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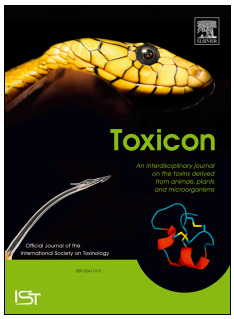

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Systemic effects of Subtilase cytotoxin produced by *Escherichia coli* O113:H21

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ABSTRACT

Subtilase cytotoxin (SubAB) is a member of the AB_5 cytotoxin family and is produced by certain strains of Shiga toxigenic *Escherichia coli*. The toxin is known to be lethal to mice, but the pathological mechanisms that contribute to Uremic Hemolytic Syndrome (HUS) are poorly understood. In this study we show that intraperitoneal injection of a sublethal dose of SubAB in rats triggers a systemic response, with ascitic fluid accumulation, heart hypertrophy and damage to the liver, colon and kidney. SubAB treated rats presented microalbuminuria 20 days post inoculation. At this time we found disruption of the glomerular filtration barrier and alteration of the protein reabsorption mechanisms of the proximal tubule. In the kidney, SubAB also triggered an epithelial to mesenchymal transition (1). These findings indicate that apart from direct cytotoxic effects on renal tissues, SubAB causes significant damage to the other organs, with potential consequences for HUS pathogenesis.

Importance: Uremic Hemolytic Syndrome is an endemic disease in Argentina, with over 400 hundred new cases each year. We have previously described renal effects of Shiga Toxin and its ability to alter renal protein handling. Bearing in mind that Subtilase Cytotoxin is an emerging pathogenic factor, that it is not routinely searched for in patients with HUS, and that to the date its systemic effects have not been fully clarified we decided to study both its systemic effects, and its renal effects to assess whether SubAB could be contributing to pathology seen in children.

1. INTRODUCTION

Hemolytic Uremic Syndrome (HUS) results from the action of multiple factors. It is defined by a triad of microangiopathic hemolytic anemia, thrombocytopenia and acute kidney failure. It´s typical form can be triggered after infection by Shiga-toxigenic *Escherichia coli* (STEC). These bacteria are known to produce Shiga toxin (Stx) type 1 (Stx1) and/or type 2 (Stx2). A new toxin, belonging, like Stx to the AB_5 family, called Subtilase cytotoxin (SubAB) was described in 2004 (2). It was initially isolated from the O113:H21 STEC strain 98NK2, which was responsible for a HUS outbreak in Australia (2, 3). Very little is known of its role in human pathology.

Although SubAB has been shown to bind with high specificity to glycans terminating in the sialic acid N-glycolylneuraminic acid (Neu5Gc) (4) its specific receptors have not yet been fully characterized. Studies show it can bind α2β1 integrins, Hepatocyte growth factor receptor (Met), NG2 and L1 cell adhesion molecule (LCAM1) (5). Once the B Subunit binds to Neu5Gc, the toxin is internalized and travels via the retrograde pathway to the endoplasmic reticulum (ER) where the catalytic A Subunit cleaves BiP (6). BiP is a major chaperone and its cleavage leads to ER stress due to accumulation of unfolded proteins. This represents a novel mechanism of action among bacterial cytotoxins. There are not many *in vivo* studies describing SubAB action. When Wang et al. (7) inoculated mice intraperitoneally (i.p.) with 5 μg of SubAB, mice developed classical HUS lesions including microangiopatic hemolytic anemia, thrombocytopenia and renal impairment.

Furthermore, the mice exhibited histopathological changes in kidney, brain, spleen and liver with significant neutrophil infiltration in liver, spleen and kidney (7).

In another study, mice inoculated i.p. with 10 µg of purified SubAB developed severe hemorrhage of the small intestine and died within 72 h. In the kidney they found hyperplasia of the juxtaglomerular apparatus without renal failure. Organs including brain, heart, lung, liver, spleen and pancreas subjected to routine histopathological analysis did not show histopathological damage (8). The aim of this study was to characterize the systemic effects caused in rats after i.p. inoculation of SubAB cytotoxin.

2. MATERIAL AND METHODS

2.1 Animals

Adult male Sprague-Dawley (SD) rats were obtained from the animal facility at the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. The rats were individually housed under controlled conditions of illumination, humidity, and temperature, with food and water being available ad libitum. Animals were allowed a minimum of 7 days to adapt to housing conditions before undergoing any manipulation.

The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the School of Medicine of University of Buenos Aires, Argentina (CICUAL; Resolution No 2356/11). All the procedures were performed in accordance with the EEC guidelines for care and use of experimental animals (EEC Council 86/609).

2.2 Experimental protocol

Male SD rats (250 ± 1 g of body weight) were randomly divided into two groups of twelve rats each. Group 1 (experimental group, n = 12) was i.p. inoculated with 10 µL (1.5 µg/µL) of SubAB that corresponds to 60 ng SubAB/g of body weight (bwt). Group 2 (control group, n = 12) was inoculated with the same volume of saline solution. Every experiment was repeated three times. Half of the animals of each group (n= 6) were killed at 2 days post inoculation. The rest of the animals were maintained for 20 days. At the moment of sacrifice rats were anesthetized (100 µg ketamine and 10 µg diazepam/g bwt, i.p.) and perfused with 4% paraformaldehyde.

SubAB cytotoxin was purified and characterized as previously described (7)

2.3 Renal function

To study renal function, rats were kept in metabolic cages for 24 h prior to sacrifice to collect urine samples. Blood samples were obtained by cardiac puncture prior to sacrifice. Plasma creatinine was assessed using a commercial kit (Wiener Lab, Rosario, Argentina). Proteinuria (pyrogallol red-molybdate [colorimetric], Alcyon 300I analyzer, Abbott Laboratories, Chicago, IL) and microalbuminuria (nephelometry IMMAGE, Beckman Coulter, Brea, CA) were also measured.

2.4 Histological and immunofluorescence studies

Kidneys, liver and distal colon were fixed in 10% neutral formalin in PBS 0.1 M (pH: 7.4). The tissue sections were dehydrated and embedded in paraffin. Sections of 5 µm were

prepared with a microtome (Leica RM 2125, Wetzlar, Germany) and mounted on 2% silane-coated slides. The sections were stained with Hematoxilin-Eosin (H-E), Periodic Acid-Schiff stain (PAS), or Mallory´s trichrome. The sections were then observed by light microscopy (Nikon Eclipse 200, NY, USA).

For immunofluorescence studies, the slides were preincubated with non immune rabbit serum in PBS (1:50) at room temperature for 30 min, followed by incubation with a polyclonal anti-megalin (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) antibody overnight in a humidified chamber at 4°C. After several washes in PBS, the slides were incubated with an anti-rabbit IgG antibody conjugated with fluorescein (1:200, Santa Cruz Biotechnology) for 1 h at room temperature in a humidified chamber. Finally, all the slides were mounted on a mixture containing PBS:glycerol (1:3), and observed in an epifluorescent microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan). Negative control was performed without primary antibody.

2.5 Electron Microscopy

Renal cortical tissue was minced into 1 mm^3 and tissues were fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 °C, rinsed, post fixed in 1 % osmium tetroxide in cacodylate buffer for 1 h at room temperature and rinsed. Then sections were dehydrated through a graded series of ethanol and infiltrated with Epon resin (Ted Pella) in a 1:1 solution of Epon and ethanol overnight. Sections were then placed in fresh Epon for several hours and embedded in Epon overnight at 60 °C.

Ultrathin sections were cut on a Reichert ultramicrotome (Depew, NY) using a diamond knife and transferred to Formvar/carbon-coated copper grids and contrasted in 2% uranyl acetate and lead nitrate. Sections were then examined using Zeiss OM 109 transmission electron microscope (Zeiss, Oberkochen, Germany).

2.6 Molecular studies

One kidney from experimental and control rats was excised prior to perfusion and homogenized in HEPES buffer (0.01 M, pH 7.4) with protease inhibitors for Western blotting SV Total RNA Isolation System (Promega, USA) buffer for RT-PCR analysis. They were then frozen at -80°C until the time of the study.

2.6.1 Western Blot

The protein concentration in samples was determined with the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, Ill., USA). Samples of 100 µg were electrophoresed using 12.5% gels and electroblotted. Blots were incubated with rabbit polyclonal anti-TGF-β1, or anti-FSP-1 antibodies (1: 1,000 ABCAM, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1: 3,000 Bio-Rad, Hercules, Calif., USA). To determine the uniformity of loading, protein blots were probed with monoclonal anti- β-actin antibody (1: 1,000 Sigma-Aldrich). Band intensities were measured using the Quantity One densitometry software package (BioRad, USA). Protein bands were normalized to their respective β-actin bands. The immunoblot analysis was carried out on three different tissue preparations from three independent experiments.

148 **2.6.2 RT-PCR**

149 Total mRNA was isolated from kidney homogenate using an SV Total RNA Isolation system
150 (Promega Co.) and reverse transcription (RT) was performed for 60 min using Moloney
151 murine leukemia virus reverse transcriptase, oligo-dT15 primer and 400 µM for each of
152 deoxyribonucleotide triphosphate (dNTP) at 42°C. Polymerase chain reaction (PCR) (35
153 cycles at 94ºC for 60 s, 58ºC for 60s and 72ºC for 60 s followed by a final extension of 10
154 min at 72ºC) was carried out using 5 µM of designed specific oligonucleotide primers with
155 a sequence common to megalin (sense 5´-CATTGACGTGGTCAACTTGG-3´ and antisense 5´-
156 CGAGCTACCGTCTTCCTTTG-3´). These primers were designed using Broad Institute,
157 Primer3, available at: http://wwwgenome. wi.mit.edu/science/software/software, on the
158 basis of GenBank sequences. Negative control was performed without the addition of
159 reverse transcriptase, to confirm the absence of genomic DNA amplification. RT-PCR
160 products obtained were cloned and sequenced. Primers were used to β-actin as internal
161 standard. Densitometry of bands was carried out using the software ImajeJ1.44 ® (Wayne
162 Rasband, National Institutes of Health, USA).

163

164 **2.7 Statistical analysis**

165 All data are presented as mean ± SEM; n = number of rats. Statistical analysis was
166 performed using the Graph Pad Prism Software 5.0 (San Diego, CA, USA). Differences
167 between experimental and control rats were analyzed by Student t-test. Statistical
168 significance was set at $P < 0.05$.

169

3. RESULTS

3.1 Macroscopical findings

Rats treated with a sublethal dose of SubAB (60 ng/g bwt) exhibited a marked increase of abdominal cavity compatible with the development of ascites at 20 days post-inoculation (Figure 1A). Puncture of the abdominal cavity showed the presence of a milky whitish liquid which rapidly coagulated. This fluid was also detected when the thoracic cavity was opened. Microscopic examination of the ascitic fluid revealed the presence of an eosinophilic material with neutrophil infiltration (Figure 1B). The liver showed a macronodular appearance with adherences (Figure 1C,D). In addition, the heart showed concentric hypertrophy (88% of animals) or eccentric hypertrophy (12%) (Figure 1 E-G). Biochemical analysis of the retrieved fluid detected cholesterol (51.5 ± 2.1 mg/dL), triglycerides (82.5 ± 19.1 mg/dL), albumin (1.4 ± 0.1 g/l) and glucose (101 ± 25.5 mg/dL).

Figure 1: Macroscopical findings in SubAB-treated rats. Abdominal distention due to fluid accumulation was evident 20 days post inoculation of SubAB. The abdominal puncture showed the presence of a milky whitish liquid of very rapid coagulation (A). Eosinophilic material with neutrophil infiltration was also detected (arrows, B). Abdominal organs presented debris and adhesion (C) and liver had nodular appearence with adherences (D).The heart showed eccentric hypertrophy (F) and concentric hypertrophy (G) compared with the control (E).

3.2 General and Renal parameters

SubAB treated-rats showed a significant increase in microalbuminuria at 20 days post inoculation (Table 1). Other parameters such as water intake, urine output, plasma creatinine and proteinuria were not altered by the toxin treatment (Table 1).

Table 1: General and renal parameters

Parameter	Control group (n=18)		SubAB, 60 ng/g bwt (n= 18)	
Time	2 days	20 days	2 days	20 days
Microalbuminuria (mg/day)	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.12 ± 0.01*
Proteinuria (mg/day)	4.1 ± 0.8	6.9 ± 1.6	3.8 ± 0.6	6.0 ± 2.2
Plasma creatinine (mg/dL)	0.75 ± 0.05	0.65 ± 0.02	0.67 ± 0.04	0.70 ± 0.03
Water intake (ml/day)	26.0 ± 3.6	39.8 ± 7.9	18.7 ± 3.2	30.7 ± 8.4
Urine output (ml/day)	23.0 ± 4.6	31.7 ± 10.1	15.79 ± 3.2	26.1 ± 6.7

*$p < 0.01$ experimental versus control group. SubAB-treated rats showed an increase in microalbuminuria 20 days post inoculation. Other parameters such as proteinuria, plasma creatinine, water intake and urine output were not altered by toxin treatment.

3.3 Histopathological damage

Histological analysis of colon and liver from rats treated with SubAB showed similar alterations at 2 and 20 days post inoculation (Table 2). On the kidney, significant

alterations were observed at 2 days and being more pronounced at 20 days (Table 2). Figure 2 shows significant lesions in colon (B-D) and liver (F-H) from SubAB-treated rats after 2 days of inoculation compared with the controls (A and E). In colon, detachment of surface colonic cells (arrowhead in B), interstitial hemorrhagic foci (asterisks in C) and Peyer´s patches activation with mononuclear cell invasion of intestinal mucosa (arrow in D) were detected. In liver, hepatocyte vacuolization (star in F), histoarchitecture distortion (G) and neutrophil cell infiltration (arrow in H) were observed.

In kidney tissue, glomeruli and tubules exhibited a significant necrosis at 2 days post inoculation of SubAB (Figure 3 B and D) compared with the controls (A and C). Glomeruli showed necrosis, mesangial proliferation and capillary thickening at 20 days post inoculation (Figure 3F). By Mallory's Trichome staining, the number of glomeruli with segmental fibrosis significantly increased after SubAB treatment (H). Quantification of number of glomeruli with fibrosis (10 fields, 200×) is showed in Figure 3I (*$p<0.001$). These damages were not observed in control glomeruli (E and G).

To further examine the glomerular lesion, electron microscopy studies were performed. Subepithelial nodules corresponding to actin filament condensation of the podocytes were detected (TC in Figure 4B), indicating a disruption in normal podocyte cytoskeleton. Foot process effacement (red arrow in C) and villous transformation (VT in B) were also present. Normal filtration barrier was observed in glomeruli from control rats (Figure 4A).

226 **Table 2: Histological alterations in rat tissues after SubAB inoculation**

Tissue/Alteration	2 days post inocuation	20 days post inoculation
Colon		
Detachment of epithelial cells	Yes	Yes
Hemorrhagic foci	Yes	No
Peyer´s patches activation	Yes	No
Mononuclear cell infiltration	No	Yes
Liver		
Hepatocyte vacuolization	Yes	Yes
Hystoarchitecture distortion	Yes	Yes
Neutrophil infiltration	Yes	Yes
Necrotic foci	No	Yes
Kidney		
Glomerular necrosis	Yes	Yes
Tubular necrosis	Yes	Yes
Mesangial proliferation	No	Yes
Capillary thickening	No	Yes
Focal and segmental fibrosis	No	Yes

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Figure 2: Ligth microscopy of colon and liver histological sections from SubAB-treated rats. Detachment of epithelial cells (arrowhead, B); hemorrhagic foci (asterisks, C) and Peyer's patches activation with mononuclear cell invasion of intestinal mucosa (arrow, D) were observed in the colon from SubAB-treated rats compared to the Control rats (A). Vacuolization of hepatocytes (black star, F); histoarchitecture distortion (G) and neutrophil infiltration (arrow, H) was observed. Normal histoarchitecture was observed in liver from control rats (E). Staining with hematoxilin-eosin (A-H).

Figure 3: Ligth microscopy of kidney histological sections from SubAB-treated rats. Necrosis in glomeruli (arrowhead, B) and renal tubules (arrowhead D) was detected at 2 days post inoculation of SubAB (60 ng/Kg). At 20 days post inoculation, glomeruli showed necrosis, mesangial proliferation, capillary thickening (black arrows in F) and segmental fibrosis (black asterisk in H). Quantification of number of glomeruli with fibrosis (10 fields, 200×) is showed in I (*$p<0.001$). Kidney sections from control rats (A, C, E, G). Staining with PAS (A-F) and with Mallory´s Trichrome (G-H) were performed.

Figure 4: Electromicroscopy of glomeruli from SubAB-treated rats at 20 days post inoculation. Ultrastructural study revealed foot process effacement (red arrow in C), villous transformation (VT in B) and actin tono filament condensation (TC in B). Control (A).

Molecular studies

3.4 TGF-β and FSP1 protein expression

Expression of TGF-β protein (12.5 kDa) was examined in kidney tissues from Control and SubAB-treated rats at 20 days post inoculation by WB (Figure 5A). The mean TGF-β levels normalized to ß-actin showed a significant increase after SubAB treatment (Figure 5B). Taking into account that TGF-ß triggers EMT (9) and that FSP1 is a marker of fibroblasts present in EMT, the expression of FSP1 (12 kDa) was examined by WB (Figure 5C). The mean FSP1 levels normalized to ß-actin were also significantly increased in rats treated with SubAB (Figure 5D).

3.5 Expression of megalin protein and mRNA

Figure 6 shows a representative immunofluorescence staining for megalin in renal proximal tubules from rats sacrificed at 20 days post inoculation of SubAB. Reduced label in SubAB-treated rats (B) was observed compared with the controls (A). In order to determine if the reduction in protein levels may be correlated with a decrease in megalin mRNA, RT-PCR analysis were performed. Figure 6C shows an expected band corresponding to megalin expression in kidney from control but not from SubAB-treated rats. The average megalin mRNA levels normalized to β-actin indicate that the decrease in megalin protein is due to a decrease in mRNA expression (Figure 6D).

Figure 5: TGF-β and FSP1 expression in renal tissue from SubAB treated-rats at 20 days post inoculation. TGF-β (A) and FSP1 (C) proteins were detected in kidney homogenates

by Western blot. Bar graphs represents the average protein levels normalized to the loading control ß-actin (B, D). n= 6 ,*p<0.001.

Figure 6: Megalin expression in kidney from SubAB-treated rats at 20 days post inoculation. Renal tissue showed a decrease in megalin expression (B) compared to controls (A) by indirect immunofluorescence and by RT-PCR (C). Bar graphs represents the average megalin levels normalized to β-actin (D, n = 6, *p< 0.001)

4. DISCUSSION

The main findings after intraperitoneal SubAB inoculation in rats were the development of ascites, cardiac hypertrophy and histological alterations in colon, liver and kidney. These findings are consistent with a systemic inflammatory response to the toxin described by Furukawa et al. after intraperitoneal inoculation of 10 µg of SubAB in mice (8). After 2 days of treatment with SubAB, we found hemorrhagic foci with mucosal erosion in the rat colon without macroscopic alterations of the small bowel. The animals did not develop diarrhea as previously described (7). Nevertheless, previous studies showed SubAB is able to inhibit the net water absorption across human colonic mucosa (10). Taking into account there have been reports of diarrhea in patients infected with bacterial strains which produce SubAB but not Stx2 (11), more studies are necessary to clarify the differences observed between the clinical setting and the observations made in animal studies.

In addition, we have detected neutrophil infiltration in several tissues in agreement with results described by Wang et al. (7). We have also observed cytoplasmic vacuoles in the hepatocytes. Vacuole formation has been previously reported in *in vitro* studies (12, 13). Lass et al. (12, 13) described vacuole formation in HeLa cells after a period of 6 h of incubation with SubAB (1µg/ml). Currently the cause underlying vacuole formation due to SubAB inoculation remains unknown although it may be linked to SubAB receptors. Little information is available regarding the precise identity and distribution of SubAB receptors (7). Further studies characterizing them could throw some light on why vacuoles were not found in other tissues.

Regarding the ascites, its development has not been previously described after the inoculation of either SubAB or Stx2. HUS patients have never been reported to develop peritoneal effusion neither has it been described in any HUS animal model. Taking into account the biochemical and histological analysis performed on the ascitic fluid, we suggest it could be related with an inflammatory response induced by the SubAB cytotoxin.

Our study is the first to describe SubAB action on the heart. We found the development of concentric cardiac hypertrophy in 88% of the animals and eccentric cardiac hypertrophy in 12%. Physiopathological mechanisms that govern the development of the hypertrophy are different, being either pressure or volume overload. HUS patients have been known to develop cardiac hypertrophy, but there aren´t enough studies on extra-renal HUS manifestations (14). As SubAB is only now emerging as a pathogenic factor, its contribution to this finding cannot be ruled out.

315 Although plasma levels of creatinine, which is a marker of kidney injury were not altered
316 in rats neither at 2 nor at 20 days they presented histopathologycal damage. In agreement
317 with our results studies performed by Vaidya et al. show that serum creatinine was a poor
318 predictor of renal injury in various structurally and mechanistically different models of
319 renal tubular injury in rats (15). There is also evidence that serum creatinine has poor
320 sensitivity when there is adequate renal reserve. In this case its levels may not be changed
321 due to compensatory increases in the function of other neprhons (16).
322 Whereas plasma creatinine is a conventional marker to monitor kidney injury in humans,
323 histopathology constitutes the gold standard for animal models (15). ~~We also found~~
324 Although plasma creatinine levels were not altered, we did find development of
325 microalbuminuria 20 days post inoculation of SubAB. This led us to further study the renal
326 protein handling mechanisms. Development of microalbuminuria could be produced by an
327 alteration in glomerular filtration and/or tubular protein reabsorption. SubAB caused
328 podocyte villous transformation, foot process effacement and actin filament condensation
329 and decreased megalin expression in proximal tubules. One of the ways in which proteins
330 can be reabsorbed in tubules is megalin dependent (17). These facts could explain the
331 presence of albumin in urine.
332 Yahiro et al. (5) identified α2β1 integrin as SubAB receptor. Podocytes bind to the basal
333 membrane by a α2β1 integrin (18, 19). Concerning SubAB effects on the podocytes, we
334 suggest that SubAB could directly affect the podocytes interacting with the α2β1 integrin.
335 Podocyte villous transformation, actin filament condensation and glomerular necrosis with
336 adherences may develop following mesangiolysis. Mesangial proliferation has been

337 associated with podocyte dysfunction and local TGF-β production, both of which are
338 present in SubAB-treated rats. We found TGF-β is increased 20 days post inoculation. TGF-
339 β would act by promoting fibroblast and mesangial cell growth and extracellular matrix
340 accumulation (20). Taking into account that TGF-ß triggers EMT (1) and that FSP1 is a
341 marker of fibroblasts present in EMT, our results suggest that SubAB may progress
342 towards tubule interstitial fibrosis (21).
343 SubAB cytotoxin produced a decrease in kidney megalin levels. This alteration could be
344 due to the increase in TGF-β involved in megalin recycling to the apical membrane of renal
345 proximal tubule cells. In previous studies, we reported that an increase of TGF-β
346 expression which caused a decrease in megalin levels was responsible of
347 microalbuminuria in Stx2-treated rats (22). The ability of SubAB to alter megalin
348 expression in renal proximal tubules from SubAB-treated rats would in part explain the
349 development of microalbuminuria.
350 Taking all this into account it is important to consider the effects produced by the toxin
351 both at 2 and 20 days. At 2 days the toxin produced a multiorganic lesion, that although it
352 was histologically observed, it did not lead to the death of the animals. At 20 days
353 postinoculation we could still find organ damage as that seen at 2 days with new
354 developments including cardiac hypertrophy, ascites and glomerular fibrosis in the kidney.
355 This shows that acute damage produced by the toxin is not self-limiting and evolves to
356 chronicity into a complex clinical picture, which could probably lead to animal death.
357 Further studies are needed to address the mechanisms underlying the observed organ
358 damage, and how the clinical picture evolves.

In conclusion we have described the action of SubAB cytotoxin on the heart developing cardiac hypertrophy, and on the liver where it elicits vacuole formation. In particular, in the kidney, SubAB is able to damage podocytes, trigger EMT and alter the renal protein handling by disruption of the filtration barrier and tubular protein reabsorption.

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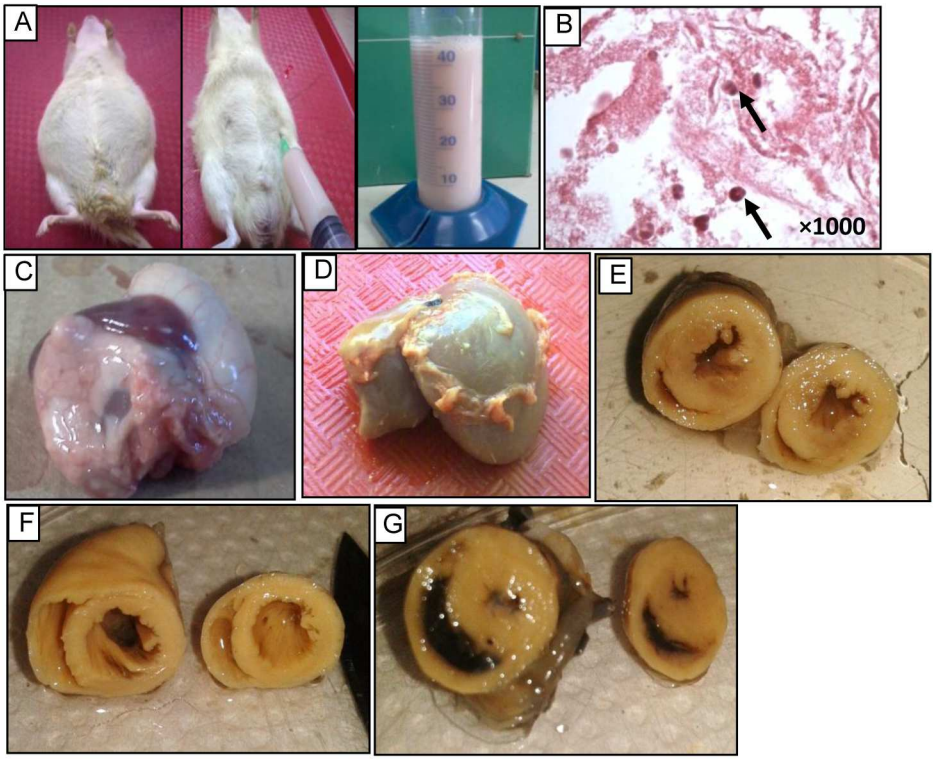

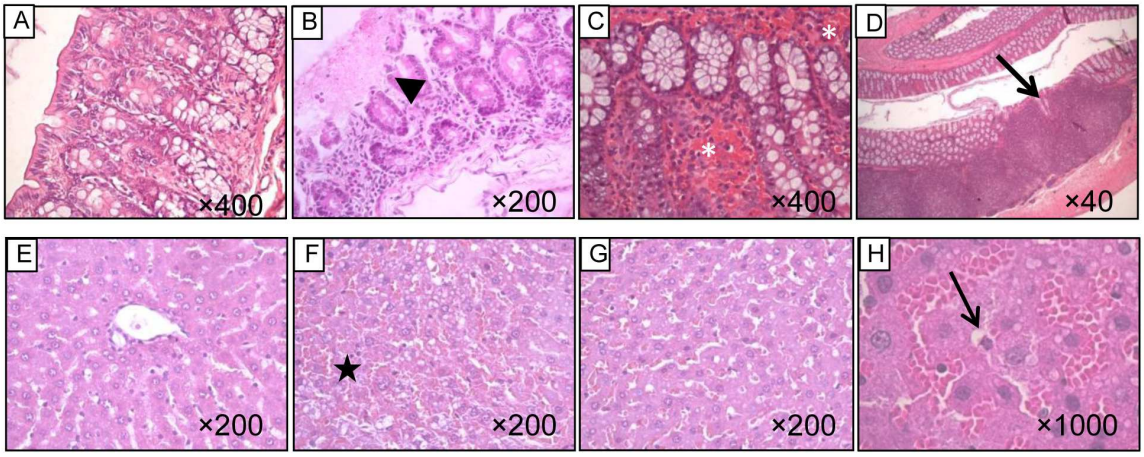

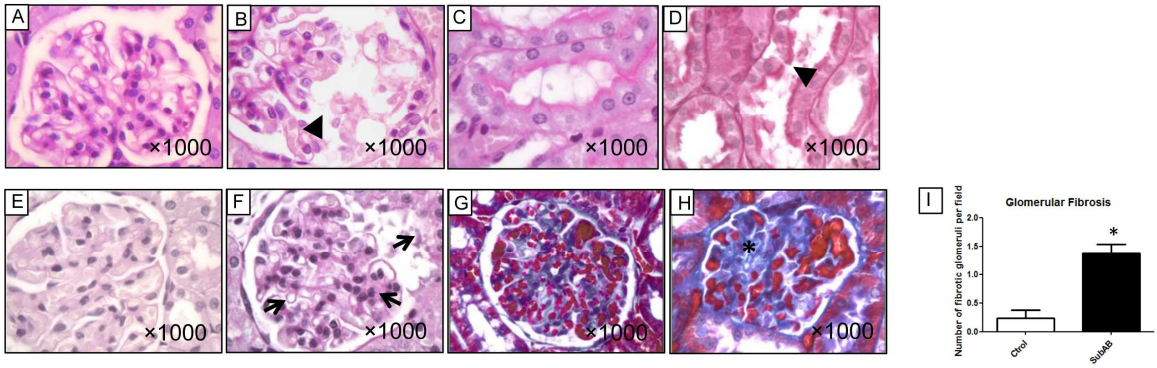

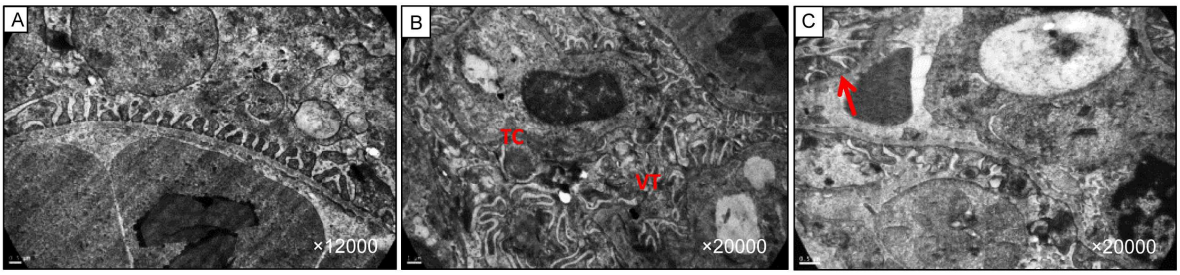

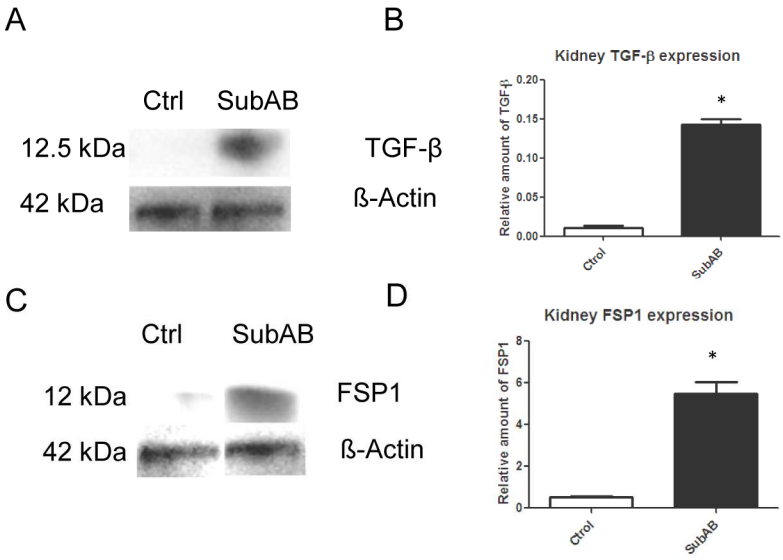

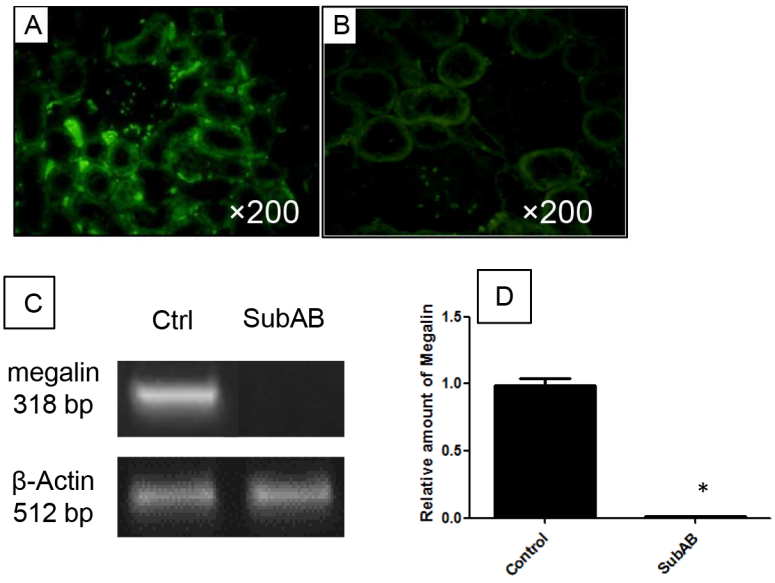

Highlights:

- We report novel effects of SubAB cytotoxin *in vivo*
- Rats develop ascites 20 days after SubAB inoculation
- SubAB is capable of triggering cardiac hypertrophy
- SubAB elicits vacuole formation in the liver
- SubAB alters podocytes cytoskeleton and tubular renal protein handling

Ethical Statement for Toxicon

I testify on behalf of all co-authors that our article submitted to Toxicon

Title: **Systemic effects of Subtilase cytotoxin produced by *Escherichia coli* O113:H21**

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1) this material has not been published in whole or in part elsewhere;
2) the manuscript is not currently being considered for publication in another journal;
3) all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

Date:20/10/2016

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