Research Article

Recombinant human soluble thrombomodulin is associated with attenuation of sepsis-induced renal impairment by inhibition of extracellular histone release

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Abstract

Multiple organ dysfunction induced by sepsis often involves kidney injury. Extracellular histones released in response to damage-associated molecular patterns are known to facilitate sepsis-induced organ dysfunction. Recombinant human soluble thrombomodulin (rhTM) and its lectin-like domain (D1) exert anti-inflammatory effects and neutralize damage-associated molecular patterns. However, the effects of rhTM and D1 on extracellular histone H3 levels and kidney injury remain poorly understood. Our purpose was to investigate the association between extracellular histone H3 levels and kidney injury, and to clarify the effects of rhTM and D1 on extracellular histone H3 levels, kidney injury, and survival in sepsis-induced rats. Rats in whom sepsis was induced via cecal ligation and puncture were used in this study. Histone H3 levels, histopathology of the kidneys, and the survival rate of rats at 24 h after cecal ligation and puncture were investigated. Histone H3 levels increased over time following cecal ligation and puncture. Histopathological analyses indicated that the distribution of degeneration foci among tubular epithelial cells of the kidney and levels of histone H3 increased simultaneously. Administration of rhTM and D1 significantly reduced histone H3 levels compared with that in the vehicle-treated group and improved kidney injury. The survival rates of rats in rhTM- and D1-treated groups were significantly higher than that in the vehicle-treated group. The results of this study indicated that rhTM and its D1 similarly reduce elevated histone H3 levels, thereby reducing acute kidney injury. Our findings also proposed that rhTM and D1 show potential as new treatment strategies for sepsis combined with acute kidney injury.

Introduction

Sepsis is a life-threatening healthcare issue caused by infection, and associated with a high risk of mortality. In sepsis, inflammatory response occurs following infection and sometimes contributes to the development of various organ dysfunctions. Kidney injury is one of the most common organ dysfunctions induced by sepsis. Once a sepsis patient develops complications of acute kidney injury (AKI), mortality is reportedly increased [1-3].

In recent years, extracellular histones have been attracting attention as mediators of death from sepsis [4]. Extracellular histones are known as damage-associated molecular patterns (DAMPs) and are released when cellular injury occurs, resulting in induction of neutrophil migration, platelet aggregation, and endothelial cell damage [5, 6]. DAMPs are likely to trigger the activation of serious inflammation [7] and result in severe tissue injury [8-12]. Suppression of the production of inflammatory mediators such as cytokines and DAMPs could thus contribute to the improvement of clinical outcomes in critically ill patients.

Thrombomodulin (TM) is a thrombin-binding anticoagulant cofactor that is expressed on the surface of endothelial cells and plays an important role in the regulation of intravascular coagulation [13]. The structure of TM consists of five domains: the N-terminal lectin-like domain (D1), a domain with six epidermal growth factor (EGF)-like structures (D2), a serine and threonine-rich domain (D3), a transmembrane domain (D4), and a short cytoplasmic domain (D5) [14]. Recent studies have shown that D1 has anti-inflammatory effects, inhibits the production of inflammatory cytokines, and also binds to high-mobility group box-1 (HMGB1), which is known DAMPs [15, 16]. However, whether administration of TM could suppress extracellular histone H3 levels and improve organ injury remains unclear. In addition, whether administration of D1 has the same effects on histone H3 levels and protective effects against kidney injury remains to be clarified. We hypothesized that

administration of TM and D1 would improve kidney injury and survival rate in sepsis-induced rats by inhibiting extracellular histone H3 release. Recombinant human soluble TM (rhTM) is homologous with the extracellular domains of TM and is used as an anticoagulant in treatment of disseminated intravascular coagulation in Japan. The purposes of this study were: 1) to investigate the relationship between extracellular histone H3 levels and kidney injury; and 2) to clarify whether rhTM and D1 could affect extracellular histone H3 levels, kidney injury, and survival rate of sepsis-induced rats.

Materials and methods

Cecal ligation and puncture (CLP)-induced sepsis model in rats

Male Wistar rats were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). The rats weighing 250-300 g were used after an acclimation period of at least 1 week before experimentation. Rats were maintained in a 12/12 h light/dark cycle to minimize noises, vibrations and odors. The animals were kept in temperatures of 22-24°C with 40-50% humidity. Food and clean water were freely accessible at all times. Therefore, we did not require all animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions. To create an in vivo sepsis-induced model, we performed CLP procedures as follows. Rats were anesthetized with isoflurane, and a 1.5-cm incision was made along the abdominal midline. Ligation was performed at a distance of 1-2 cm from the blind-ending cecum, avoiding the mesenteric vessels of the cecum. The distal cecum was then punctured once with an 18-G needle, and a small amount of feces was gently squeezed out. The cecum was returned to the abdominal cavity, and layers of the abdominal incision were sequentially sutured. After CLP, rats were subcutaneously injected with 10 mL/kg of saline. Rats were deprived of food and free access to water after CLP.

rhTM and D1 were used for experimental studies at the indicated dose, which was provided by Asahi Kasei Pharma (Tokyo, Japan). The molecular weights of rhTM and D1 are 52,171 Da and 20,191 Da, respectively. The dose of D1 in this study was thus calculated as 0.36 of the rhTM dose by conversion according to relative molecular weights for equivalent titration.

Blood samples were collected by cardiac puncture at each time point under general anesthesia and centrifuged at 1500×*g* for 10 min. The supernatant after centrifugation was collected and stored at -80°C until further analyses.

All experimental protocols described in the present study were approved by the Institutional Animal Care and Use Committee of Sapporo Medical University (Permit No. 19-023). The stuffs who conducted this experiment had taken special seminar and training in animal care or handling, which was approved by the Committee of our University. All efforts were made to minimize suffering. In the present study, as the humane end point for rats, a state of respiratory urgency, continuous laying, and convulsion were judged as indicative of a moribund state and treated with euthanasia. There were no animals died before meeting criteria for euthanasia. Once animals reached endpoint criteria, they were euthanized immediately. Euthanasia was performed by irreversible cardiac or respiratory arrest due to deep anesthesia with isoflurane. Animal health and behavior were observed carefully every 30 minutes after CLP procedure. Additionally, we considered an animal welfare every 4 h until 24 h. A total of 104 rats were used in this study and 20 rats of them were euthanized by the endpoint criteria.

Determination of histone H3 levels after CLP

We investigated changes in circulating histone H3 levels after CLP. Blood samples were collected from rats under general anesthesia. We observed the rats every 4 h until 24 h

113 after the end of the CLP procedure. Thus, the duration of the experiment was set at 24 h. We
114 determined the timing of elevations in histone H3 levels. Commercial enzyme-linked
115 immunosorbent assay kits were used for measurement of histone H3 levels (Shino-Test,
116 Kanazawa, Japan) according to the instructions from the manufacturer.
117

118 Administration of rhTM after CLP or optimal dose of rhTM for 119 affecting histone H3 levels after CLP

120 We administered several doses of rhTM after CLP to determine the optimal dose of
121 rhTM for affecting histone H3 levels. We set four groups: a control group that received 2 mL
122 of vehicle; and low-, middle-, and high-dose groups that received rhTM at 1 mg/kg, 3 mg/kg,
123 and 6 mg/kg, respectively. Blood samples were obtained at the time point of significant
124 increases in histone H3 levels after the CLP procedures in each group and histone H3 levels
125 were compared between these four groups.
126

127 Administration of D1 after CLP

128 To evaluate the efficacy of D1 on histone H3 levels, vehicle and a calculated dose of
129 D1 based on the optimal dose of rhTM were administered as a bolus injection into the tail
130 vein of rats after CLP. Blood samples were collected from rats under general anesthesia at the
131 time point of a significant increase in histone H3 levels after CLP. Histone H3 levels were
132 then determined and compared between vehicle- and D1-treated groups.
133

134 Histopathologic analysis of kidney

135 Kidney tissue specimens were obtained after euthanasia by deep anesthesia and were
136 submerged in 10% formaldehyde neutral buffer solution. Fixed tissues were embedded in

137 paraffin, cut at intervals of 3 μm, and stained with hematoxylin and eosin (HE). The number
138 and size of injured areas in the whole kidney were determined.
139

140 Immunohistochemical staining of the kidney and quantification

141 Immunohistochemical staining for histone H3 was performed using rabbit polyclonal
142 antibody (Lot. 001; Shino-Test). After antigen retrieval and blocking, sections were
143 preincubated with 10% normal goat serum for 20 min. Sections were incubated with a 1:500
144 dilution of the primary antibody histone H3 for 15 min. Sections were then incubated with
145 Bond™ Polymer Refine Detection (Leica Biosystems, Newcastle-Upon-Tyne, UK). The
146 chromogen was 3,3'-diaminobenzidine (DAB), followed by counterstaining with hematoxylin.
147 Staining intensity was graded as: 0, negative; 1, weak; 2, moderate; or 3, strong. A grade of 2
148 or 3 was considered positive for immunohistochemical staining, whereas a grade of 0 or 1 was
149 considered negative. We counted the positive cells in a randomly selected microscopic field at
150 high magnification (×400). Quantitative analysis of histone H3 expression was performed
151 using histological images that were digitally obtained using a light microscope in order to best
152 reflect overall immunostaining of the specimen on each slide. Camera control with constant
153 setup conditions was maintained throughout the course of the study. Adobe Photoshop
154 software (Adobe Systems, Mountain View, CA) was used to process acquired images. For
155 quantification, positive signals were selected using a constant threshold in each image at high
156 magnification (×400). All immunohistochemical specimens were evaluated by a
157 board-certified pathologist (Department of Pathology, Sapporo Medical University School of
158 Medicine, Japan).
159

160 Survival analysis

To evaluate the effects of rhTM and D1 on survival rate after CLP, rats were divided into three groups: a control group of rats administered vehicle after CLP; an rhTM group of rats administered the optimal dose of rhTM after CLP; and a D1 group of rats administered D1 (0.36 of rhTM dose) after CLP. Survival of the rats was monitored for 24 h. We analyzed and compared survival rates of rats between these three groups.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis between groups was performed by Tukey's multiple comparison test. Survival rate was analyzed by the Kaplan-Meier method using log-rank testing. Analyses were performed with Graph Pad Prism version 6.0h software (GraphPad Software, San Diego, CA). The level of significance was set at 0.05 for all analyses.

Results

Serial changes in extracellular histone H3 levels after CLP procedure

Circulating histone H3 level began to significantly increase at 16 h after CLP and continued to increase up to 24 h (Fig 1). Based on this result for this timing, we decided to evaluate extracellular histone H3 levels at 16 h after CLP in the following studies.

Fig 1. Circulating histone H3 level after CLP procedure. Circulating histone H3 level elevated over time. n = 5 per group. † $p \leq 0.01$ vs. 0 h.

Effect of rhTM on histone H3 levels after CLP

185 Significant differences were found in circulating histone H3 levels at 16 h after CLP
186 among the four groups. Histone H3 levels in middle-dose (3 mg/kg) and high-dose (6 mg/kg)
187 rhTM groups were significantly lower than that in the control group administered vehicle only
188 (Fig 2). Therefore, in the following experiment, we used rhTM dose at 3 mg/kg for evaluation
189 of rhTM efficacy on decreasing histone H3 level, improving survival rate and protective
190 actions on the kidney.
191
192 **Fig 2. Effectiveness of rhTM for reducing histone levels.** Circulating histone H3 levels
193 were significantly lower with administration of middle- or high-dose (3 mg/kg or 6 mg/kg)
194 rhTM compared to control group administered vehicle. n = 5 per group. † $P \leq 0.01$ vs. vehicle.
195

196 Effect of D1 on histone H3 levels after CLP

197 The administration dose of D1 was set at 1.09 mg/kg converted according to the
198 molecular weight of rhTM. Administration of D1 significantly reduced circulating histone H3
199 levels, similar to rhTM (Fig 3). No significant difference in reduction of histone H3 levels
200 was seen between rhTM-treated and D1-treated groups.
201
202 **Fig 3. Effect of lectin-like domain 1 (D1) of rhTM on reduction of histone H3 levels in**
203 **sepsis-induced rats.** n = 6 per group. ‡ $P \leq 0.001$ vs. vehicle.
204

205 Effect of rhTM and D1 on serum creatinine levels

206 Serum creatinine levels were significantly lower in rhTM- and D1-treated rats than in
207 vehicle-treated rats (Fig 4). No significant difference in serum creatinine level was seen
208 between rhTM and D1 groups.
209

210 **Fig 4. Serum creatinine level in rats after CLP procedure.** Administration of rhTM and D1
211 improved serum creatinine levels. n = 6 per group. † $P \leq 0.01$ vs. vehicle.
212

213 Survival rate

214 The survival rate at 24 h after CLP was 40% in the rhTM-treated group and 20% in the
215 vehicle-treated group. The difference in survival rate between the two groups was significant
216 according to Kaplan-Meier analysis (p=0.048) (Fig 5A). In the D1-treated group, the survival
217 rate for 24 h after the CLP procedure was 40%, compared with 20% in the vehicle-treated
218 group. Kaplan-Meier analysis revealed significant prolongation of survival in the D1-treated
219 group compared with the vehicle-treated group (p=0.038) (Fig 5B).
220

221 **Fig 5. Survival curves in rats after CLP procedure.** A) Rats were administered rhTM after
222 CLP. B) Rats were administered D1 after CLP. n = 10 per group. * $P<0.05$ vs. vehicle.
223

224 rhTM and D1 improves histopathologic findings of the kidney

225 After CLP in rats, scattered degeneration foci were widely observed in tubular
226 epithelial cells and were widely distributed throughout the whole kidney in rats in the
227 vehicle-treated group (Fig 6A-C). We observed that more severe degenerations were mainly
228 observed in the tubular epithelium, suggesting peripheral circulatory insufficiency (Fig 6A).
229 We found glomerular swelling in the cortical area, showing swelling of the glomerular
230 vascular endothelial cells and nuclei (data not shown). On the other hand, in rats treated with
231 rhTM and D1, tubular structure was maintained and degeneration was observed in small areas.
232 As a result of quantification, administration of rhTM and D1 significantly decreased numbers
233 and sizes of areas of degeneration in the kidney compared with non-treated rats (Fig 6B, C).
234 In rats from the sham group, immunostaining for histone H3 was mainly observed in

235 the cortical area. However, in rats from the vehicle-treated group, less immunostaining for
236 histone H3 was observed compared with the sham group (Fig 7A). Rats with administration of
237 rhTM and D1 showed more immunostaining for histone H3 compared with the vehicle-treated
238 group. We performed quantitative analysis of histone H3 expression in the cortical area for
239 each group (Fig 7B). The number of positive cells in the non-treated group was significantly
240 decreased compared with the sham group. Administration of rhTM and D1 significantly
241 increased the number of positive cells compared with the vehicle-treated group. Our data
242 suggested that soluble TM can rescue sepsis-induced renal tubular damage.
243
244 **Fig 6. Histopathologic examination of kidney stained with HE.** A) Histologic findings in
245 rat kidney. Renal tissue was obtained 16 h after CLP and stained with HE. Arrowheads
246 indicate degeneration. Representative images are shown at a magnification of ×200. B)
247 Quantitation of kidney degeneration in the four groups is shown (number of degenerations). n
248 = 6 per group. C) Quantitation of kidney degeneration in the four groups (size of
249 degenerations). n = 6 per group. † $P \leq 0.01$ vs. vehicle. ‡ $P \leq 0.001$ vs. vehicle. Scale bars =
250 200 μm.
251
252 **Fig 7. Histopathologic examination of kidney with immunohistochemical staining for**
253 **histone H3.** A) Immunohistochemical findings in rat kidney. Renal tissue was obtained 16 h
254 after CLP and stained with antihistone H3 peptide polyclonal antibody. Representative images
255 are shown at a magnification of ×200. B) Quantitation of positive cells for
256 immunohistochemical staining in the four groups is shown. n = 6 per group. † $P \leq 0.01$ vs.
257 vehicle. ‡ $P \leq 0.001$ vs. vehicle. Scale bars = 200 μm.
258

259 # Discussion

260 The present study showed two important findings. First, circulating histone H3 levels
261 elevated over time and were associated with kidney injury diagnosed pathologically and from
262 renal function, and with high mortality in rats with intraabdominal infection-induced sepsis.
263 Second, we found that administration of rhTM and D1 dramatically reduced circulating
264 histone H3 levels, protected against kidney injury, and improved mortality in comparison with
265 non-treated rats with intraabdominal sepsis. From the present results, administration of rhTM
266 appears to be associated with renal protective effects via attenuation of histone H3 levels and
267 consequent improvement of survival rate in sepsis rats. The lectin-like domain of rhTM,
268 which does not show anticoagulant activity, is suggested to play a central role in
269 anti-inflammatory activity. rTM, and the D1 domain in particular, thus holds promise as a
270 drug for the treatment and prevention of septic AKI.

271 Nuclear histone proteins have been reported to be released into the extracellular space
272 and act as major mediators of death in sepsis [4, 17]. In our sepsis model, extracellular histone
273 H3 levels increased significantly from 16 h after CLP. At that time, renal tissue showed
274 extensive degeneration and vacuolation with increased histone H3 levels. However, no
275 findings of acute tubular necrosis were identified. These findings show the early stage of AKI
276 in pathological evaluations.

277 Results of histological findings demonstrated microcirculatory disturbance, showing
278 the ischemic injury of kidney. Moreover, mortality rates of CLP-induced sepsis rats in the
279 non-treated group, in which histone H3 levels were high, were significantly higher than in the
280 rhTM and D1 treated groups. These results suggest that elevated histone H3 levels might be
281 associated with kidney injury and prognosis in intraabdominal sepsis-induced rats.

282 rhTM has been reported to bind to DAMPs, resulting in reduction of DAMP levels
283 through inactivation by neutralization or decomposition. This elimination of released histone
284 H3 has been demonstrated to improve mortality in experimental investigations [18-20]. rhTM

285 comprises the following three structural regions: the lectin-like domain, the EGF-like domain,
286 and a serine-threonine-rich region. The lectin-like domain of the three structures has an
287 anti-inflammatory effect and a binding effect on HMGB1, a known DAMP [21]. Another
288 study showed that rhTM (particularly the lectin-like domain) protects the kidney from
289 ischemia-reperfusion injury via activated protein C (APC)-dependent and APC-independent
290 mechanisms [22]. On the other hand, endothelial dysfunction during ischemic injury caused
291 by inflammatory response results in microvascular congestion, leading to microcirculation
292 abnormalities [23, 24]. Endothelial cells play an important role in preventing intravascular
293 thrombus formation and maintain microcirculation [25]. However, the inhibitory effect of
294 rhTM and D1 on circulating histone H3 level remains poorly understood. We therefore
295 hypothesized that regulation of extracellular histone H3 by administration of rhTM and D1
296 would prevent sepsis-induced endothelial dysfunction, improve disturbances in
297 microcirculation, and result in improved renal injury and prognosis in a rat sepsis model.

298 In our experimental study, CLP induced a significant increase in circulating
299 extracellular histone H3 levels and induced AKI as evaluated pathologically and from serum
300 creatinine levels. Results of immunohistochemical staining of rat renal cells also demonstrated
301 decreased expression of histone H3. These data suggest that CLP-induced histone H3
302 elevation is associated with induction of AKI in rats.

303 Our results demonstrated that administration of rhTM and D1 inhibited CLP-induced
304 histone H3 elevation and mitigated CLP-induced AKI. Moreover, administration of both
305 rhTM and D1 improved prognosis of CLP-induced rats. Furthermore, results of
306 immunohistochemical staining clearly demonstrated increased expression of histone H3 in
307 rats that received either rhTM or D1, preventing histone H3 from being released to the
308 extracellular space. These results suggest that rhTM and D1 might have the main effect of
309 inhibiting the release of histone H3. On the other hand, no significant differences were evident

310 between the rhTM-treated group and the D1-treated group in terms of the effects on inhibiting
311 histone H3, improving renal injury, and prognosis. The main domain of rhTM that exerts
312 anti-inflammatory action thus appears likely to be the lectin-like domain, D1.

313 The present study clearly demonstrated an association between increased histone H3
314 levels and kidney injury, and indicated that administration of rhTM and D1 reduced histone
315 H3 levels and protected renal tissue. However, the following issues remain to be clarified in
316 future studies: 1) the mechanisms underlying suppression of histone H3 by rhTM and D1; and
317 2) effects of rhTM and D1 on other organ injuries that were not examined in this study.
318 Further studies are thus needed in the future to clarify the mechanisms regulating histone H3
319 and protecting against organ injuries by rhTM and D1.

320 In this study, rhTM and D1 were administered at the time of CLP production. This
321 differs from real clinical situations. Furthermore, we found that suppression of the increase in
322 histone H3 levels is associated with improvement of kidney injury. This shows the possibility
323 that AKI is associated with increased serum histone H3 levels. In the pathological
324 examination of renal tissue, AKI was induced by CLP and a significant decrease in
325 intracellular histone protein levels was identified. However, whether the decrease in
326 intracellular histone H3 levels following AKI and the increase in serum histone H3 levels are
327 related remains unclear. In general, leukocytes activated by inflammation are known to
328 release histone H3, resulting in the elevation of histone H3 levels in sepsis [26]. Ito et al. [27]
329 reported that depletion of leukocytes by anticancer drug-induced bone marrow suppression
330 resulted in inhibition of histone H3 production in a mouse model of sepsis. Therefore, the
331 increase in histone H3 levels in sepsis is thus highly possible to be derived from leukocytes.
332 Conversely, since this study showed pathological findings that CLP induced disappearance of
333 intracellular histone H3 in degenerated renal cells, damaged renal cells were considered to
334 release intracellular histone H3 into the extracellular space and this mechanism is partially

associated with increased serum histone H3 levels in sepsis rats.

In the future, further studies are required to clarify the relationship between disappearance of intracellular histone protein in renal tubular cells and increases in blood concentrations.

Conclusions

CLP induced increases in extracellular histone levels, with this elevation peaking in the late phase of sepsis followed by the peak in tumor necrosis factor (TNF)-α level. CLP also induced AKI and this elevation of extracellular histone is thought to be related to CLP-induced AKI. rhTM and its lectin-like domain D1 were found to similarly reduce these elevated histone H3 levels, thereby protecting against CLP-induced AKI and significantly improving the survival rate of sepsis rats. Based on these facts, rhTM is considered to have anti-inflammatory activity through the suppression of histone H3 levels, and thus may have renal protective activity. Furthermore, since the D1 domain, which has no anticoagulant action among the domains of rhTM, has the same effects on reducing histone H3 levels as rhTM did, these findings suggest that the D1 domain may play a crucial role in anti-inflammatory activity among the domains of rhTM. The results of our study suggest that rhTM and D1 domains may be useful drugs for treating sepsis complicated by AKI. Our results also may provide a rationale for further clinical studies of rhTM and its lectin-like domain as a potential drug for the treatment of septic AKI.

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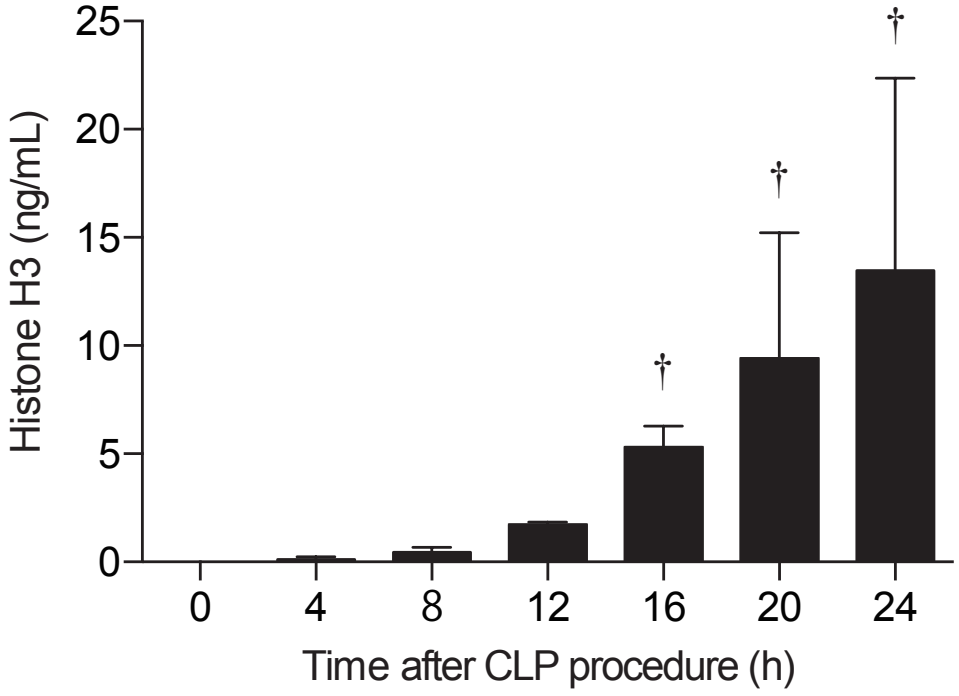
25
20
15
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Histone H3 (ng/mL)
†
†
†
0
4
8
12
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24
Time after CLP procedure (h)

Fig 1.

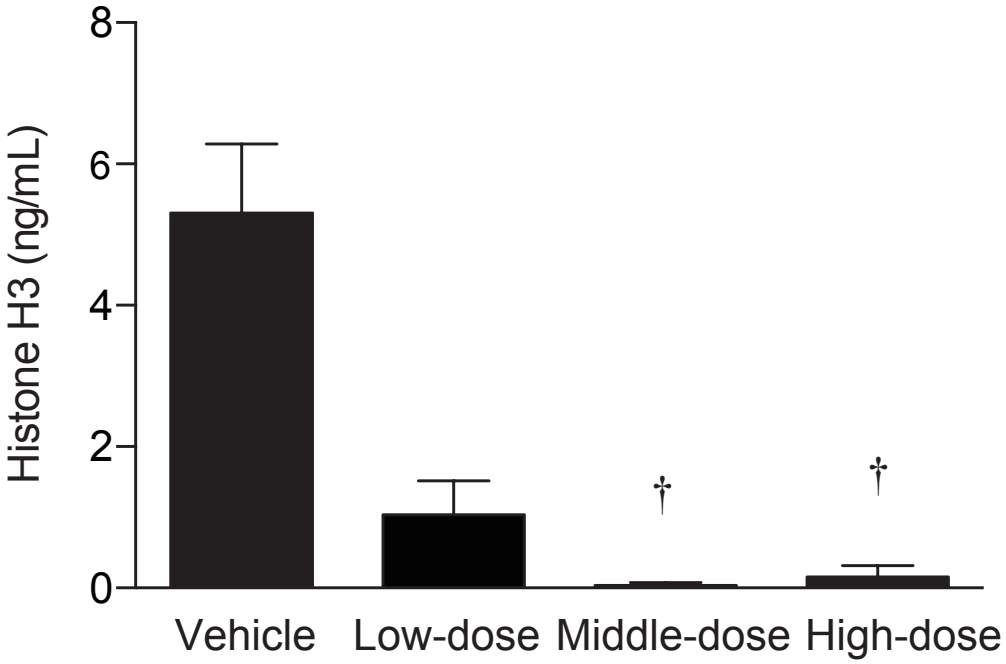

8
6
4
2
0
Histone H3 (ng/mL)
†
†
Vehicle
Low-dose
Middle-dose
High-dose

Fig 2.

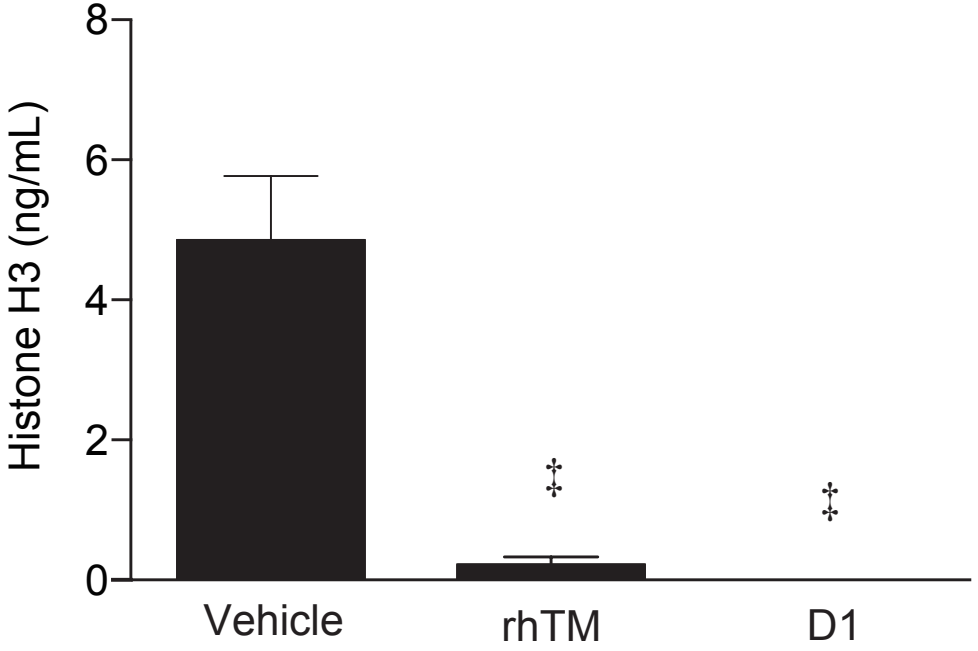


Fig 3.

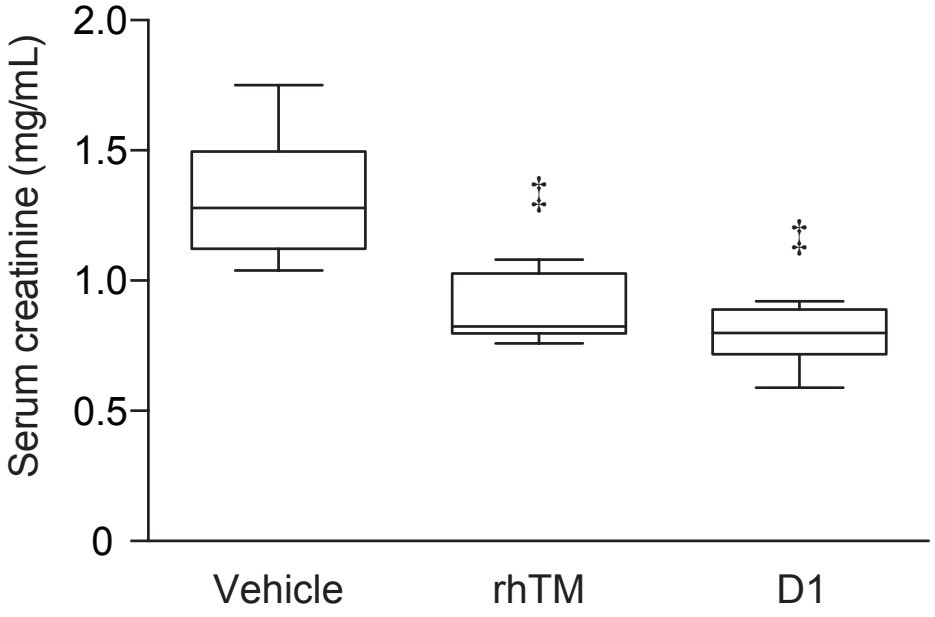
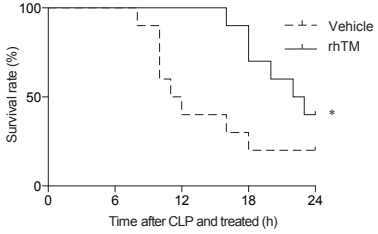


Fig 4.

A


100
50
0
Survival rate (%)
0
6
12
18
24
Time after CLP and treated (h)
Vehicle
rhTM
*

B

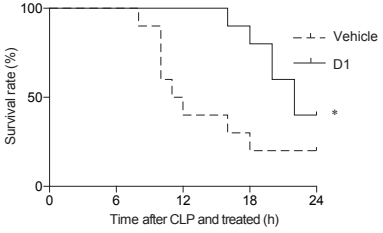

100
50
0
Survival rate (%)
0
6
12
18
24
Time after CLP and treated (h)
Vehicle
D1
*

Fig 5.

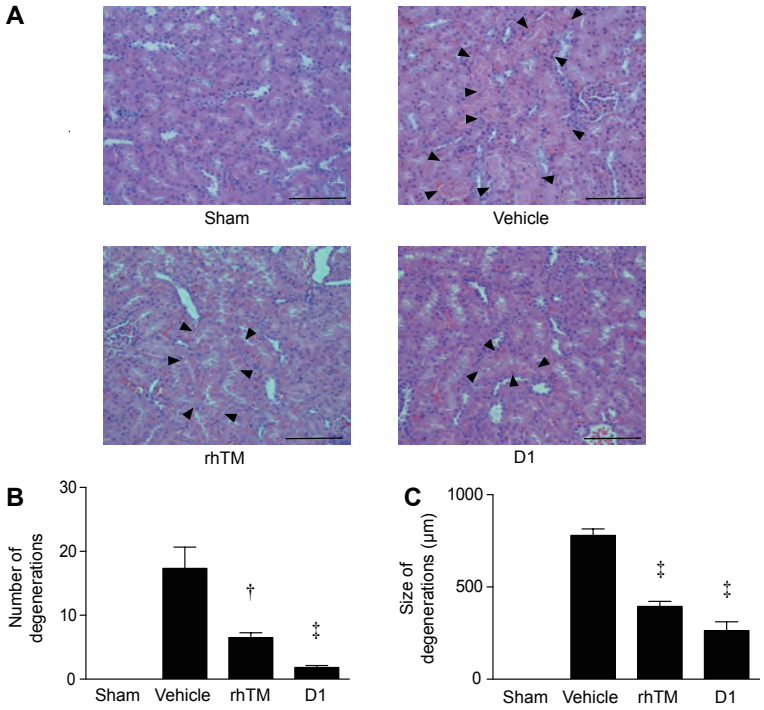

A
Sham
Vehicle
rhTM
D1
B
Number of degenerations
30
20
10
0
†
‡
Sham
Vehicle
rhTM
D1
C
Size of degenerations (μm)
1000
500
0
‡
‡
Sham
Vehicle
rhTM
D1

Fig 6.

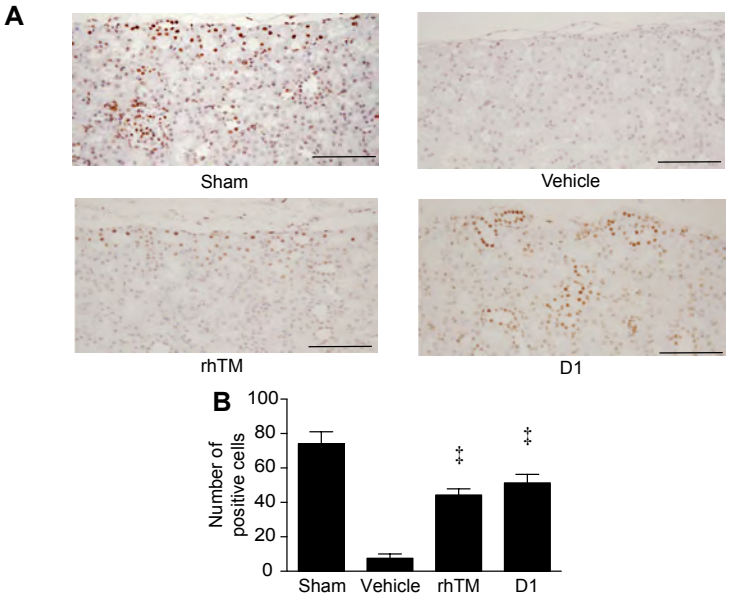

A
Sham
Vehicle
rhTM
D1
B
Number of positive cells
100
80
60
40
20
0
‡
‡
Sham
Vehicle
rhTM
D1

Fig 7.