Alpha7 cholinergic-agonist prevents systemic inflammation and improves survival during resuscitation

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Abstract

Severe haemorrhage is a common cause of death despite the recent advances in critical care. Conventional resuscitation fluids are designed to re-establish tissue perfusion, but they fail to prevent inflammatory responses during resuscitation. Our previous studies indicated that the vagus nerve can modulate systemic inflammation *via* the alpha7 nicotinic acetylcholine receptor (α7nAchR). Here, we report that the alpha7nAChR-agonist, GTS, restrains systemic inflammation and improves survival during resuscitation. Resuscitation with GTS rescued all the animals from lethal haemorrhage in a concentration-dependent manner. Unlike conventional resuscitation fluids, GTS inhibited the production of characteristic inflammatory and cardiodepressant factors including tumour necrosis factor (TNF) and high mobility group B protein-1 (HMGB1). Resuscitation with GTS was particularly effective in restraining systemic TNF responses and inhibiting its production in the spleen. At the molecular level, GTS inhibited p65RelA but not RelB NF-κB during resuscitation. Unlike non-specific nicotinic agonists, GTS inhibited serum protein TNF levels in both normal and splenectomized, haemorrhagic animals. Resuscitation with GTS inhibited poly(ADP-ribose) polymerase and systemic HMGB1 levels. Our studies suggest that GTS provides significant advantages as compared with non-specific nicotinic agonists, and it could be a promising anti-inflammatory supplement to improve survival during resuscitation.

Keywords: haemorrhagic shock • cytokines • TNF • HMGB1 • inflammation • alpha7nAchR • nicotinic receptors • resuscitation

Introduction

The innate immune system is an essential component of our defences to trauma and injury. But at the same time, it is one of the principal causes of morbidity and mortality in critical care [1–3]. This outcome is especially dramatic in severe haemorrhage, because after losing over 50% of the blood volume, the system is unable to re-establish tissue perfusion. Thus, resuscitation fluids are classically designed to restore circulatory volume and tissue perfusion but they fail to prevent inflammatory responses during resuscitation. Characteristic inflammatory cytokines such as tumour necrosis factor (TNF) and high mobility group B protein-1 (HMGB1) contribute to lethal cardiovascular shock during

resuscitation [2–4]. TNF diffuses in the bloodstream and produces a fatal cardiovascular collapse [4]. TNF is a sufficient and necessary mediator of 'haemorrhagic shock' because: (*i*) it is found in patients and experimental models of 'haemorrhagic shock'; (*ii*) TNF may contribute to the lethality of haemorrhagic shock; and (*iii*) TNF neutralization attenuates cardiovascular shock. In addition to TNF, recent studies indicated that HMGB1 plays an important role in haemorrhagic shock. HMGB1 was originally identified as a nuclear DNA-binding protein that functions as a structural co-factor. However, during cellular damage or necrosis, HMGB1 is liberated into the extracellular milieu where it functions as an inflammatory cytokine [5–7]. Extracellular HMGB1 sustains inflammatory responses [5] producing abrupt cardiac standstill [8], intestinal derangement [9] and acute lung injury [10]. For these reasons, there is a major interest in inhibiting the production of these factors during resuscitation.

The nervous system is an important regulator of the immune system, and neuronal anti-inflammatory mechanisms have been selected by evolution to modulate inflammatory responses [1].

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These mechanisms can provide a major advantage for novel pharmacological anti-inflammatory strategies to control systemic inflammation [1, 11]. We reported that the parasympathetic nervous system restrains systemic inflammation *via* the vagus nerve [1, 11–13]. Electrical stimulation of the vagus nerve inhibits serum TNF levels in wild-type but not in alpha7nAChR-knockout mice [14, 15]. However, the anti-inflammatory potential of this mechanism is limited by the capacity of the vagus nerve to release acetylcholine and the neuronal innervation of specific organs [15]. Thus, we studied the anti-inflammatory potential of acetylcholine, the principal neurotransmitter of the vagus nerve. Acetylcholine inhibits the production of both TNF and HMGB1 in macrophages *via* the alpha7 nicotinic acetylcholine receptor (nAChR) [12–14]. *In vivo*, treatment with nicotine, a more selective cholinergic agonist, inhibits serum TNF and HMGB1 levels and improves survival in experimental models of systemic inflammation [12, 16]. Nicotine has been used in several clinical trials including inflammatory bowel disorders [17], but its therapeutic potential is limited by its collateral toxicity. We proposed that alpha7nAChR-agonists could avoid the collateral toxicity of nicotine [1, 11, 13]. GTS is a characteristic alpha7nAChR-agonist, has been used in clinical trials and has proven to be less toxic than nicotine [18, 19]. GTS has been used in clinical trials to target neuronal alpha7nAChR in the brain of patients with Alzheimer's disease [20]. These studies showed a limited effect of GTS on the central nervous system potentially due to its limited ability to cross the blood–brain barrier. From an immunological perspective, this characteristic is an advantage to use GTS in the periphery and avoid secondary effects on the central nervous system. In contrast to nicotine, GTS has no effect on locomotor activity or on dopamine turnover indicating that it is less toxic than nicotine [18, 19]. Thus, we reasoned that GTS would represent a promising supplement to control systemic inflammation during resuscitation.

Recently, we reported that similar to vagus nerve stimulation, nicotine inhibits TNF and HMGB1 production from macrophages *via* alpha7nAChR [12]. Here, we tested the ability of the alpha7-agonist, GTS, to control systemic inflammation during resuscitation. Hextend was used as the control resuscitation fluid because recent studies indicate that it prevents multiple organ injury [21] and improves short-time survival as compared with saline solution [22]. Hextend is a novel plasma volume expander containing 6% hydroxyethyl starch in Ringer's lactate [23]. Hetastarch creates oncotic pressure, which is normally provided by blood proteins and permits retention of intravascular fluid. Our current study indicates that resuscitation with Hextend supplemented with GTS rescued all the animals from lethal haemorrhage and improved survival in a concentration-dependent manner. GTS inhibited the production of cardiodepressant factors including both HMGB1 and TNF. It was particularly effective at inhibiting TNF production in the spleen and modulating splenic NF-kB during resuscitation. Unlike non-specific nicotinic agonists, GTS inhibited systemic TNF levels in both control and splenectomized animals. Resuscitation with GTS inhibited hepatic poly(ADP-ribose) polymerase and systemic HMGB1 levels. Our studies suggest that GTS

is a promising supplement to control systemic inflammation during resuscitation and improve survival in severe haemorrhage.

Materials and methods

Animal experiments

Adult male Sprague–Dawley (350–450 g) rats were purchased from Harlen Sprague–Dawley (Indianapolis, IN, USA) and allowed to acclimate for 7 days, housed at 25°C on a 12-hr light/dark cycle. Animals were randomly grouped and assigned to a specific experiment. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee of the North Shore University Hospital, and the New Jersey Medical School. Investigators were blinded to the experimental treatment.

Haemorrhage

Animals were anaesthetized by inhalation of isoflurane (5% induction, 2% maintenance; Minrad, Buffalo, NY, USA) and subjected to surgical catheter placement into the femoral artery and vein under sterile conditions. To avoid blood clot and maintain catheter patency, the catheters were flushed with 1% heparin solution immediately before placement. Heparin was not used *in vivo* during the experiment or observation period in agreement with previous studies [24]. After the catheter implantation, the blood pressure and the heart rate were recorded for 15 min. to establish a physiological baseline prior the haemorrhage procedure. Then, the animals were subjected to haemorrhage over 15 min. to reach a mean arterial blood pressure (MAP) of 35–40 mmHg and subsequent maintenance of this blood pressure by continued blood withdrawal for another 15 min. After the shock phase, resuscitated animals received specific resuscitation treatment over 40 min. with a total volume of 15 ml/kg (equivalent to 1000 ml of Hextend in a 70 kg man). The femoral artery was catheterized with a catheter R-FAC (Braintree Scientific, Inc., Braintree, MA, USA) previously flushed with 1% heparin in 0.9% saline to allow spontaneous bleeding due to arterial blood pressure. Shed blood was considered lost and it was not reinfused. After resuscitation treatment, the animals were kept anaesthetized for 2 hrs to record heart rate, blood pressure and blood chemistry. The control animals were also anaesthetized with isoflurane for blood collection. Then, the anaesthesia was stopped and the animals were allowed to recover from anaesthesia and housed individually in regular cages. Splenectomy was performed as we described [15]. Briefly, the spleen was identified following a midline laparotomy incision, and removed after appropriate blood vessel ligation. Sham animals underwent laparotomy without splenectomy.

Blood chemistry and cytokine measurement

Activation blood was collected at 2 hrs after the haemorrhagic shock and analysed using the i-Stat blood analyzer (Abbot Laboratories, IL, USA). Lung function was assessed by analysing blood gases including total and partial carbon dioxide (TCO_2, PCO_2), bicarbonate (HCO_3), pH and the base excess of extracellular fluid (BEecf). The organ function tests include anion sodium, potassium, chloride, the anion gap (AnGap), total plasma protein

(TP), and blood urea nitrogen (BUN). Blood chemistry also included glucose, haematocrit and haemoglobin. The systemic inflammatory status was assessed by analysing critical pro-inflammatory cytokines and cardiodepressant factors such as TNF and HMGB1. The major organs were collected and immediately sliced and washed with PBS at 4°C. TNF concentrations in serum and major organs were determined by ELISA (R&D Systems, Inc., Minneapolis, MN, USA). HMGB1 was analysed by Western blot as previously described [12].

NF-κB and PARP analyses

Homogenates were normalized by protein concentration and the activation of IκBα was analysed by Western blot by using anti-IκBα polyclonal antibody ab7217 and anti-phosphorylated-IκBα(phospho S32 + S36) monoclonal antibody [clone39A1431] ab12135 (Abcam; Cambridge, MA, USA). Transcriptional activity of splenic p65RelA was analysed by TransAM DNA-Binding ELISA (Active Motif; Cambridge, MA, USA) following manufacturer's instructions. The results were confirmed by EMSA from nuclear extracts performed as we previously described [12]. PARP activity in organ homogenates was analysed using a commercially available kit (R&D Systems, Cat#4677–096K) following the manufacturer's instructions. Protein levels were measured using the Bradford method (Bio-Rad Lab Inc., Hercules, CA, USA) and PARP activity was normalized to protein content.

Statistical analyses

All data in the figures and text are expressed as mean ± standard deviation (SD). Statistical analyses were performed using the one-way ANOVA with multiple pairwise comparisons with the Bonferroni's adjustment for multiple hypothesis testing. The Student's t-test was used to compare mean values between two experimental groups. Statistical analyses of survival were determined using the logrank test. P-values <0.05 were considered statistically significant.

Results

Alpha7nAChR-agonist improved survival during resuscitation

Haemorrhagic shock required withdrawing 21 ± 4.3 ml blood/kg body weight, and the maintenance of that blood pressure for another 15 min. required another 7 ± 2.7 ml blood/kg body weight. Animals without resuscitation treatment (NR; $n = 10$) did not re-establish normal blood pressure (Fig. 1A), and all animals died within the first 5 hrs after haemorrhage with an average time of death of 161 ± 26 min. (Fig. 1B). Resuscitation with 15 ml/kg Hextend allowed the animals to recover normal blood pressure and re-establish tissue perfusion, but still, 90% of the animals died within the first 5 hrs after haemorrhagic shock ($P < 0.001$, logrank test Hextend *versus* NR; $n = 9$). Resuscitation with Hextend supplemented with 350 uM GTS rescued all the animals from lethal haemorrhage ($P < 0.05$, logrank survival test *versus*
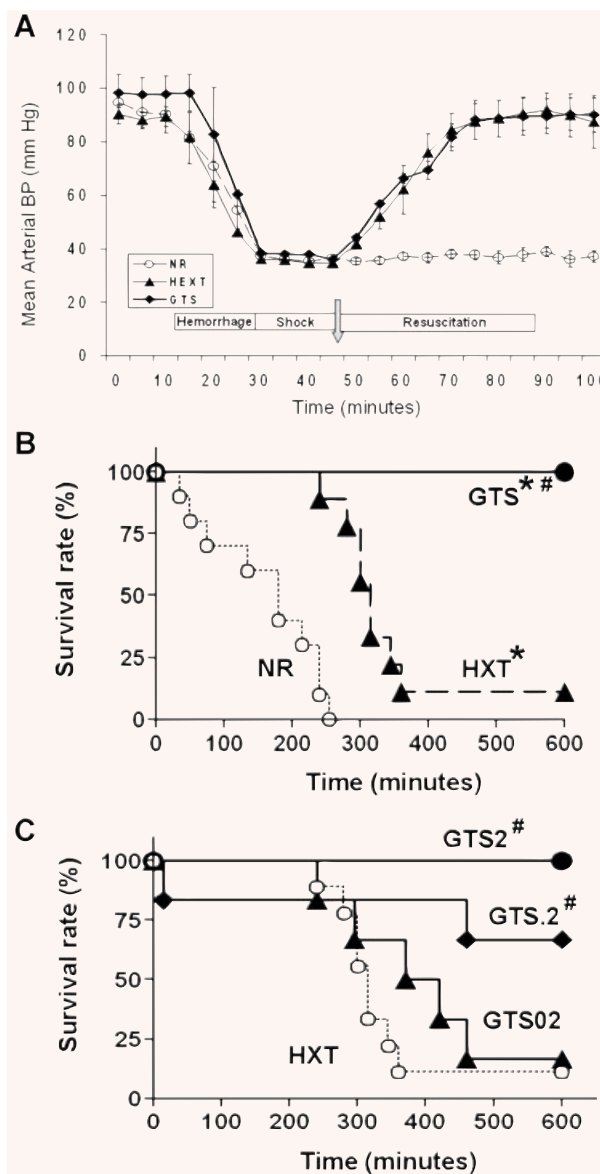
Fig. 1 Resuscitation with alpha7nAChR-agonist improves survival in severe haemorrhage. Adult male Sprague–Dawley haemorrhagic rats received no resuscitation (NR) or were resuscitated with 15 ml/kg (i.v.) Hextend (HEXT) or Hextend supplemented with GTS (GTS). (**A**) Arterial blood pressure was recorded during haemorrhage and the resuscitation treatment. (**B**) All animals without resuscitation treatment (NR; $n = 10$) died within the first 5 hrs after haemorrhage with an average time of death of 161 ± 26 min. Resuscitation with Hextend allowed the animals to restore normal blood pressure and tissue perfusion, and yet 90% of the animals died ($n = 9$). Resuscitation with Hextend supplemented with GTS (GTS) statistically protected all the animals ($n = 7$/group). (**C**) Treatment with 2 (GTS2), 0.2 mg/kg (GTS.2), but not 0.02 mg/kg (GTS02) GTS induced a statistically significant protection as compared with Hextend solution alone. $P < 0.05$ survival logrank test *versus* NR (*) or HXT(#).

Hextend; $n = 7$). GTS improved survival in haemorrhagic shock in a concentration-dependent manner (Fig. 1C). Treatment with 2 or 0.2 mg/kg but not 0.02 mg/kg GTS induced a statistically significant protection as compared with Hextend solution alone. Treatment with 0.2 mg/kg GTS protected 67% of the animals with haemorrhagic shock, whereas 0.02 mg/kg GTS protected 17% of the animals ($n = 7$/group). Several animals died during the haemorrhagic shock and three animals died during the resuscitation treatment. One animal died during resuscitation with Hextend alone, one during resuscitation with Hextend+GTS at 2 mg/kg and another one during resuscitation with Hextend+GTS 0.2 mg/kg. All these animals were excluded from our statistic analyses as many of these deaths were attributed to a particular problem with the anaesthesia system. Our analyses excluded the animals that died during the surgical procedure and counted only those animals that completed the resuscitation treatment. All non-survival animals died within the first 8 hrs after haemorrhagic shock. All the animals that passed this critical period survived the haemorrhagic shock, and no late deaths were found, although the animals were followed for up to 7 days. These results suggest that GTS induced a lasting protection and it did not merely delay the pathologic onset.

Alpha7nAChR-agonist prevented systemic inflammation during haemorrhage

Characteristic pathological markers of haemorrhage were assessed by blood chemistry. Animals without resuscitation were characterized by uraemia, metabolic acidosis and hyperglycaemia (Fig. 2). Animals without resuscitation showed characteristic high levels of BUN. Although resuscitation with Hextend or Hextend supplemented with GTS improved survival, they did not prevent uraemia (Fig. 2A). Haemorrhagic animals without resuscitation also showed metabolic acidosis that was associated with elevated anion gap (AnGap) levels, low levels of chloride and bicarbonate (Fig. 2B–E). Both resuscitation with Hextend, or Hextend supplemented with GTS prevented metabolic acidosis and re-established normal values of AnGap and bicarbonate but not hypochloraemia. Haemorrhage also causes a characteristic hyperglycaemic response that was significantly prevented by both resuscitation with Hextend and Hextend supplemented with GTS (Fig. 2F). Together, these results indicated that the therapeutic potential of GTS was not due to these mechanisms.

Because our previous studies indicated that the vagus nerve controlled systemic TNF responses *via* alpha7nAChR, we reasoned that the alpha7-agonist will produce a similar effect. Indeed, the most significant effects of GTS were in inhibiting systemic TNF levels. Haemorrhage induced a systemic TNF response that was not affected by resuscitation with Hextend (Fig. 3A). However, resuscitation with Hextend containing GTS inhibited this lethal response, and these animals had circulating TNF levels similar to those in control mice. Albeit these results appear not to

be statistically significant with Bonferroni's corrections, resuscitation with Hextend alone reduced TNF levels in the lung, liver and spleen, though not to the extent of GTS, and yet there was little influence upon serum and cardiac TNF. Because the effects of Hextend have been previously described, we have performed conservative statistical analyses to focus on the effects of Hextend+GTS as compared with Hextend alone. As compared with Hextend, resuscitation with GTS blunted TNF levels in all of the organs, but particularly in the spleen, liver and the heart. Because our studies indicated that both acetylcholine and nicotine inhibit NF-κB in macrophages *via* alpha7nAChR [12, 14], we wondered whether GTS would inhibit this pathway during resuscitation. Canonical p65RelA and alternative RelB binding to DNA were analysed by TransAM DNA-Binding ELISA (Fig. 4). Nuclear extracts from major organs were collected at 2 hrs after haemorrhage, which is approximately 30 min. before the average time of death for the control animals without resuscitation treatment. Resuscitation with Hextend did not affect p65RelA in the liver but increased its activation in the heart and the spleen. Haemorrhage induced a significant activation of both pathways but conventional resuscitation further activated p65RelA but not RelB. These results concur with recent studies indicating that resuscitation promotes systemic inflammatory responses. Resuscitation with Hextend supplemented with GTS inhibited p65RelA NF-κB in most of the organs but not in the heart. Again, the most significant results with GTS were found in the spleen. These effects were specific for p65RelA NF-κB as RelB was not affected by either resuscitation with Hextend or GTS (Fig. 4D).

Our results suggest that GTS may inhibit systemic and cardiac TNF levels by inhibiting TNF transcription in the spleen similar to what we described in experimental models of sepsis [15]. We analysed mRNA TNF levels in the heart and the spleen by real-time PCR (Fig. 5). Haemorrhage increased mRNA TNF levels in both the heart and the spleen as analysed by real-time PCR. As compared with Hextend, GTS inhibited NF-κB and decreased mRNA TNF levels in the spleen but not in the heart. In order to determine whether the inhibition of splenic TNF is sufficient to account for the anti-inflammatory potential of GTS, we analysed the pharmacological implications of the spleen during resuscitation.

Pharmacological implications of the spleen in resuscitation

Our previous studies indicated that both vagus nerve stimulation and treatment with nicotine attenuated systemic TNF levels in sham but not in splenectomized animals [15]. We studied the pharmacological implications of the spleen during resuscitation using splenectomized animals with haemorrhage. Blood chemistry analyses showed that haemorrhage in splenectomized animals induced similar uraemia, metabolic acidosis and hyperglycaemia as compared to normal animals (data not shown). Again, the most significant effects of GTS were found in systemic
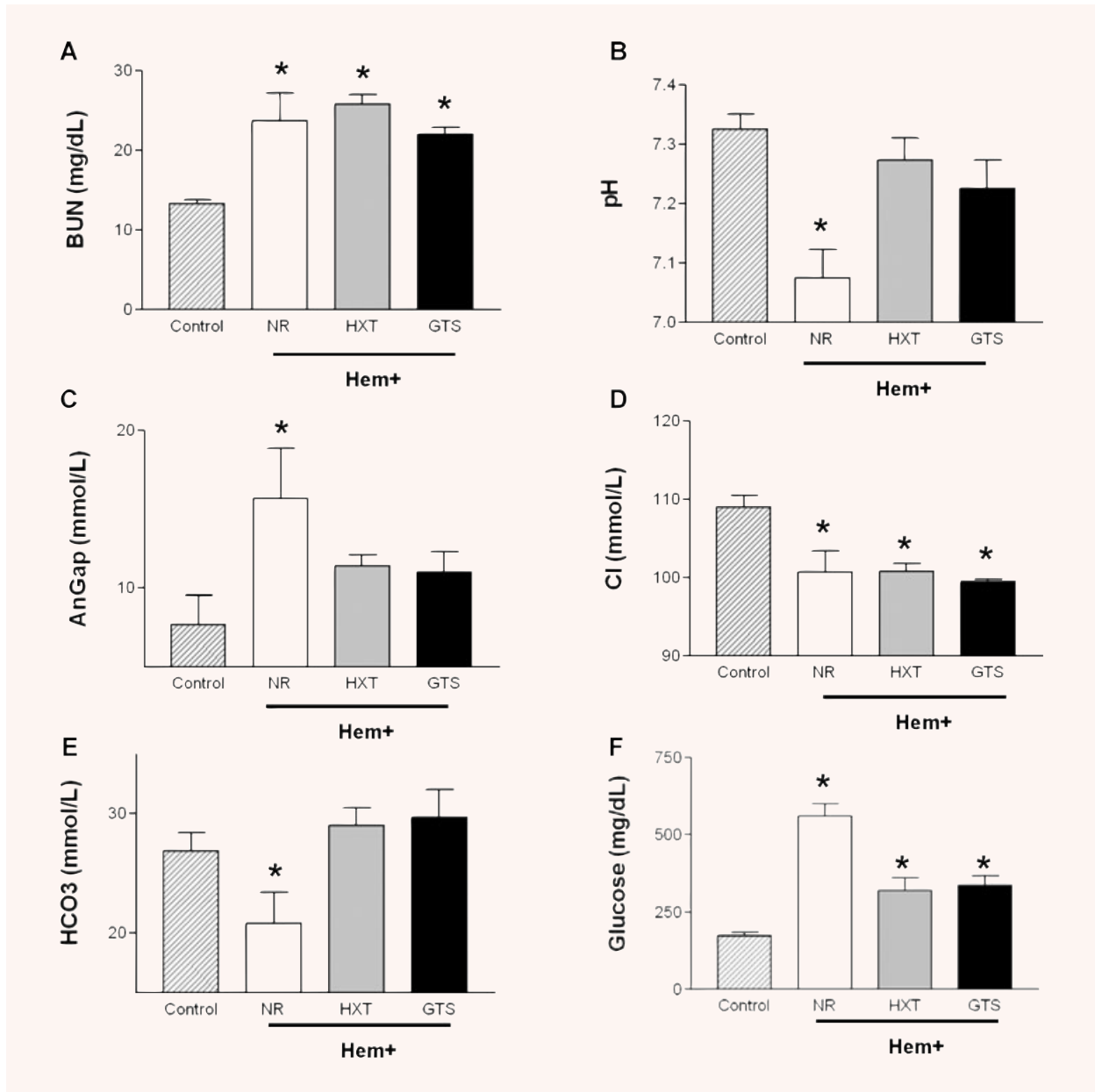
Fig. 2 Blood chemistry analyses during resuscitation. Blood from control adult male Sprague–Dawley rats, or haemorrhagic animals without resuscitation treatment (NR) or resuscitated with 15 ml/kg (i.v.) Hextend (HXT) or Hextend containing GTS (GTS) was collected at 2 hrs after the haemorrhagic load to analyse (**A**) blood urea nitrogen (BUN), (**B**) pH, (**C**) the anion gap (AnGap), (**D**) chloride (Cl), (**E**) bicarbonate (HCO_3) and (**F**) Glucose. * represents $P < 0.01$ *versus* control ($n = 4$/group; one-way ANOVA with Bonferroni's corrections).

TNF levels. Unlike systemic inflammation during endotoxemia, splenectomy actually exacerbated serum TNF levels during resuscitation (Fig. 6A). These higher serum TNF levels correlated with higher TNF levels in the liver but not in the lung or the heart. These results reveal a fundamental difference between the systemic inflammatory responses in endotoxemia and haemorrhage. The pattern of TNF expression during resuscitation in splenectomized animals is similar to that of non-splenectomized animals. More significant, GTS significantly inhibited systemic TNF levels in splenectomized animals (Fig. 6B). However, the anti-inflammatory
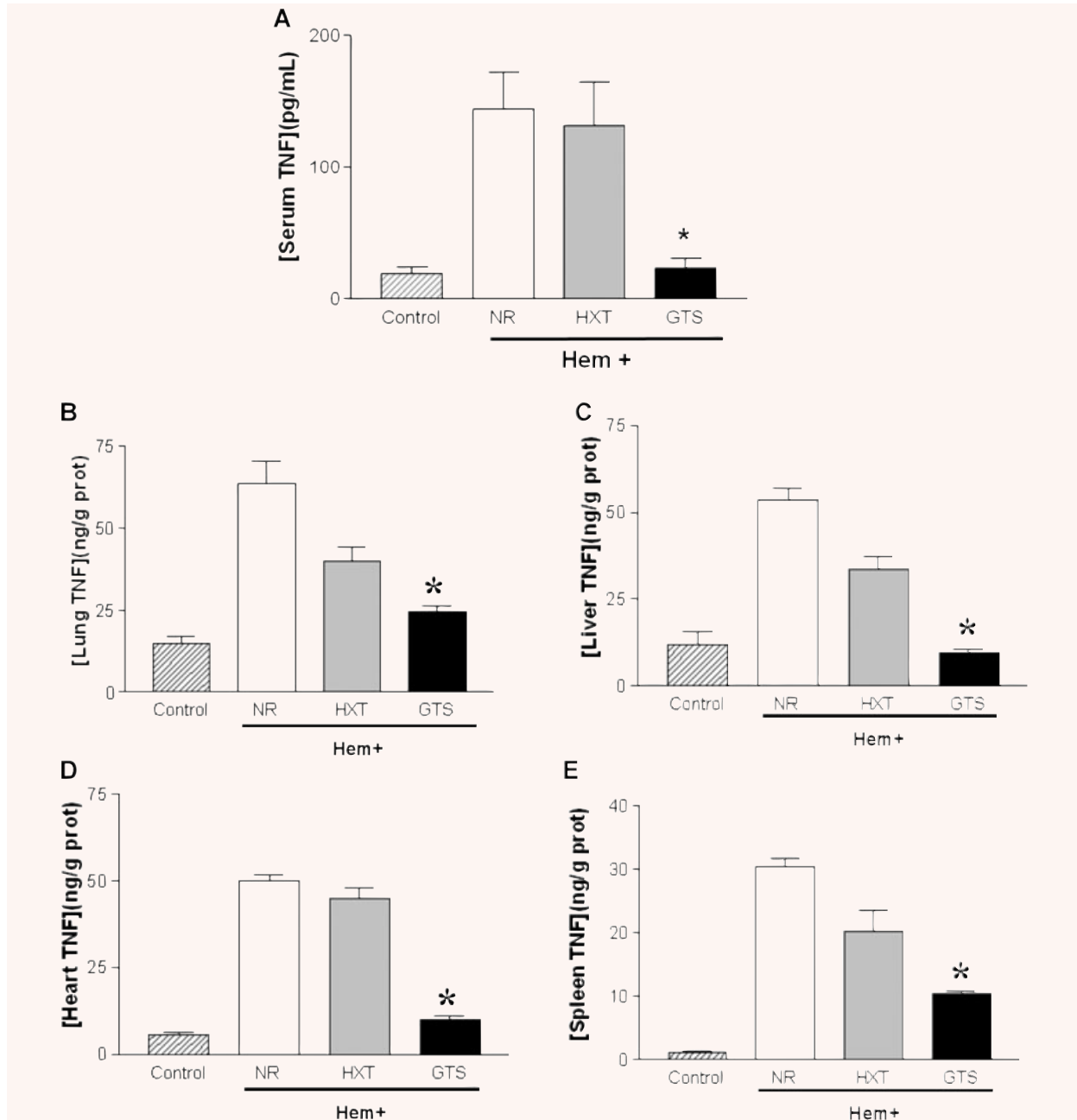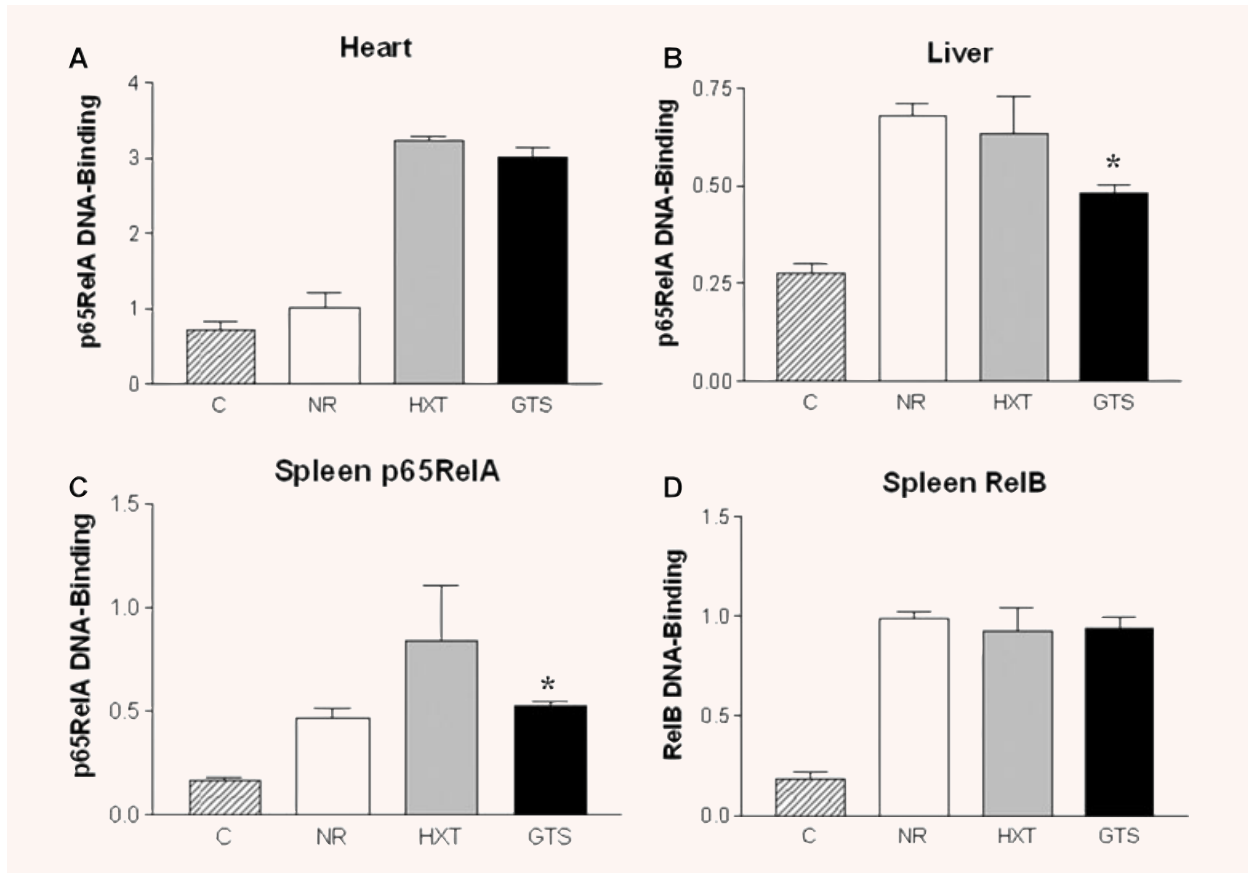
Fig. 3 GTS inhibits systemic TNF response during resuscitation. Blood and organs from control or haemorrhagic animals without (NR) or with resuscitation with Hextend (HXT) or Hextend supplemented with GTS (GTS) were analysed at 2 hrs to determine TNF protein concentration in the (**A**) serum, (**B**) lung, (**C**) liver, (**D**) heart and (**E**) spleen. * represents $P < 0.05$ *versus* HXT ($n = 4$/group; one-way ANOVA with Bonferroni's corrections).

potential of GTS was lower than in non-splenectomized animals particularly in the liver. However, unlike vagus nerve stimulation or treatment with nicotine, GTS significantly inhibited serum TNF levels as well as TNF levels in the lung and the heart of splenec-tomized animals. These results indicate that GTS might provide a significant advantage as compared with non-specific cholinergic agonists to control systemic inflammation particularly in those patients with an injured or compromised spleen.

Fig. 4 GTS inhibits splenic RelA but not RelB NF-κB *in vivo* during resuscitation. Organs from control (C) or haemorrhagic animals without (NR) or with resuscitation with Hextend (HXT) or Hextend supplemented with GTS (GTS) were collected at 2 hrs to analyse NF-κB during resuscitation in the (**A**) heart, (**B**) liver and (**C, D**) spleen. Both canonical p65RelA (**A–C**) and alternative RelB (**D**) NF-κB binding to DNA were analysed by TransAM DNA-Binding ELISA. Results are provided in arbitrary but relative units among the different samples and organs. * represents $P < 0.05$ *versus* HXT (n = 5/group; one-way ANOVA with Bonferroni's corrections).

Alpha7nAChR-agonist inhibited systemic HMGB1 response and PARP activation during haemorrhage

HMGB1 is a characteristic marker of cellular damage that can act as a cardiodepressant factor causing acute lung injury and abrupt cardiac standstill [7, 25]. Haemorrhage induced a systemic HMGB1 response that was not prevented by resuscitation with Hextend (Fig. 7A). However, one of the most significant effects of GTS was inhibiting systemic HMGB1 response during resuscitation. These results were particularly significant as no serum HMGB1 was found in those animals resuscitated with Hextend containing GTS. Because recent studies associated HMGB1 with liver damage during reperfusion [5, 26] and PARP appears to regulate HMGB1 secretion [27] and contributes to haemorrhagic shock [28–30], we analysed the effects of GTS on hepatic PARP activity. Haemorrhage activates hepatic PARP, and resuscitation with Hextend did not

prevent it. However, resuscitation with Hextend supplemented with GTS inhibited PARP activation (Fig. 7B). Similar results were observed in other organs such as the spleen (data not shown). It is noteworthy that both serum HMGB1 levels and hepatic PARP activity correlated and the activity levels in animals resuscitated with GTS were similar to that in control animals.

Discussion

Conventional resuscitation fluids are designed to re-establish blood pressure and tissue perfusion, but they fail to prevent lethal inflammatory responses during resuscitation. For instance, resuscitation with Hextend re-established blood pressure and tissue perfusion, but still 90% of the animals died within the first 6 hrs
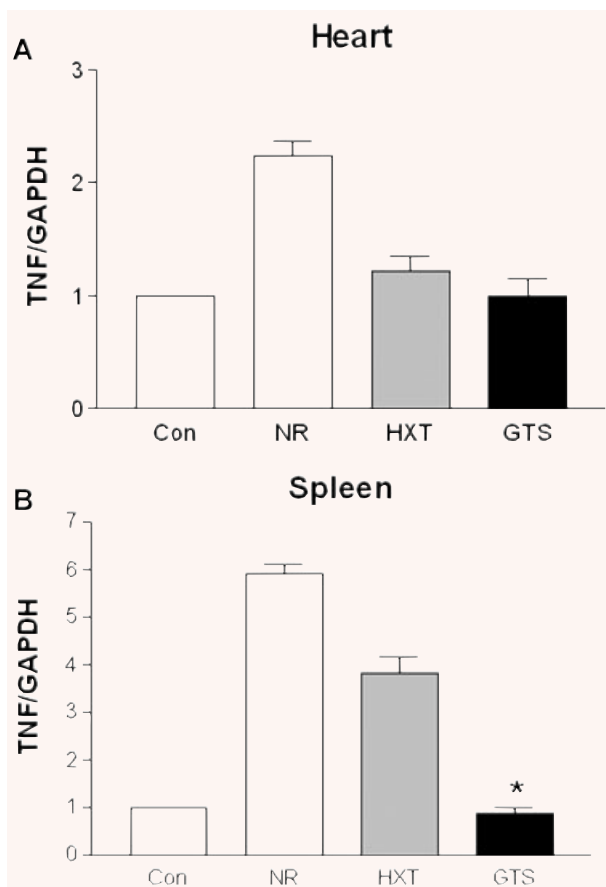
Fig. 5 GTS inhibits TNF transcription in the spleen during resuscitation. Organs from control or haemorrhagic animals without or with resuscitation were analysed at 2 hrs after haemorrhage. TNF RNA in the (**A**) heart and (**B**) spleen was analysed by real-time PCR. * represents $P < 0.05$ *versus* HXT (n = 4/group; one-way ANOVA with Bonferroni's corrections).

after haemorrhage. Here, we report that all the animals resuscitated with Hextend supplemented with GTS survived lethal haemorrhagic shock. Our results are particularly significant for three reasons. First, animals were subjected to severe haemorrhage with approximately 50% of blood loss where all the control animals died within the first 5 hrs after haemorrhage. Second, shed blood was considered lost and it was not reinfused. Our animals were treated with a small volume of resuscitation of 15 ml/kg that represents approximately 50% of the shed blood volume. Moreover, our studies use Hextend as control solution. This is a critical consideration as recent studies indicate that resuscitation with Hextend prevents multiple organ injury [21] and improves survival as compared with saline [22]. Third, the animals were followed for up to 7 days to analyse total survival including late deaths. GTS did not affect basic physiological parameters. Both resuscitation with Hextend or Hextend with GTS re-established

blood pressure, prevented metabolic acidosis and hyperglycaemia. The most significant result correlating with the therapeutic potential of GTS was its anti-inflammatory potential to restrain systemic TNF and HMGB1 levels.

Physiological anti-inflammatory mechanisms represent efficient systems selected by evolution to control inflammation and provide significant opportunities for novel anti-inflammatory strategies [1, 11, 13]. The vagus nerve restrains systemic inflammation in wild-type but not in alpha7-knockout mice [14, 15]. Both, acetylcholine and nicotine control the production of inflammatory cytokines in macrophages *via* the alpha7 nicotinic receptor [12, 14]. Treatment with nicotine inhibits serum TNF and HMGB1 levels and improves survival in experimental models of systemic inflammation [12]. Despite its clinical implications, the anti-inflammatory potential of cholinergic agonists is underestimated due to the collateral toxicity of nicotine including addiction, tachycardia and arrhythmia. We proposed that alpha7nAChR-agonists might represent a promising pharmacological strategy to control systemic inflammation and avoid collateral toxicity [1, 11, 13]. This hypothesis is consistent with recent studies indicating that individual cholinergic receptors mediate specific properties of nicotine. Thus, α7nAChR-agonists are expected to avoid nicotine-induced addiction [31], locomotor activity [32], tumourigenesis [33], autonomic dysfunction or allodynia [34], which appear to be mediated by other nicotinic receptors namely β2nAChR [32], α3nAChR [33], α4nAChR [31] or α5nAChR [34], respectively. GTS (3-[(2,4-dimethoxy)benzylidene]-anabaseine) is a characteristic alpha7nAChR-agonist, already used in clinical trials and proven less toxic than nicotine [18, 19]. GTS had no effect on locomotor activity in mice or dopamine turnover in rats. In contrast, nicotine produced a biphasic effect on locomotor activity [18]. These results prompt the potential use of GTS to avoid collateral toxicity of unspecific agonists [35, 36].

GTS inhibited systemic HMGB1 levels during resuscitation. Originally described as an intracellular protein, HMGB1 can be released into the extracellular milieu where it functions as a proinflammatory cytokine [2, 5, 37, 38]. Extracellular HMGB1 acts as a pro-inflammatory cytokine that activates immune cells and sustains inflammatory responses [5] contributing to abrupt cardiac standstill [8], acute lung injury [10] and intestinal derangement [9]. HMGB1 appears to be a pharmacologic target for haemorrhagic shock as: (a) Serum HMGB1 levels are increased in patients with haemorrhagic shock [39]; and (b) Inhibition of HMGB1 activity with neutralizing antibody significantly decreased liver damage after ischaemia and reperfusion [26]. Our previous studies supported HMGB1 as a late pharmacologic target for systemic inflammation, because it appears in the serum at 18–24 hrs after the induction of sepsis. However, this study now shows 'early' serum HMGB1 levels at 2 hrs after haemorrhage [40, 41]. Recent studies also reported 'early' extracellular HMGB1 release after hepatic ischaemia/reperfusion [26]. These differences between 'late' secretion in sepsis and 'early' release in haemorrhage may be explained by two different mechanisms of HMGB1 release/ production [5]. The first mechanism may be a time-consuming 'active secretion' from immune cells to act as a pro-inflammatory
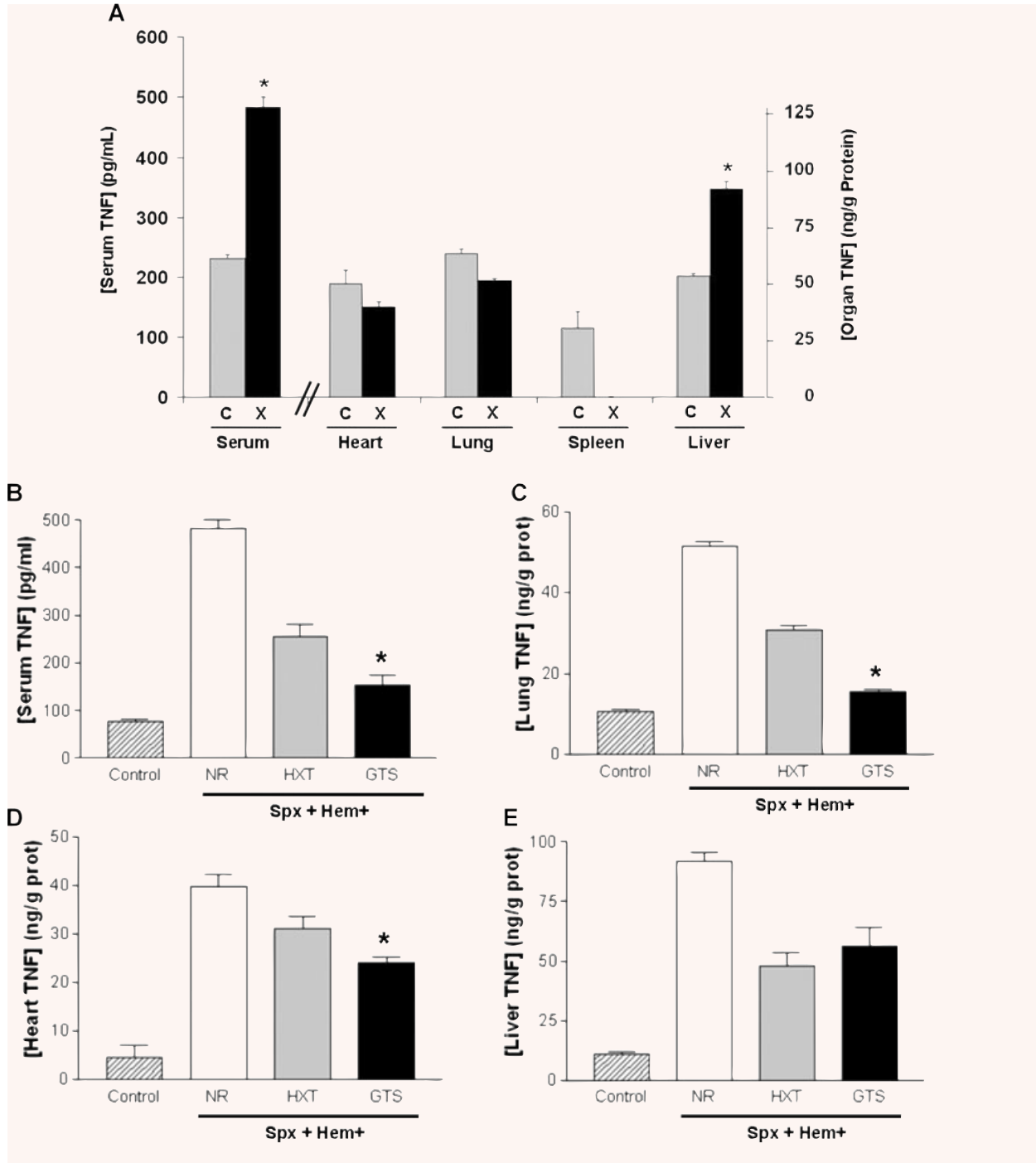
Fig. 6 GTS attenuates systemic TNF levels in splenectomized animals after resuscitation. (**A**) Serum TNF levels were analysed in blood collected from non-splenectomized (c) or splenectomized (x), haemorrhagic animals. Higher serum TNF levels in splenectomized animals correlated with higher TNF levels in the liver but not in the lung or the heart. * represents $P < 0.05$ (Student's t-test). Haemorrhagic splenectomized animals were subjected to the different treatments and TNF concentrations were determined in the (**B**) serum, (**C**) lung, (**D**) heart and (**E**) liver. * represents $P < 0.05$ *versus* HXT ($n = 4$/group; one-way ANOVA with Bonferroni's corrections).
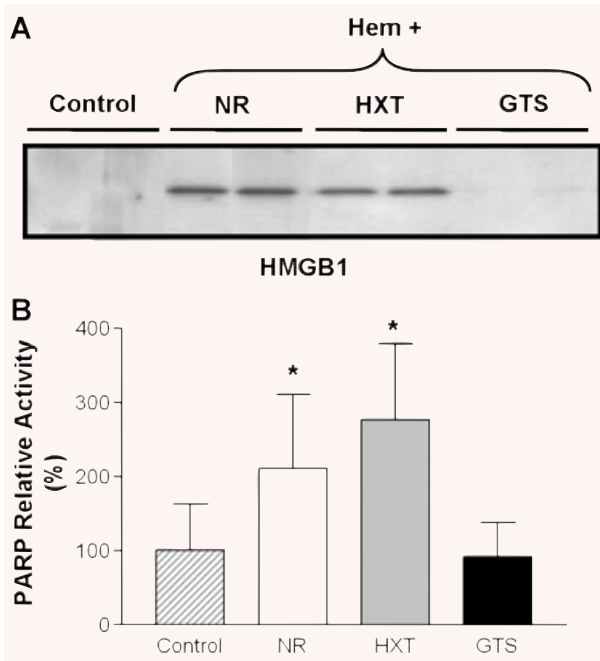
Fig. 7 Alpha7nAChR-agonist inhibits systemic HMGB1 response. (**A**) Blood from control or haemorrhagic animals with or without resuscitation were analysed at 2 hrs after haemorrhage to determine serum HMGB1 levels. (**B**) GTS inhibits PARP activity in the liver, a major source of HMGB1 during haemorrhage. * represents $P < 0.05$ *versus* control ($n = 4$/group; one-way ANOVA with Bonferroni's corrections).

cytokine during an immunologic challenge such as sepsis [42]. The second mechanism may be the 'passive release' of HMGB1 from damaged or necrotic cells during haemorrhage. In this haemorrhagic scenario, HMGB1, an intracellular protein, represents an optimal signal to recognize tissue damage and initiate reparative responses [43]. From an immunological perspective, HMGB1 represents a characteristic 'necrotic marker' or damage-associated molecular pattern (DAMP) molecule [44, 45]. This emerging family of specific intracellular proteins represents optimal chemotactic markers selected by the innate immune system to recognize tissue damage and initiate reparative responses [46, 47]. From a pathological perspective, HMGB1 can be used as a marker for cellular necrosis and tissue injury. In this sense, resuscitation with Hextend with GTS, but not Hextend alone, prevented serum HMGB1 levels supporting its therapeutic effects.

Our previous studies indicate that nicotine inhibits endotoxin-induced p65NF-κB activation in RAW264.7 macrophage cells *via* alpha7nAChR [40, 41]. Here, we report that *in vivo*, GTS, inhibits p65NF-κB activation in the spleen but not in the heart during resuscitation as compared with Hextend alone. However, GTS is no different than NR in altering p65RelA, and both are less than Hextend, yet the pattern of TNF and p65RelA did not correlate as would be expected. A potential explanation is that the expression

of alpha7nAChR may limit the effect of GTS to particular cells. This effect could be overlooked by analysing total extract of organs. Future studies are needed to study the regulation of p65RelA by GTS in specific cell types. This mechanism appears to be specific for p65RelA NF-κB as RelB was not affected by GTS. Haemorrhage induced a significant activation of both pathways but conventional resuscitation further activated p65RelA but not RelB. Because GTS specifically inhibited p65RelA, it can be used to further study the different implications of the conventional p65RelA and the alternative RelB NF-κB pathway in resuscitation. The activation of p65RelA NF-κB in the heart and the spleen during resuscitation did not correlate with increased mRNA TNF levels in these organs. These potential discrepancies can be due, at least in part, to the implications of other factors required for TNF transcription. In addition to NF-κB, GTS also inhibited the activation of poly(ADP-ribose) polymerase (PARP) providing the first evidence of the regulation of PARP by the alpha7nAChR. This effect has significant implications because PARP contributes to haemorrhagic shock as PARP inhibition provides marked survival benefit and protects from organ injury [28–30, 48]. Of special note is the correlation between PARP inhibition and systemic HMGB1. PARP inhibition can explain the regulation of systemic HMGB1 as PARP contributes to cell death *via* necrosis [49, 50] and PARP inhibitors prevent HMGB1 release in necrotic cells [27]. Furthermore, because PARP also regulates NF-κB activation and inflammatory cytokines [30], PARP inhibition may mediate the anti-inflammatory effects of cholinergic agonists *via* the alpha7nAChR.

Our previous studies indicated that the spleen is a major source of serum TNF and inhibition of TNF production in the spleen can prevent cardiovascular shock in endotoxemia [15]. The vagus nerve and non-specific cholinergic agonists prevent systemic inflammation during endotoxemia in normal but not in splenectomized animals [15]. These results have significant clinical implications suggesting that non-specific cholinergic agonists may not be efficient in patients with injured or compromised spleen. In sepsis, splenectomy moderated systemic TNF levels and abolished the anti-inflammatory potential of unspecific cholinergic agonists [15]. Here we report that in contrast to septic shock, splenectomy failed to prevent systemic inflammation in haemorrhagic shock. Actually, serum TNF levels during haemorrhagic shock were significantly higher in splenectomized than in normal animals. These higher serum TNF levels in splenectomized animals correlated with higher TNF levels in the liver. One potential explanation is that splenectomy may prevent the regulation of hepatic TNF production induced by the splenic release of anti-inflammatory cytokines such as TGF-β into the splenic and portal vein. In any case, these results reveal a fundamental difference between the systemic inflammatory response in experimental sepsis and haemorrhage. Indeed, Hextend reduced splenic TNF in normal animals (Fig. 3) and yet there was little influence upon serum and cardiac TNF levels. These results may argue against a splenic TNF aetiology as the mechanism for mortality, unless there is a TNF threshold, below which one may observe survival benefit. Resuscitation with Hextend attenuates systemic TNF levels in

splenectomized but not in control rodents. Similar to nicotine, GTS induced a stronger anti-inflammatory effect in normal rodents. But unlike nicotine, GTS efficiently attenuated serum TNF levels and prevented systemic inflammation in both normal or splenectomized animals. From a pharmacological perspective, these results suggest that GTS could provide a significant advantage as compared with unspecific cholinergic agonists to control systemic inflammation including patients with injured or compromised spleen. Taken together, our studies suggest that GTS could restrain the production of critical inflammatory and cardiodepressant factors during resuscitation. Future studies are needed to confirm the specificity of GTS in immune cells as well as its anti-inflammatory signalling pathway and the implications of alpha7nAChR in inflammatory responses and other cytokines during haemorrhage and resuscitation.

Acknowledgements

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