Ximenia americana heteropolysaccharides ameliorates inflammation and visceral hypernociception in murine caerulein-induced acute pancreatitis: involvement of CB2 receptors

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Abbreviations: TPL-Xa, Total polysaccharide of *X. americana*; AP, acute pancreatitis; MPO, myeloperoxidase; CGRP, Calcitonin Gene-Related Peptide; SP, substance P; TRPV1, Transient Receptor Potential Vanilloid 1; CB1, cannabinoid receptor type 1; CB1, cannabinoid receptor type 2; NF- κB, Nuclear factor-κB; NMR, nuclear magnetic resonance.

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Abstract

Background: This study aimed to characterize and investigate the anti-inflammatory and anti-hypernociceptive effects of the total polysaccharide of *X. americana* (TPL-Xa) bark and the of cannabinoid receptors in a mouse model of acute pancreatitis-induced by caerulein

Methods: TPL-Xa was characterized by ^{1}H and ^{13}C RMN spectroscopy. Animals received TPL-Xa (10 mg/kg, i.v.) 30 min before and after caerulein (50 μg/kg, 10x, i.p.) administration. To evaluate the involvement of cannabinoid receptors, AM281 (3 mg/kg, s.c.) and AM630 (1 mg/kg, s.c.) were administered 30 min before TPL-Xa. Plasma levels of amylase and lipase, pancreatic myeloperoxidase (MPO), histology, visceral hypernociception and motor coordination were evaluated 11 and 24 h after acute pancreatitis (AP). *Results*: TPL-Xa, containing a heteropolysaccharide composed of glucose, galactose, arabinose, rhamnose, fucose and galacturonic acid, reduced amylase and lipase levels, MPO activity, acinar cell necrosis, edema and neutrophil infiltration. TPL-Xa increased the threshold of visceral hypernociception, that was reversed by AM630, an antagonist of cannabinoid receptors type 2 (CB2). In addition, TPL-Xa did not alter the animals motor coordination.

Conclusions: TPL-Xa contains heteropolysaccharides that inhibits the inflammation and hypernociception in the experimental model of caerulein-induced AP, by a mechanism involving type CB2 receptors.

Keywords: pancreas inflammatory nociception; medicinal plant polysaccharides; structural characterization; cannabinoid receptors.

1. Introduction

Acute pancreatitis is an inflammatory pancreas disorder, mainly caused by the presence of gallstones and alcohol abuse [1,2]. Its incidence varies from 5 to 80 per 100.000 individuals per year [3] and is manifested by elevated serum levels of pancreatic enzymes and acute pain [4,5]. The pain management is still a major challenge, since there are patients refractory to the current therapies, a lack of

investigation of visceral pain pathophysiology [6] and side effects of the standard treatment with opiates [7].

The cannabinoid system has emerged as an important antinociceptive pathway, since the cannabinoid receptors are distributed in anatomical regions (medulla dorsal horn, periaqueductal gray matter) involved in pain transmission and modulation [8]. It has been demonstrated that the activation of cannabinoid receptors (CB1, CB2) reduces hypernociception in a model of irritable bowel syndrome [9], and in models of acute [10–12] and chronic pancreatitis [13]. These protective effects were also observed *in vitro* models of pancreatic acinar cell injury [14,15]. Consequently, substances that interfere with other nociceptive pathways, such as the cannabinoid system, have been considered as alternatives for pain modulation [16].

Plant polysaccharides are molecules known for their modulatory effects on the immune system [17], such as inflammation [18,19] and nociception. The antinociceptive effects were demonstrated in the mice visceral nociception induced by acetic acid for the polysaccharides of *Thladiantha dubia* [20], *Solanum betaceum* [21,22], *Solanum lycopersicum* [23]. In addition, polysaccharide rich fractions isolated from *Ximenia americana* barks, a plant popularly used as anti-inflammatory and analgesic [24,25,26]. Also, in the model of caerulein-induced experimental acute pancreatitis the polysaccharide of lemon (low-methoxyl pectin) attenuated the inflammatory response and improved intestinal barrier integrity [27]. The total polysaccharides fraction (TPL-Xa: 8.1% yield) presented 43% carbohydrate (21% uronic acid) and resulted in two main fractions after chromatography (FI: 12%, FII: 22% yield). FII showed better homogeneity/purity, content of 44% carbohydrate, including 39% uronic acid, arabinoseand galactose as major monosaccharides, and infrared spectra with peaks in carbohydrate range for COO−groups of uronic acid [24].

This study aimed to analyze the structural features of total polysaccharides of *X. americana* barks and to evaluate their anti-inflammatory and antinociceptive effects in the model of acute pancreatitis induced by caerulein in mice. The involvement of cannabinoid receptors in their beneficial effects was also evaluated.

2. Materials and Methods

2.1. Chemicals

Caerulein and the reagents for dosage of MPO (myeloperoxidase) were obtained from Sigma Aldrich (St. Louis, MO-USA); ketamine and 2% xylazine König S/A (Hurlingham, Buenos Aires, Argentina); antagonists of cannabinoid receptors CB1 (AM281) and CB2 (AM630) from Tocris Bioscience (Ellisville, MD, EUA); commercial kits for amylase from Labtest (Lagoa Santa, MG, Brazil) and lipase from Bioclin (Belo Horizonte, MG, Brazil) and diazepam from TEUTO S/A (Anápolis, GO, Brazil).The remaining drugs and reagents were of analytical grade.

2.2. Polysaccharides extraction and structural characterization

Bark of *X. americana* L (voucher n° 46794/Herbarium Prisco Bezerra - Federal University of Ceará), collected at Custódio- Quixadá, Ceará, Brazil, were washed, dried at 40 °C and macerated into powder. Five grams of dry powder were suspended in absolute methanol for depigmentation, extraction with 0.1 M NaOH and precipitation in ethanol, resulting in the total polysaccharides (TPL-Xa: 8.1% yield, containing 43% total carbohydrates, including 21% uronic acid and 6.5% proteins) [24].

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) of TPL-Xa was performed in the Fourier transform Bruker Avance DRX 500 spectrometer (USA, California), equipped by reverse detection probe, operating at 499.9 MHz (^{1}H) or 125 MHz (^{13}C), in the window spectral ratio of 20 ppm for ^{1}H or 200 ppm for ^{13}C. TPL-Xa (32 mg) was solubilized in D_2O (3% m/v). The analysis was carried out at 24 ºC and residual water signal at 4.79 and chemical shifts (δ) expressed in ppm.

2.3. Animals

Male Swiss mice (20-25 g) were housed in a climate-controlled room and maintained at 22-26 °C in a 12 h light/12 h dark cycle, fed with standard chow, water *ad libitum* and allowed to acclimatize for a minimum of 1 week. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of Ceará (nº 3202316/2014; 06/2014).

2.4. Acute pancreatitis model and treatment with TPL-Xa

Acute pancreatitis was induced by ten injections of caerulein (50 μg/kg), administered by intraperitoneal (i.p.) route at hourly intervals [12]. The control animals received saline i.p. Animals were treated with TPL-Xa (10 mg/kg or saline by intravenous (i.v.) route 30 min before the first, and 30 min after the last injection of caerulein. To evaluate the involvement of cannabinoid receptors in the TPL-Xa effects, AM281 (CB1-specific antagonist, 3 mg/kg and AM630 (CB2-specific antagonist, 1 mg/kg) were administered by subcutaneous (s.c.) route in non-treated or treated animals 30 min before TPL-Xa. After the 11^{th} and 24^{th} hours following the first injection of caerulein, mice were anesthetized (ketamine and 2% xylazine) for collection of blood samples and sacrifice. The pancreas was rapidly removed and frozen at -80 °C for evaluation of myeloperoxidase activity, or fixed in formalin (10%) for histopathological analysis.

2.5. Serum amylase and lipase

Blood samples were taken and centrifuged at 3500 rpm for 10 min. The serum amylase and lipase levels were measured by colorimetric method using commercial kits. The values of amylase and lipase were expressed as units of enzyme U/dL and U/L, respectively.

2.6. Pancreatic myeloperoxidase activity

Samples of pancreatic tissue were homogenized in 0.5% hexa-decyl-trimethyl-ammonium bromide (50 mg of tissue/500 μl) and centrifuged (40000 × g, 20 min, 4 ºC). Supernatants were incubated in 96-well plates (10 μL) with a mixture of 5 mg O-dianisidine, 15 μL 1% H_2O_2, 3 mL phosphate buffer and 27 mL H_2O and measured at 450 nm for 1 min in microplate reader (BMG FLUOstar OPTIMA) [28]. One unit of MPO activity was defined as that degrading 1 mmol of peroxide per min at 25 ºC and expressed as U/mg of tissue.

2.7. Histopathological analysis

Mice were euthanized at 11 and 24 h after pancreatitis induction. The pancreas was excised, fixed with 10% buffered formalin for 24 h, embedded in paraffin, cut into

5 µm thick sections and stained with hematoxylin-eosin (HE). The assessment of pancreatic edema, inflammatory cell infiltrate and acinar necrosis were graded with scores ranging from 0 to 3 [29] under light microscopy.

2.8. Mechanical visceral hypernociception (von Frey test)

Mice were placed in clear acrylic box with raised platforms of wire mesh, 15 min before the test. The abdominal hypernociceptive reaction (licking of the abdomen, abdominal and/or whole-body withdrawal) was evoked by application of a gradual pressure (g) using polypropylene tip (0.5 mm^2 contact area) coupled to a hand-held force transducer (Electronic von Frey Aesthesiometer; Insight). Hypernociception was evaluated at baseline (time zero - mean of three measurements) and 11 and 24 h after the first dose of caerulein [30].

2.9. Rota-rod test

After 30 min of TPL-Xa treatment, the animals locomotor function was assessed. Diazepam (5 mg/kg, i.p.) was used as a positive control. Mice had been selected 24 h prior to the test, excluding those that did not remain on the Rota-rod (22 r.p.m.) for at least two consecutive periods of 60 s. The time in which animals remained on the apparatus was recorded [31].

2.10. Western blot analysis

The Pancreatic tissue was macerated in lysis buffer, after centrifugation, the proteins were dosed by the BCA method (Sigma-Aldrich). For immunoblotting, the samples were subjected to 10% SDS-PAGE and homogeneous transfer to nitrocellulose membranes. Membranes were blocked with TBS-T buffer and 5% milk for 1 h at room temperature. For protein detection, blotted membrane was incubated with the specific anti-CB2 (1:200; SC) overnight at 4-8º C. Membranes were incubated for one hour at room temperature with conjugated secondary antibodies (anti-rabbit, 1:2500; Santa Cruz). The blots will be revealed using hemiluminescence technique (ECL plus system).

2.11. Statistical analysis

Parametric results are presented as mean ± S.E.M and analysed by One-way analysis of variance (ANOVA) followed by the Bonferroni t-test. Histological analyses were presented as median (maximum and minimum) and analysed by Kruskal-Wallis followed by a Dunn test. Values of $p<0.05$ were considered significant.

3. Results

3.1. ^{1}H and ^{13}C NMR spectra of TPL-Xa

The ^{1}H NMR spectra of TPL-Xa revelead a large signal of chemical displacement (δ) in 3.83 ppm, attributed to the group -O-CH3, and a minor signal at 1.17 ppm, suggestive of a methyl group linked to L-fucose monomer [32]. Other signals were also reveled: at 1.25 and 1.31 ppm corresponding to methyl groups of L-rhamnose unit; at 1.91 ppm indicated the presence of galacturonic acid acetyl groups [33] and at 5.40 and 5.25 ppm, corresponding, respectively, to H1 of unsubstituted/substituted α-L-arabinose. Besides, signals between 5.09-5.19 ppm are possibly related to H1 α-D-glucose and α-D-galactose [32,34,35], and 3.0-4.5 ppm are attributed to the hydrogens (H2-H6) of α-D-glucose, α-D-galactose or α-L-arabinose [32]. In the ^{13}C NMR spectra, signals at 107.47 and 106.89 ppm possibly correspond to anomeric carbons of unsubstituted/substituted α-L-arabinose, respectively, and those at 99.66 and 95.72 ppm correspond to C1 of the pyranoside ring of α-D-glucose and α-D-galactose, respectively [32,36]. The signal at 57.38 ppm could be attributed to -O-CH_3 bound to galacturonic acid carboxylate [34]. Signals between 55.0-85.0 ppm could be carbon chains (C2-C6) of α-D-glucose, α-D-galactose and α-L-arabinose [35,36], and at 16.44 ppm attributed to L-fucose.

3.2. Inhibitory effect of TPL-Xa on pancreatic enzymes and inflammatory parameters

Caerulein induced acute pancreatitis was characterized by a significant increase in the levels of serum amylase and lipase at the 11^{th} and the 24^{th} hour after the first caerulein injection compared to saline. TPL-Xa reduced the elevated levels of amylase at the 11^{th} h by 28.5% (Fig. 2A) and at the 24^{th} h by 26.6%, while lipase levels were

reduced only at the 11th h by 52% (Fig. 2B). Caerulein also caused pancreatic tissue damage (edema, inflammatory infiltration and acinar cell necrosis) (Fig. 3 A-D) and increased MPO activity (Fig. 4). Administration of TPL-Xa markedly reduced the histological parameters at the 11th h: edema, inflammatory infiltration, acinar cell necrosis and total scores. TPL-Xa reduced the MPO activity by 56% at the 11th h (Fig. 4A), but caused no alteration at 24th h (Fig. 4B).

3.3. TPL-Xa inhibits visceral hypernociception without alteration of locomotor function

The acute pancreatitis induced by repeated doses of caerulein reduced the threshold of nociceptive responses at the 11th h and 24th h. TPL-Xa increased the threshold of visceral hypernociception by 42% only at the 11th h after first caerulein injection (Fig. 5A). TPL-Xa did not alter the latency of animal permanence in the Rota-rod. The sedative agent diazepam decreased this latency (Fig. 5B).

3.4. Involvement of CB2 cannabinoid receptors in the anti-hypernociceptive effect of TPL-Xa.

The threshold of hypernociception in animals with acute pancreatitis was not altered by the administration of selective CB1/CB2 antagonists (Fig. 6B;D). However, the administration of AM630 (selective antagonist of CB2 receptors), but not of AM281 (selective antagonist of CB1 receptors) reversed the TPL-Xa protective effect (Fig. 6 B;D). In addition, AM 630 reversed the protection of TPL-Xa observed in total histopathological scores (Fig. 6E) and the inhibition of CB2 receptor expression (Fig. 6F).

Before treatments (time zero of the von Frey test) no differences were observed between groups (Fig. 6A;C).

4. Discussion

In this study a heteropolysaccharide was identified in the polysaccharide extract obtained from the medicinal plant *X. americana* barks (TPL-Xa). This heteropolysaccharide was shown to be composed of α-D-glucose, α-D-galactose, α-L-

arabinose, α-rhamnose, L-fucose and galacturonic acid, and reduced the inflammatory parameters of caerulein-induced acute pancreatitis in mice.

The ^{1}H and ^{13}C NMR spectra of TPL-Xa corroborates other data demonstrated by chemical analysis, agarose gel electrophoresis, monosaccharide composition and infrared spectra previously showed for the two major polysaccharide rich fractions isolated from TPL-Xa. This fractions revealed the presence of uronic acid (8-39%), arabinose (39-57%), rhamnose (4-7%), galactose (16-20%) and glucose (7-35%) in its monosaccharide composition and infrared spectra demonstrated peaks in carbohydrate range for COO^{-} groups of uronic acid [24]. Importantly, some studies have related the presence of these acidic compounds of the cell wall of plants with some biological activities, such as, antitussive, antioxidant, anti-inflammatory and anticoagulant [37,38,39]. Thus, it is possible to speculate that the presence of uronic acid residues (21%) in the *X. americana* heteropolysaccharide may be contributing to the inhibitory effect of TPL-Xa on inflammatory parameters of acute pancreatitis induced by cerulein. However, additional trials to assess the relationship structure-activity are still required. Furthermore, in other studies, phytochemical screening for major constituents for ethanol and water extracts of *Ximenia americana* barks demonstrated also the presence of saponins, tannins, flavonoids, alkaloids, phenolics, glycosides, resins, quinones and terpenoids [40,41].

TPL-Xa protected the inflammatory damage seen by the decrease of histological alterations (edema, neutrophil infiltration and acinar necrosis) and MPO activity, a common indirect marker of neutrophil infiltration [28] and oxidative stress [42]. The model of hyperstimulation of murine exocrine pancreas with caerulein, a cholecystokinin analogue, is one of the most widely used and the best characterized model of acute pancreatitis due to its high applicability, rapid induction, reproducibility and low invasiveness [43]. Hyperstimulation of isolated mouse pancreatic acinar cells with cholecystokinin causes sustained cytosolic calcium overload and intracellular trypsin activation resulting in cell death [44]. One of the consequences of pancreatic enzymatic activation is a local inflammatory response, characterized by oedema and neutrophil infiltration, the latter closely associated to the development of caerulein-induced acute pancreatitis [45].

Our data suggest that the protective action of TPL-Xa in pancreatic inflammation may particularly involve reduction of neutrophil infiltration, an effect already demonstrated for plant polysaccharides [18,19,46,47]. In addition, previous data

has demonstrated in mice that the ethanolic extract of *X. americana* bark reduced cell migration in carrageenan-induced peritonitis in mice [48]. These data all together support the anti-inflammatory use of *X. americana* bark in folk medicine [25].

In the present study, we also demonstrated that TPL-Xa attenuated the visceral hypernociception in caerulein-induced AP via mechanisms that involve cannabinoid receptors, since the pretreatment with CB2 receptor antagonist (AM630) partially reversed the antinociceptive effect of TPL-Xa.

The cannabinoid system modulates acute and chronic pain from several diseases [49,50]. Previous studies suggested the involvement of endocannabinoids in acute pancreatitis [12,51]; HU210, a potent central cannabinoid (CB1) and peripheral cannabinoid (CB2) receptor agonist, inhibited inflammation and pancreatic hypernociception in the caerulein-induced model of AP in mice [12]. Therefore mice lacking of CB1 in primary nociceptors developed higher hypernociception to abdominal mechanical stimuli after pancreatic inflammation [51]. The CB2 receptor subtype has a widespread distribution in the immune system [52], including endocrine and pancreas exocrine cells [53] and CB2 receptor agonists have significant antinociceptive actions in neuropathic [54] chronic [55] and inflammatory [56] pain models. Additionally, activation of CB2 receptors may exert anti-inflammatory effects via reduction of NF-κB [57]. However, no specific data are available in exocrine pancreas. In fact, the role of the cannabinoid system in the development of AP and the relevance of endocannabinoid receptor subtype involvement remains unclear [58]. Consistent with the previous study, we found an increase in CB2 expression in pancreatic tissue with cerulein-induced AP [12]. In our evaluation, the effect was decreased by the treatment with TPL-X and the fate was lost after the treatment with the CB2 receptor antagonist, justifying the effect found in the Frey test.

Despite the importance of pain in pancreatitis, its pathophysiological mechanisms are still poorly understood. However, it is recognised that this type of pain is characterized as being recurrent, hyperalgesia intense and long-lasting [59] and is associated with decrease in the threshold to mechanical stimulation of the abdominal region, an area of referred pain [30]. In the present study, TPL-Xa attenuated visceral hypernociception induced by pancreatitis in mice, increasing the threshold to mechanical stimuli (von Frey test). This action of TPL-Xa on inflammatory pain and hypernociception in acute pancreatitis is consistent with the wellknown activities of plant polysaccharides in the immune system [60,61], including anti-inflammatory

[18,19,62]. Studies in mice have recently demonstrated antinociceptive activities in visceral inflammatory pain of crude polysaccharide extracts of *Thladiantha dubia* Bunge [20] and polysaccharides isolated from *Solanum betaceum* Cav. fruit (tamarillo) and *Solanum lycopersicum* L [21–23]. Furthermore, the inhibitory effect of the aqueous extract and polysaccharide fractions of *X. americana* barks has been previously demonstrated in classic models of nociception, including in the visceral nociception induced by acetic acid [24,63]. Furthermore, this data is consistent with the popular use of *X. americana* in painful conditions, such as stomachache and headache [25,64].

It is important to highlight, that the systemic treatment of animals with TPL-Xa did not alter locomotor activity, a common adverse side effect of analgesic drugs. Therefore, polysaccharides obtained from natural sources represent a structurally diverse class of macromolecules known to modulate a variety of biological responses, causing low toxicity [17].

In conclusion, the total polysaccharides obtained from *X. americana* bark contain a heteropolysaccharide that possesses anti-inflammatory and antinociceptive effects in caerulein experimental acute pancreatitis, actions involving cannabinoid type 2 receptors.

Conflict of interest

The authors declare no competing financial interest.

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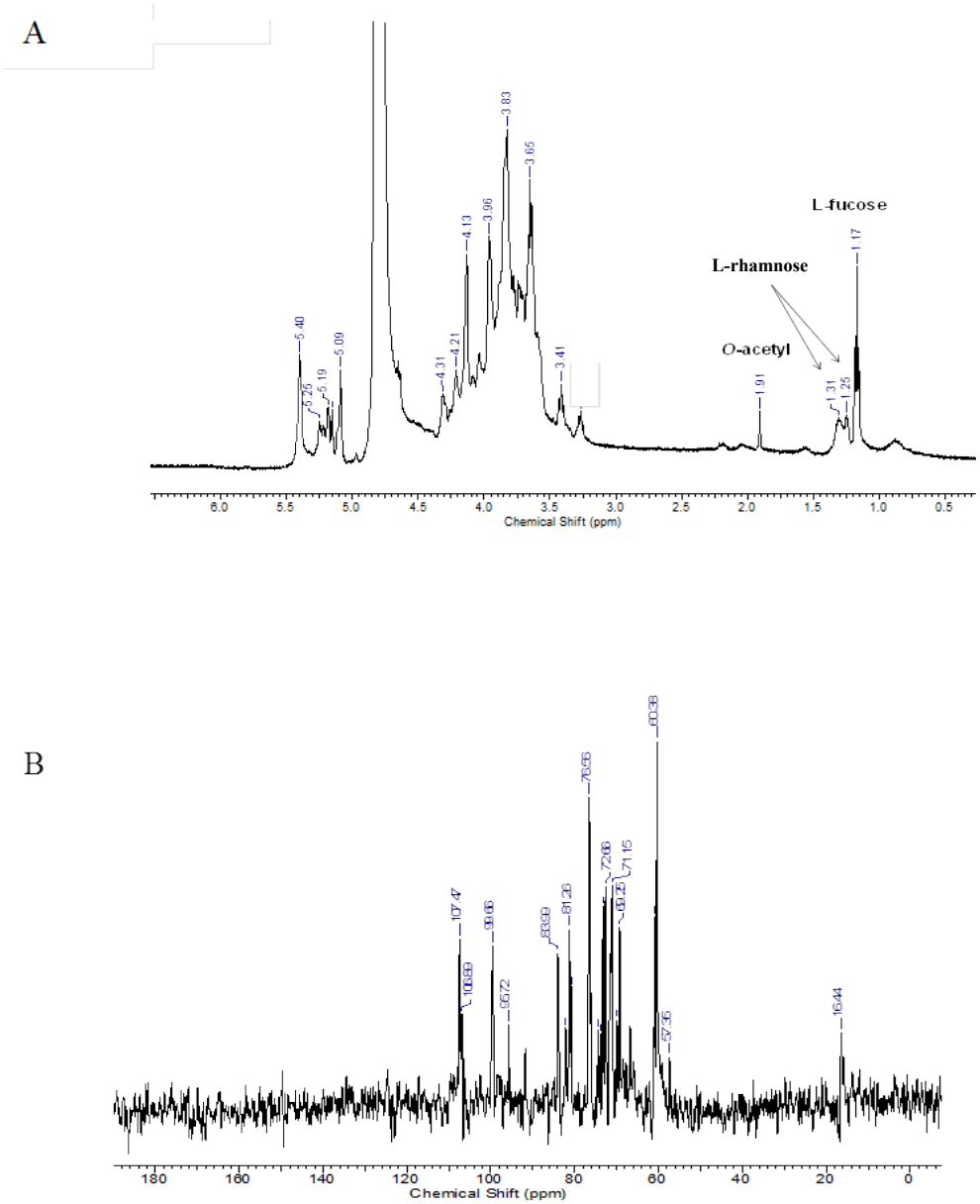

Fig. 1 NMR of TPL-Xa. ^{1}H (A) and ^{13}C (B).

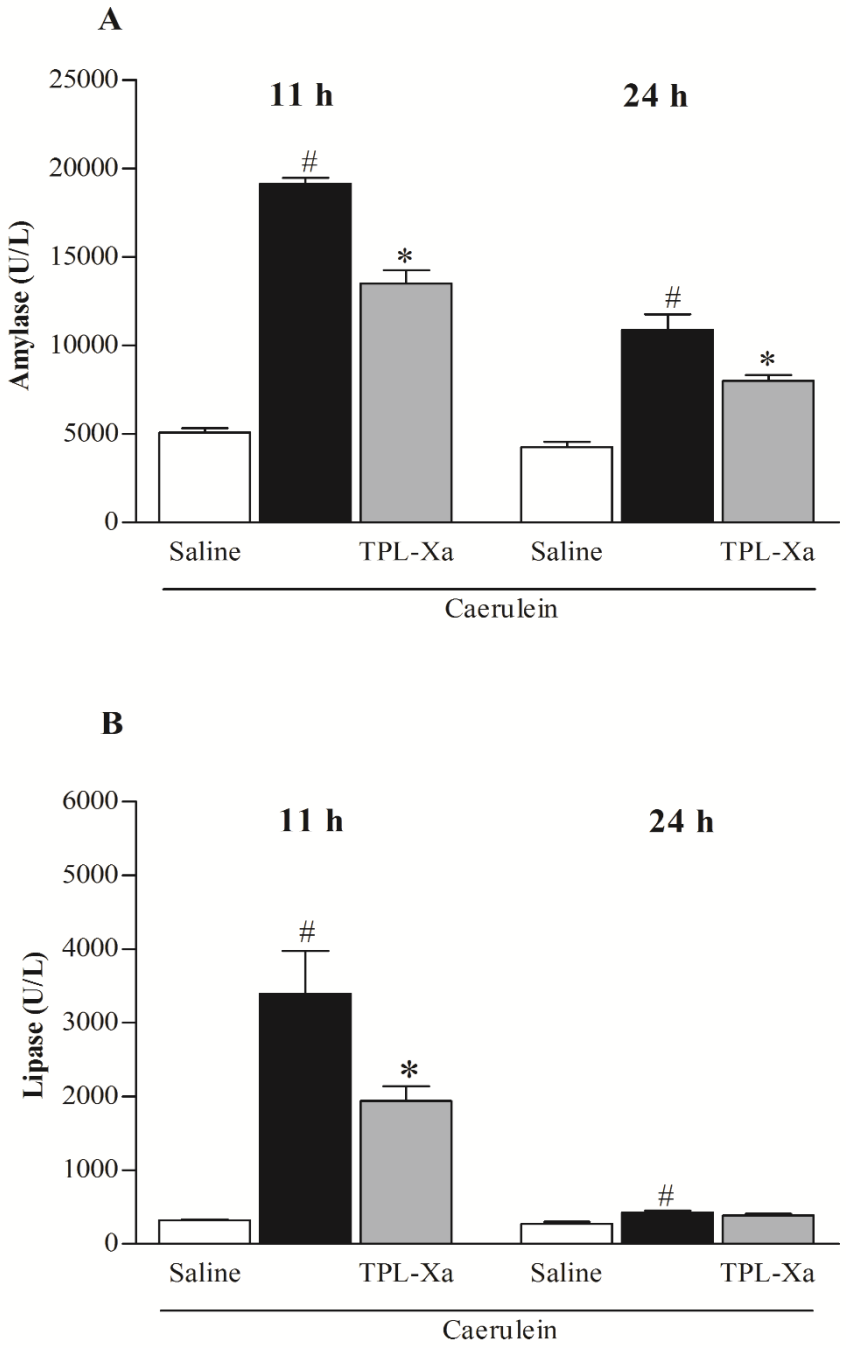

Fig. 2 TPL-Xa reduces pancreatic enzymes. Mice received 10 i.p. injections of caerulein (50 μg/kg) or saline at 1 h intervals. TPL-Xa (10 mg/kg) or saline was administered i.v. 30 min before the first and 30 min after the 10th dose of caerulein. Mice were sacrificed 11 h and 24 h after the caerulein first injection. Blood samples were taken and the plasma levels of amylase (A) and lipase (B) were measured. Mean ± S.E.M. (n = 8). ANOVA and Bonferroni test, *$p<0.05$ compared to caerulein; #$p<0.05$ compared to saline.

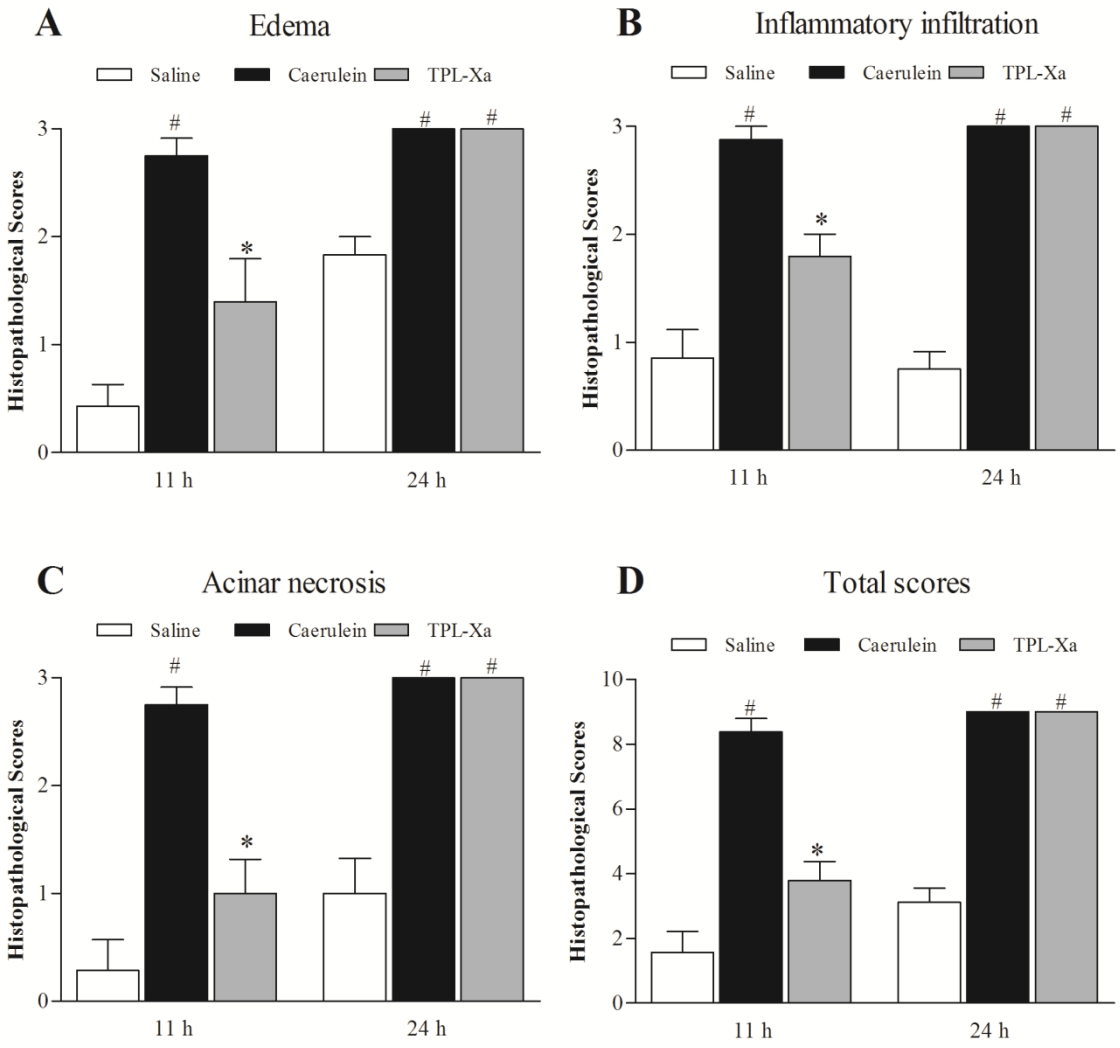

Fig. 3 TPL-Xa inhibits histological damage in acute pancreatitis. Mice received 10 i.p. injections of caerulein (50 µg/kg) or saline at 1 h intervals. TPL-Xa (10 mg/kg) or saline was administered i.v. 30 min before the first and 30 min after the 10th dose of caerulein. Control animals received saline (i.p.) instead of caerulein. Mice were sacrificed at 11 h and 24 h after the first injection of caerulein for histopathological evaluation: (A) Edema; (B) inflammatory infiltration; (C) acinar cell necrosis; (D) total scores. Values are expressed as median (maximum and minimum) (n=6). ANOVA, Kruskal-Wallis followed by Dunn test. *$p<0.05$ compared to caerulein; #$p<0.05$ compared to saline.

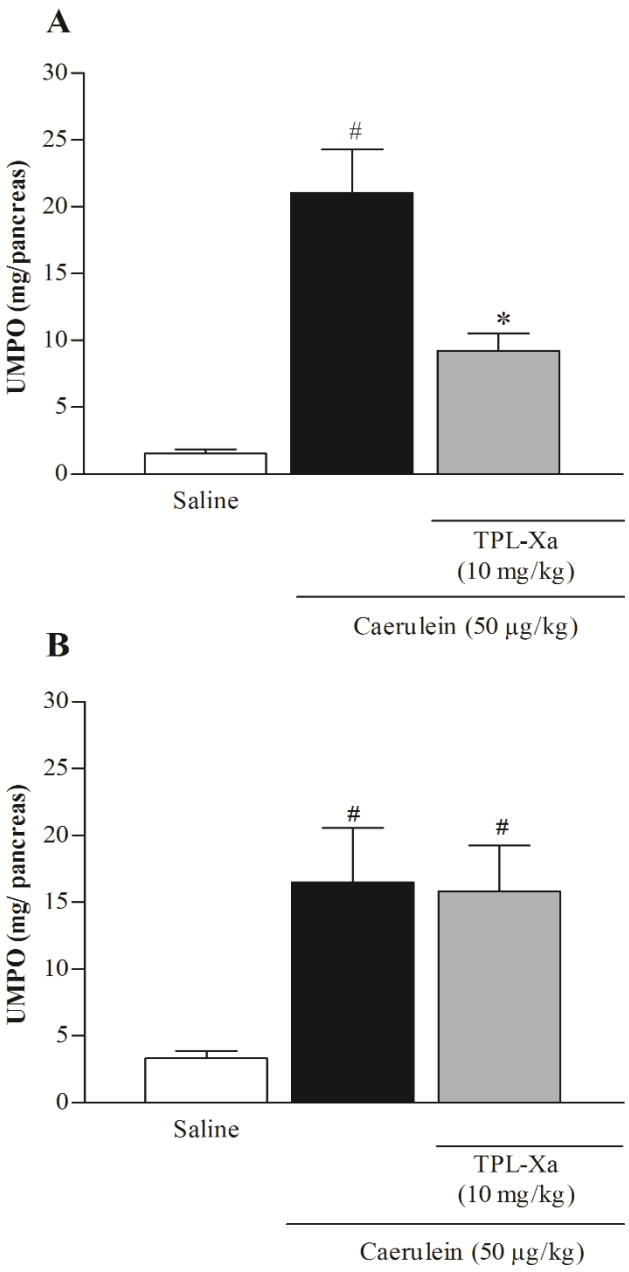

Fig. 4 TPL-Xa inhibits MPO activity in acute pancreatitis. Mice received 10 i.p. injections of caerulein (50 µg/kg) or saline at 1 h intervals. TPL-Xa (10 mg/kg) or saline was administered i.v. 30 min before the first and 30 min after the 10th dose of caerulein. Control animals received saline (i.p.) instead of caerulein. Mice were sacrificed (A) 11 h and (B) 24 h after the first injection of caerulein for evaluation of MPO activity. Values are expressed as units of MPO (UMPO) per milligram of tissue. Mean ± S.E.M. (n = 8). ANOVA and Bonferroni test. *$p<0.05$ compared to caerulein; #$p<0.05$ compared to saline.

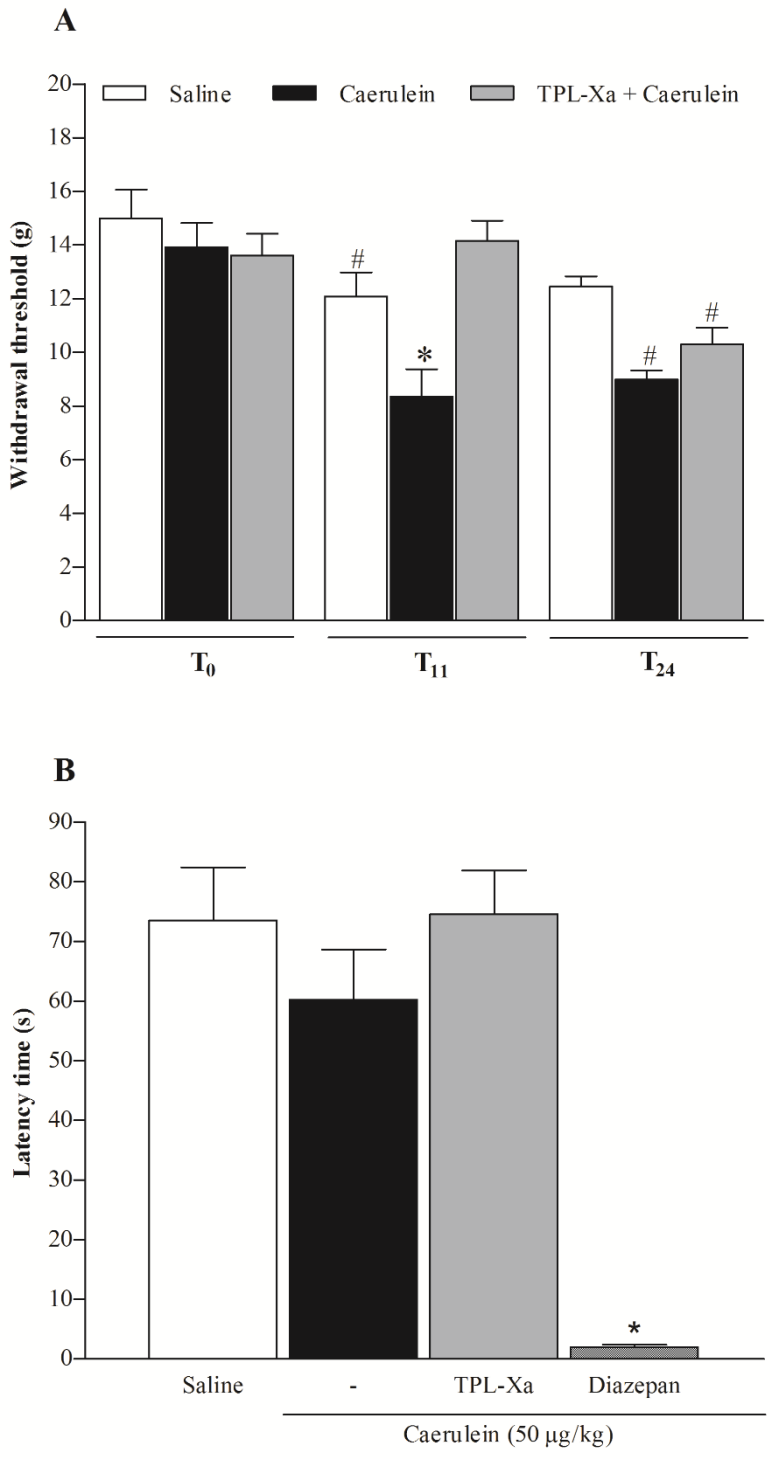

Fig. 5 TPL-Xa inhibits visceral hypernociception without alteration of locomotor activity. Mice received 10 i.p. injections of caerulein (50 µg/kg) or saline at 1 h intervals. TPL-Xa (10 mg/kg, i.v.), saline (i.v.) or diazepam (5 mg/kg, i.p.) was administered 30 min before the first and 30 min after the 10th dose of caerulein. (A) Visceral hypernociception was evaluated by the von Frey test at the 11th and 24th h after the first injection of caerulein. (B) Locomotor function was evaluated at the 11th h in the rota-Rod test. Mean ± S.E.M. (n = 8). ANOVA and Bonferroni test. *$p<0.05$ compared to caerulein; #$p<0.05$ compared to saline.

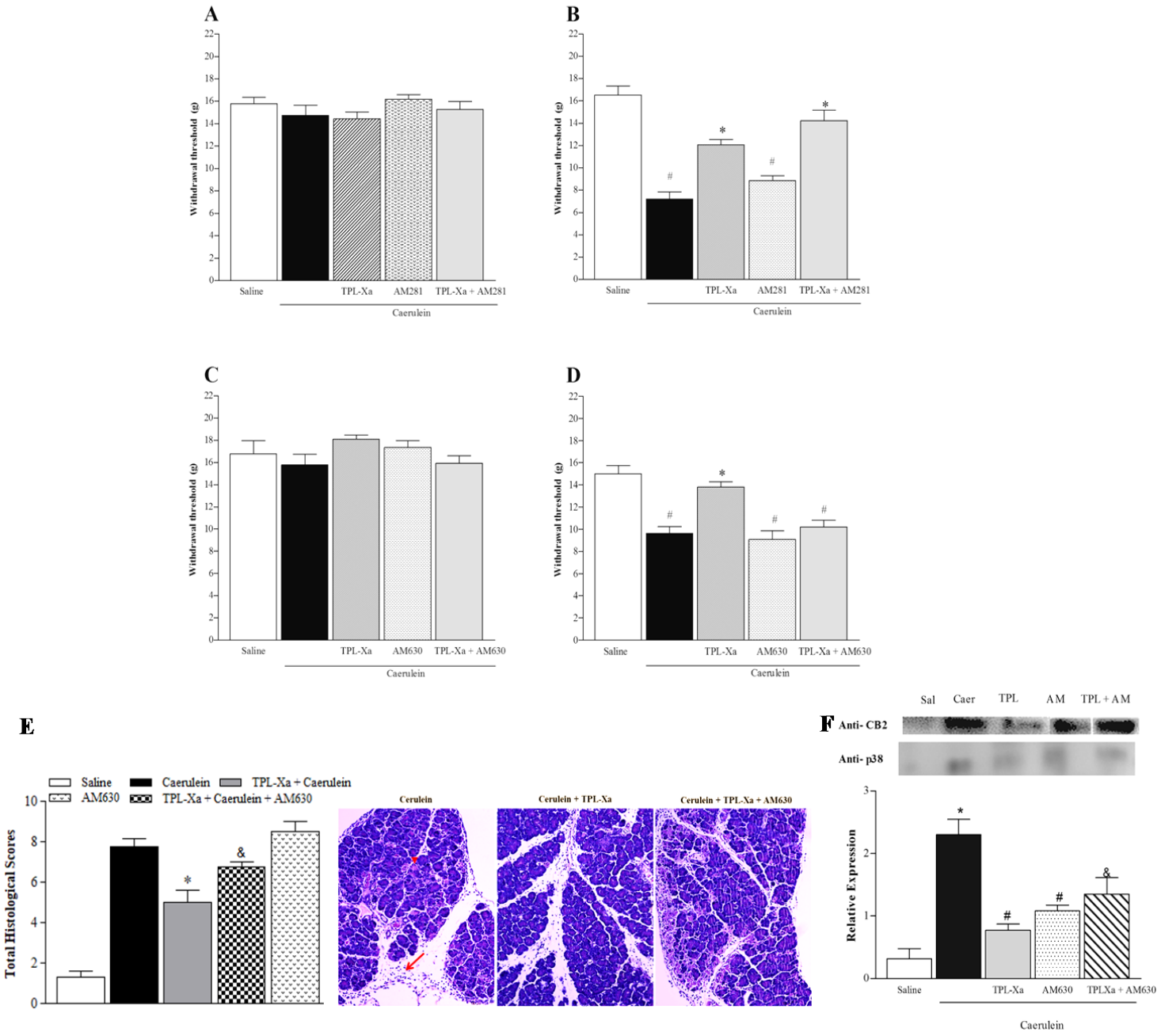

Fig. 6 Involvement of CB2 cannabinoid receptor in the anti-hypernociceptive effect of TPL-Xa. Mice received 10 i.p. injections of caerulein (50 µg/kg) or saline at 1 h intervals. (A; C) Non-treated animals evaluated at baseline (time zero); (B) Animals pre-treated with saline (i.v., TPL-Xa (10 mg/kg; i.v.), AM281 (3 mg/kg, s.c.) or TPL-Xa + AM281; (D, E and F) Animals pre-treated with saline (i.v.), TPL-Xa, AM630 (1 mg/kg, s.c.) or TPL-Xa + AM630. (D) Withdrawal threshold (E) showing total histological scores, in the photomicrographs intense neutrophil infiltrate (red arrow) and necrosis (arrowhead). Hypernociception, histological analyses and expression of CB2 was evaluated 11 hours after the first injection of caerulein. Photomicrographs of pancreas tissue (100X magnification). Mean ± S.E.M. (n= 8). ANOVA and Bonferroni test. *p<0.05 compared to caerulein; #p<0.05 compared to saline and (&) p<0.05 compared to TPL-Xa + caerulein.