells. Bowman's space (thick arrow) is minimal or nonexistent. Glomeruli show adhesion zones (arrow head) between the glomerular visceral and parietal layer of Bowman capsule. The capillaries (asterisk) are occupied by amorphous material slightly eosinophilic. Original magnification: A) x250; B) x400.

C) and D) Tubular damage. The proximal tubules are dilated (hash) lined by epithelial cells with clear cytoplasm and diffuse apical edges with protrusion of the nuclei. The much dilated distal tubules show fibrinoid (cross) adhered to the apical edge and with, also, intraluminal location. Original magnification: C) x250; D) x400.

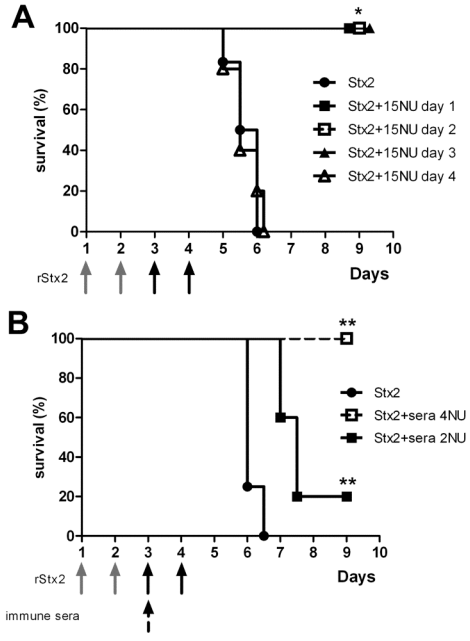

Fig. (3). Protection by BLS-Stx2B immune serum.

Determination of the therapeutic window in the split-dose model. Mice were injected with the split-dose model (n=6) and with a high single dose of immune sera (15 NU, i.v.) on days 1 (n=3), 2 (n=3), 3 (n=3) or 4 (n=4). Grey arrows indicate a dose of 0.5 ng/mouse and black arrows indicate a dose of 1 ng/mouse of rStx2. *P=0.0127 vs Stx2. Log-Rank test.

Determination of the protective dose. Mice were injected with the split dose model (n=4) and with 4 NU (n=6) or 2NU (n=6) of immune serum on day 3 (i.v.). **P=0.0013 4 NU vs Stx2 and P=0.0027 2 NU vs Stx2. Log-Rank test.

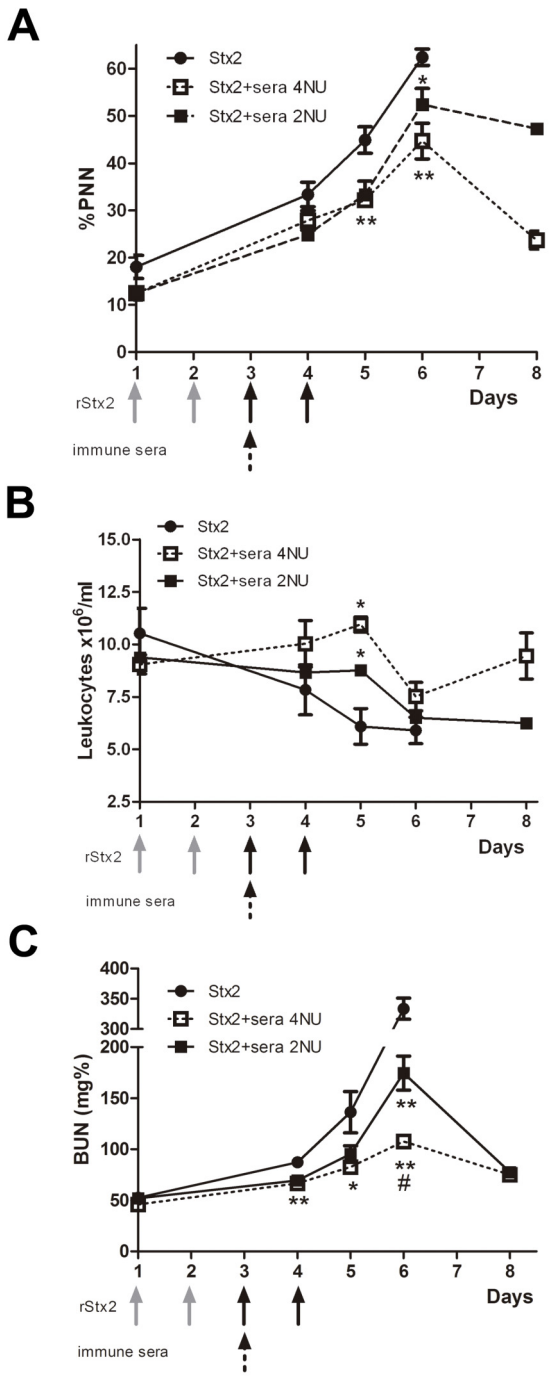

Fig. (4). Clinical parameters associated to Stx2-damage

Mice were injected with the split-dose model and with 4 NU or 2NU of immune serum on day 3 (i.v.). Grey arrows indicate a dose of 0.5 ng/mouse and black arrows indicate a dose of 1 ng/mouse of rStx2. Dashed arrows indicate the time of immune serum administration (day 3).

and B) Systemic signs of Stx2-associated toxicity. Mice were bled and total and differential counts of leukocytes were assayed. Each time point represents the mean±SEM for 6 mice/group. A) Relative number of PMN cells. *P<0.05 and **P<0.005 vs Stx2 at the same time point. B) Absolute numbers of total leukocytes. *P<0.05 vs Stx2 at the same time point. ANOVA

C) Renal Stx2-induced toxicity. Mice were bled and BUN was measured. Each time point represents the mean±SEM for 6 mice/group. *P<0.05 and **P<0.005 vs Stx2 at the same time point. # P<0.005 vs Stx2+2 NU at the same time point. ANOVA

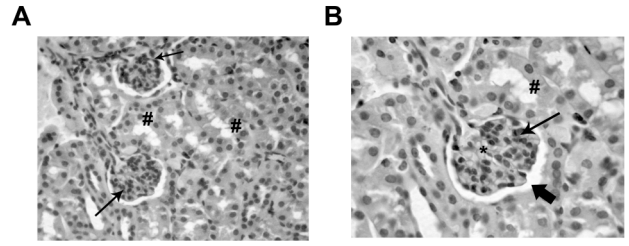

Fig. (5). Histological studies of kidneys.

Mice were injected with the split-dose model and with 4 NU of immune serum on day 3 (i.v.). Mice were euthanized at day 8 and tissues were fixed and stained with hematoxylin and eosin. Images were acquired using a C. Zeiss III photomicroscope. Figures show slightly dilated proximal tubules (hash), lined by epithelial cells with eosinophilic cytoplasm and preserved apical edges. The dilated distal tubules do not show intraluminal fibrinoid material. The glomeruli (thin arrow), with regular sizes and shapes, show microscopically visible Bowman's space (thick arrow) without adhesion zones. Permeable glomerular capillaries are seen (asterisk). Original magnification: A) x250; B) x400.

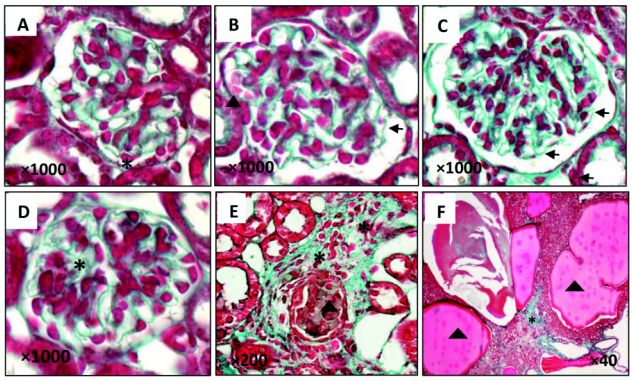

Fig. (6). Long-term Stx2-associated renal damage

Mice were injected with the split-dose model and with 4 NU of immune serum on day 1, 2 or 3 (i.v.). Three months after Stx2-intoxication, mice were euthanized and kidneys excised.

A) Normal glomerulus with conserved podocytes (black asterisk).

B) and C) Slight Renal alterations. Mice treated with 4NU serum at day 1 (B) or 2 (C) showing slight alterations of renal histoarchitecture, with areas of epithelial adherence (black arrowhead) and loss of podocytes (black arrow).

D)-F) Chronic renal failure. One out of six mice treated with 4 NU of immune serum at day 3 developed lesions characteristic of chronic renal failure. D) Focal segmental glomerulosclerosis (black asterisk). E) Areas of interstitial (black asterisk) and glomerular (black arrowhead) fibrosis with loss of glomerular tuft. F) Dilated and cystic renal tubules with endoluminal proteinaceous material (black arrowhead) and interstitial fibrosis (black asterisk). Original magnification: A-D) x1000; E) x200; F) x40.

These results demonstrated the existence of a therapeutic window that allows the administration of effective neutralizing agents to rescue host from severe and irreversible injury. Although the damage previous to administration of the immune serum cannot be prevented, if the antibody is effective enough to neutralize the remaining Stx2, it allows mice to survive and recover, returning to normal biochemical parameters within days after challenge.

Evaluation of Long-Term Damage in Treated Mice

One of the most important sequelae of HUS is the long-term renal insufficiency. Thus, considering that neutralizing therapy was

administered after Stx2 had already exerted its toxicity, we evaluated long-term renal integrity by histopathological studies in surviving treated mice. Mice treated with the four doses of Stx2 and 4NU of immune serum at day 1, 2 or 3 were euthanized three months after intoxication and their kidneys excised.

All surviving mice treated with 4 NU of immune serum on day 1 (n=3) showed kidneys with conserved histoarchitecture (Fig. **6B**). Mice treated with 4NU of immune serum at day 2 (n=6) showed slight alterations in renal histoarchitecture, mainly the loss of podocytes (Fig. **6C**). Mice treated with neutralizing serum at day 3 (n=6) showed the same slight alterations as mentioned before. However, one out of six mice in this group showed local segmental glomerulosclerosis (Fig. **6D**), areas of interstitial and glomerular fibrosis, loss of glomerular tuft (Fig. **6E**), and dilated renal tubules with endoluminal proteinaceous material and interstitial fibrosis (Figure 6F). Altogether, these lesions are indicative of the development of chronic renal damage. It is important to highlight that all non Stx2-intoxicated aged-matched control mice showed conserved renal histoarchitecture (n=6; Fig. **6A**).

DISCUSSION

In human disease, 10 to 15% of STEC infected children progress to HUS between 1 to 7 days after the initial onset of gastrointestinal signs [25]. Since the concentration of Stx2 in blood from HUS patients has never been determined, it still remains elusive precisely when Stx2 reaches systemic circulation. This event should probably occur after diarrhea onset and some days before the appearance of the first characteristic signs of HUS: thrombocytopenia, anemia and acute renal failure, or central nervous involvement [26]. In this regard, hospitalization and volume expansion of children with high presumption of STEC infection has been recommended for limiting disease duration and/or pathogen dissemination. This early intervention, before HUS development, could therefore be beneficial for both individual patients and affected communities [27].

Nowadays, there are no specific therapies for HUS, and therapy for HUS patients is primarily supportive. Diagnostic reagents have recently been developed for early detection of Stx [28] and antibodies (chimeric and humanized) have been developed for potential therapy [29-32]. However, it is unclear whether it would be effective to apply anti-Stx therapies to humans after the clinical signs of systemic Stx2-induced damage have been developed, though these agents are protective in HUS mouse models by STEC-infection [33, 34]. For these reasons, we aimed to develop a lethal mouse model of daily and incremental Stx2 split-doses, which can better reproduce the clinical situation in humans, to test more accurately new therapeutic agents.

In the present study, we have developed a model of HUS in mice consisting of multiple sublethal doses of Stx2 administrated over a period of 4 days, in such a way that each dose (even each of the two low doses) is necessary to induce an accumulative damage that leads to death. This protocol gives the possibility to partially block Stx2 toxicity and evaluate the pathophysiologic ongoing consequences.

An additional advantage of this model is the development of glomerular damage. In fact, endothelial cell injury and thrombosis have been difficult to reproduce in mouse models by a high single dose of Stx2 [35-38], but histological examination of kidneys from these serially Stx2-injected mice showed glomeruli seriously affected and capillaries occupied by fibrinous material. These characteristics are commonly observed in HUS patients, and have been similarly reported in mice receiving an alternative sublethal split-doses model of Stx2 [39].

On the other hand, García et al [40] compared the effects of a single injection of a high dose of Stx2 or the same dose split in several and consecutive injections in rabbits and demonstrated a more aggressive and worse evolution in the group of one dose compared to the group of split-doses. These findings are also in line with Siegler et al [41] who concluded that, in the primate model, disease development is modulated by the rate of Stx1 administration, and it is speculated that the quantity and rate of Stx absorption from the gut is one determinant of disease severity in humans. These observations might reflect that if the absorption rate of Stx2 is too low or during a short period of time, the toxin would be cleared preserving tissues from irreversible damage. On the contrary, when Stx2 absorption rate is upper a given threshold (in terms of amount of Stx and/or exposure timing) tissue damage is large enough to make evolution towards HUS irreversible

Administration of the anti-Stx2 immune serum, which was developed in mice after immunization with the BLS-Stx2B chimera [19], was able protect mice against death and reverse the clinical manifestations of Stx2 intoxication in a dose-dependent manner, when serum was delivered as late as together with the third dose of Stx2. However, although treatment resulted in the rescue of all mice from the acute lethal effect, one out of six mice developed chronic renal lesions indicating that the treatment should be administered as soon as possible to avoid long-term irreversible renal damage induced by Stx2.

In conclusion, the split-dose model presented several advantages in comparison to the high single dose model, and is useful to study novel therapeutics on acute and long-term Stx2-dependent kidney damage.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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