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Neuronal nitric oxide synthase is involved in vascular hyporeactivity and multiple organ dysfunction associated with haemorrhagic shock

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Abbreviations: ARL, ARL17477; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase; HS, haemorrhagic shock; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; MAP, mean arterial pressure; MPO, myeloperoxidase; 7NI, 7-nitroindazol.

ABSTRACT

Severe haemorrhage can lead to global ischaemia and haemorrhagic shock (HS), resulting in multiple organ failure (MOF) and death. Restoration of blood flow and reoxygenation is associated with an exacerbation of tissue injury and inflammatory response. The neuronal nitric oxide synthase (nNOS) has been implicated in vascular collapse and systemic inflammation of septic shock; however the role of nNOS in HS is poorly understood. The aim of this study is to evaluate the role of nNOS in the MOF associated with HS.

Rats were subjected to HS under anaesthesia. Mean arterial pressure was reduced to 30 mmHg for 90 min, followed by resuscitation with shed blood. Rats were randomly treated with 2 chemically distinct nNOS inhibitors (ARL 17477 1 mg/kg and 7-nitroindazol 5 mg/kg) or vehicle upon resuscitation. Four hours later, parameters of organ injury and dysfunction were assessed.

HS was associated with MOF development. Inhibition of nNOS activity at resuscitation protected rats against the MOF and vascular dysfunction. In addition, treatment of HS-rats with nNOS inhibitors attenuated neutrophil infiltration into target organs and decreased the activation of NF-κB, iNOS expression, NO production and nitrosylation of proteins. Furthermore, nNOS inhibition also reduced the levels of pro-inflammatory cytokines TNF-α and IL-6 in HS-rats.

In conclusion, two distinct inhibitors of nNOS activity reduced the MOF, vascular dysfunction and the systemic inflammation associated with HS. Thus, nNOS inhibitors may be useful as an adjunct therapy before fluids and blood administration in HS patients in order to avoid the MOF associated with reperfusion injury during resuscitation.

Keywords: nitric oxide, nNOS, iNOS, nitrosylation, organ injury and dysfunction, haemorrhage, resuscitation.

INTRODUCTION

Trauma is the main cause of death for people under 35 years, with more than 5 million deaths every year around the world. Severe haemorrhage associated with trauma can lead to a state of global ischaemia and haemorrhagic shock (HS), resulting in multiple organ failure (MOF) and death. Unfortunately, the chances for survival after trauma have not significantly improved for decades (1). Prehospital HS treatment should be focused on the maintenance of adequate mean arterial pressure (MAP) along with organ perfusion until arrival at the hospital. Vasopressors may also be required to maintained tissue perfusion when the fluid resuscitation does not achieve the expected goal (2).

HS is one of the most common types of shock and causes an ischaemia-reperfusion-type injury (3). The restriction of blood supply to organs after bleeding causes tissue hypoxia (ischaemia), and the restoration of blood flow and reoxygenation is associated with an exacerbation of tissue injury and a profound inflammatory response (reperfusion injury) (4). The excessive generation of free radicals [such as superoxide anion and nitric oxide (NO)] play a key role in post-ischaemic tissue injury (5).

There is now good evidence that an enhanced formation of NO contributes to the pathophysiology of septic and haemorrhagic shock. Most studies indicate that the expression of inducible NO synthase (iNOS) is the key source of the observed enhanced NO formation (6-9). However, there is recent evidence that the activation of the neuronal isoform of NOS (nNOS) may also play an important role in the pathogenesis of the systemic inflammatory response in sepsis (10-14). Most notably, activation of nNOS importantly contributes to the vascular dysfunction in sepsis (14) and sepsis in nNOS-gene knockout animals is associated with the reduced release of pro-inflammatory cytokines as well as reduced oxidative and nitrosative stress (12, 15). However, we know very little about the roles of nNOS in the vascular decompensation, organ injury/dysfunction and inflammation associated with HS.

Therefore, the aim of the present study was to investigate the role of nNOS in the organ injury and dysfunction associated with a relevant model of HS in the rat. As both specificity and selectivity of any drug may be an issue especially when using inhibitors of NOS, we have compared the effects of 2 chemically distinct and potent nNOS inhibitors, namely ARL 17477 (ARL) and 7-nitroindazol (7NI). ARL is 17 times more selective for nNOS than eNOS (IC_{50} are 1 and 17 μM, respectively; 16). In vitro, 7NI is an equipotent inhibitor of all isoforms of NOS, but in vivo 7NI exhibits a marked selectivity for the nNOS, possibly as a result of differential cellular uptake (17). Our results show that the nNOS inhibition at the onset of resuscitation protects rats against the MOF and vascular dysfunction associated with HS thorough the attenuation of NF-κB activation and consequent reduction of NF-κB -dependent proteins (such as iNOS and pro-inflammatory cytokines), and through the attenuation of protein nitrosylation by NO-derived species.

METHODS

Animals

The animal protocols used in this study were approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London (PPL: 70/7348) in accordance with the derivatives of Home Office guidance on Operation of Animals (Scientific Procedures Act 1986) published by Her Majesty's Stationery Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Haemorrhagic Shock

This study was carried out on male Wistar rats (Charles River Ltd, Margate, UK) weighing 230 g to 280 g. The animals were housed in a temperature-controlled environment with a 12 h light/dark cycle and received a standard diet and water *ad libitum.* Haemorrhagic shock (HS) was

performed as previously described (18). Briefly, rats were anaesthetized with sodium thiopentone (120 mg/kg i.p., maintained using 10 mg/kg i.v.) and cannulation of trachea, bladder, jugular vein, and femoral and carotid arteries were performed. Body temperature was monitored by a rectal thermometer (36 ± 0.5 ºC) by means of a homoeothermic blanket system (Harvard Apparatus, Natick, MA). Blood was withdrawn through carotid artery in a rate of 1 mL/min to achieve a mean arterial pressure (MAP) of 30 ± 2 mmHg, which was recorder through the femoral artery with a pressure transducer coupled to a PowerLab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). The MAP was maintained at 30 ± 2 mmHg either by further withdrawal of blood during the compensation phase or administration of shed blood during the decompensation phase. The total volume of shed blood was 9,46 ± 1,40; 8,72 ± 0,44 and 9,72 ± 1,40 mL for HS+Vehicle, HS+ARL and HS+7NI, respectively ($p>0.05$). The volume of re-infused blood for maintenance of MAP at 30 ± 2 mmHg was 2,37 ± 0,54; 1,69 ± 0,69 and 1,79 ± 0,43 mL for HS+Vehicle, HS+ARL and HS+7NI, respectively ($p>0.05$). After 90 min of initiation of haemorrhage or when 25% of the shed blood had to be re-injected to sustain MAP at 30 ± 2 mmHg, resuscitation was performed over a period of 5 min with the remained shed blood mixed with 100 IU/mL heparinized saline. The same volume of blood re-infused for maintenance of MAP (see above) was replaced by Ringer's lactate on resuscitation. In addition, 1 h after the resuscitation with the blood, an infusion of Ringer's lactate was started (1.5 mL/kg/h; i.v.) and this fluid replacement was maintained throughout the whole experiment. Two hours after onset of resuscitation, a dose-response curve for norepinephrine (NE; 1, 3 and 10 nmol/kg) was performed in all experimental groups. Four hours after resuscitation, blood was obtained for measurement of lactate (Accutrend Plus Meter, Roche Diagnostics, West Sussex, UK), serum cytokines and assessment of organ injury. Also, at this time, kidney and liver were harvested for further analysis. Blood samples were centrifuged to separate serum from which creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) were measured within 24 h (IDEXX Laboratories Ltd, West Yorkshire, UK). The last 3 h urine

was obtained for the estimation of the creatinine clearance. Sham-operated rats were used as control and underwent identical surgical procedures but without haemorrhage or resuscitation.

Experimental design

Rats were subjected to surgery and randomly divided into the following 6 groups (n = 8-10 per group): (i) sham + vehicle; (ii) sham + ARL 17477 (ARL; 1 mg/kg); (iii) sham + 7-nitroindazol (7NI; 5 mg/kg); (iv) HS + vehicle; (v) HS + ARL (1 mg/kg) and (vi) HS + 7NI (5 mg/kg). ARL was dissolved in PBS and 7NI was dissolved in 10% DMSO. Control groups (sham and HS) were performed with 10% DMSO or PBS, and since no difference was found between them, they were grouped [sham + vehicle (PBS n=4 and 10% DMSO n=4) and HS + vehicle (PBS n=5 and 10% DMSO n=5)]. The doses of 7NI and ARL used in our study were based on previous studies that have been shown to inhibit nNOS activity in vivo (14, 16, 19; see also introduction).

Western blotting

Western blotting was performed as previously described (18). Briefly, kidney (cortex + medulla) and liver samples were homogenized and centrifuged. After electrophoresis, the proteins were transferred to a polyvinyldenediflouoride (PVDF) membrane, which was incubated with a primary antibody [mouse anti-total IκBα (1:1000); mouse anti-IκBα pSer$^{32/36}$ (1:1000); rabbit anti-NF-κB p65 (1:1000); rabbit anti-iNOS (1:200); rabbit anti-nNOS (1:1000)]. Membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (1:2000) and developed with ECL detection system. The membranes were stripped and incubated with β-actin monoclonal antibody (1:5000) and subsequently with an anti-mouse antibody (1:10000) to assess gel-loading homogeneity. The immunoreactive bands were visualized by autoradiography and the densitometric analysis was performed using Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA).

Nitrite and nitrate (NOx)

Briefly, nitrate was converted to nitrite using nitrate reductase in the presence of co-factors. The total nitrite in plasma was assayed by adding Griess reagent [0.05% (wt/vol) naphthalethylenediamine dihydrochloride and 0.5% (wt/vol) sulphanilamide in 2.5% (vol/vol) phosphoric acid] to each sample and read at 550 nm. NOx concentration, as nitric oxide synthesis estimation, was calculated by comparison with standard solution of sodium nitrate prepared in water.

Immunohistochemistry for nitrotyrosine and myeloperoxidase (MPO)

Briefly, 4 μm sections of kidney and liver samples were obtained. For nitrotyrosine, the sections were blocked with PBS / 1% BSA (PBA) for 1 h and then incubated with 2% goat serum in PBA for 30 min. For myeloperoxidase, the sections were blocked with 10% goat serum for 15 min. The slides were then incubated with rabbit anti-nitrotyrosine antibody (1:2000; Merck Millipore, Darmstadt, Germany) or with rabbit anti-myeloperoxidase antibody (1:25; Cat# ab9535, Abcam, Cambridge, UK) and then incubated with labelled polymer-HRP antibody (Dako EnVision+ System-HRP-DAB, K4010). DAB chromogen solution was used and a counter-staining was performed with Harris haematoxylin. Images were acquired using NanoZoomer Digital Pathology Scanner (Hamamatsu Photonics K.K., Japan) and analysed using the NDP Viewer software. The relative quantification of nitrotyrosine immunostaining was achieved through densitometry analysis using NIH ImageJ 1.36 imaging software (NIH, Bethesda, MD, USA) and it is expressed as arbitrary units. The number of MPO positive cells was counted in 10 randomly selected fields (x 400) in a double-blinded manner.

Cytokines determination

Serum cytokines TNF-α and IL-6 were determined using commercial immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer protocol.

Reagents

ARL 17477 dihydrochloride was purchased from Tocris Bioscience (R&D systems Europe, Abingdon, UK). Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). All stock solutions were prepared using nonpyrogenic PBS. Ringer's lactate was from Baxter Healthcare. The bicinchoninic acid protein assay kit and SuperBlock blocking buffer were from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Antibodies (anti-total IκBα, anti-IκBα $pSer^{32/36}$, anti-NF-κB p65, anti-iNOS and anti-nNOS) were from Cell Signalling Technology Inc. (Beverly, MA, USA), anti-nitrotyrosine was from Merck Millipore (Darmstadt, Germany) and anti-myeloperoxidase was from Abcam (Cambridge, UK).

Data analysis

All values are expressed as mean ± SEM. Statistical analysis was carried out using Graph Prism 5.03 (Graph Pad Software, San Diego, CA). Data were assessed by one or two-way ANOVA followed by Bonferroni's post hoc test. A p value of less than 0.05 was considered to be significant.

RESULTS

Inhibition of nNOS protects against organ injury and dysfunction associated with HS

When compared to sham-operated rats, HS-rats exhibited decreased increased levels of serum creatinine (Figure 1A) and a significant decrease in creatinine clearance (Figure 1B). HS-rats also had elevated plasma concentrations of aspartate aminotransferase (AST; Figure 1C), alanine aminotransferase (ALT; Figure 1D) and creatine kinase (CK; Figure 1E) indicating liver and muscular injury, respectively. When compared to sham-operated rats, HS-rats treated with vehicle also had elevated concentrations of blood lactate (Figure 1F), indicating global tissue hypoxia. The treatment of HS-rats with the nNOS inhibitors ARL or 7NI, significantly attenuated the plasma

creatinine increase and the reduction on creatinine clearance, and the liver and muscle injury associated with HS. The nNOS inhibitors ARL and 7NI also attenuated tissue hypoxia, as observed by lower lactate levels when compared to HS vehicle group. The treatment of sham-rats with nNOS inhibitors had no significant effect when compared to sham vehicle group (Figure 1).

Inhibition of nNOS prevents circulatory failure and vasoconstrictor hyporeactivity associated with HS

When compared to sham-operated rats (open circles), HS-rats treated with vehicle (closed squares) exhibited a significant reduction in MAP after resuscitation (Figure 2A). Administration of ARL at resuscitation failed to attenuate the reduction in MAP caused by HS (closed triangle and open diamond, respectively). In contrast, inhibition of nNOS with 7NI at resuscitation totally impaired the development of circulatory failure in HS rats (closed diamond). The MAP from sham animals treated with ARL or 7NI were not different from sham vehicle group (data not shown).

In addition to the circulatory failure, we have also investigated the vasoconstrictor reactivity to NE. Two hours after resuscitation, HS-rats developed a substantial reduction in NE (3 and 10 nmol/kg) response (Figure 2B; black bars) when compared with sham rats (open bars). The treatment of HS-rats at resuscitation with ARL or 7NI was able to prevent the development of NE hyporeactivity significantly (Figure 2B). The administration of NE in sham rats treated with ARL or 7NI had no significant effect when compared to sham vehicle group (data not shown).

Inhibition of nNOS reduces neutrophil accumulation associated with HS

As both ARL and 7NI reduced the organ injury associated with HS, we investigated the mechanisms involved in organ protection in renal and liver biopsies. When compared with sham rats, HS-rats treated with vehicle showed an increase in MPO positive cells in kidney and liver, indicating

an accumulation of neutrophils in these tissues. The administration of ARL or 7NI significantly decreased neutrophils accumulation in both organs (Figure 3).

Inhibition of nNOS attenuates NF-κB activation associated with HS

When compared with sham-operated rats, kidney (Figures 4A and C) and liver (Figures 4B and D) from rats subjected to HS exhibited a significant increase in the phosphorylation of IκBα on $Ser^{32/36}$ (Figures 4A and B) and translocation of the NF-κB subunit p65 to the nucleus (Figures 4C and D). The treatment of HS-rats with ARL or 7NI attenuated the phosphorylation of IκBα on $Ser^{32/36}$ and translocation of the NF-κB subunit p65 to the nucleus associated with HS in both kidney and liver (Figure 4). Treatment of sham animals with both nNOS inhibitors caused no difference in NF-κB activation when compared with sham vehicle group (data not shown).

Inhibition of nNOS attenuates iNOS expression and NOx production associated with HS

As iNOS is a NF-κB-dependent gene, and it is also involved in the development of circulatory failure and hyporeactivity to vasoconstrictors associated with HS and sepsis, we have also investigated the effects of nNOS inhibition on later iNOS expression and NOx production. When compared with sham-operated rats, kidney and liver from HS-rats exhibited a significant increase in iNOS expression (Figures 5A and B, respectively), and an increase in serum NOx production (Figure 6). The treatment of HS-rats with nNOS inhibitors ARL or 7NI attenuated the increase of iNOS expression associated with HS (Figure 5A and B), as well as NOx production (Figure 6). In contrast, no difference among the groups in nNOS expression was noticed in kidney or liver (Figures 5C and D).

Inhibition of nNOS attenuates nitrosylation of proteins associated with HS

As we observed a reduction in iNOS expression as well as in NOx production in HS-rats treated with nNOS inhibitors, we determined the tyrosine nitration in kidney and liver tissue by

immunohistochemistry. When compared with sham-operated rats (Figures 7A and H), kidney and liver of HS-rats exhibited a marked staining for nitrotyrosine (Figures 7B and I, respectively). Treatment of HS-rats with ARL or 7NI attenuated the staining for nitrotyrosine both in kidney and liver (Figures 7G and N). No difference in nitrotyrosine staining was noticed between sham animals treated with vehicle or with nNOS inhibitors (Figure 7).

Inhibition of nNOS attenuates pro-inflammatory cytokines production associated with HS

When compared with sham-operated rats, the pro-inflammatory cytokines TNF-α (Figure 8A) and IL-6 (Figure 8B) were significantly increased in the serum of HS-rats treated with vehicle. The treatment of HS-rats with ARL or 7NI attenuated the production of both cytokines. No significant change in cytokine production was noticed in sham rats treated with vehicle or with nNOS inhibitors (Figure 8).

DISCUSSION

The main findings of the present study are that the inhibition of nNOS (with two potent inhibitors of nNOS) at resuscitation protected rats against the vascular decompensation and the multiple organ injury/dysfunction associated with HS. Inhibition of nNOS activity was associated with: (i) reduction of neutrophil migration to organs, (ii) attenuation of NF-κB activation, (iii) reduction of NF-κB-dependent protein expression (including iNOS and the pro-inflammatory cytokines TNF-α and IL-6) and (iv) attenuation of protein nitrosylation by NO-derived species.

The vascular decompensation in HS is associated with an enhanced formation of NO (6) and the induction of iNOS induced by preconditioning with LPS aggravates the organ dysfunction associated with HS (18). Non-selective inhibition of NOS preserves myocardial function (20) and increases survival in HS (21), while the selective inhibition of iNOS attenuates circulatory failure

and the organ injury/dysfunction associated with HS (7, 22). Although iNOS has been claimed to be the main source of NO production, the neuronal isoform of NOS may have a more prominent role in sepsis and HS. The nNOS-derived NO plays a key role in the pathophysiology of lung injury (10), MOF (11) and vascular dysfunction (14) induced by sepsis.

Our data show that nNOS inhibition at the onset of resuscitation attenuates the organ injury and dysfunction associated with HS. To our knowledge, this is the first study that investigates the role of nNOS in MOF associated with this critical condition. In addition, as hypotension and hyporeactivity to vasoconstrictors are strongly associated with NO production (8, 9, 23), the inhibition of nNOS also impaired the vascular hyporeactivity associated with HS. This result is in accordance with previous studies that show that nNOS has an important role in the vascular dysfunction in sepsis and endotoxemia (12, 14).

To investigate the mechanisms by which nNOS inhibition afforded organ protection in HS, we first evaluated the role of the neutrophils in the main organs affected by HS (kidney and liver). Neutrophils play an important role in the pathogenesis of MOF after HS, and cause direct local cytotoxic cellular effects through degranulation and release of substances such as myeloperoxidase, reactive oxygen species and cytokines. HS-rats have increased number of neutrophils (MPO+ cells) in kidney and liver tissues. Interestingly, both nNOS inhibitors (ARL and 7NI) decreased the number of neutrophils in both shock organs. This was indeed a curious finding as NO inhibits rolling and adhesion of neutrophils in inflammation and sepsis (24). However, this matter is still unresolved as iNOS knockout animals or animals treated with the different NOS inhibitors show a reduction in neutrophil migration to inflammatory sites in several models of inflammation, including HS (22, 25). Indeed, the role of NO on neutrophil influx seems to be dependent on the tissue and also on the stimulus (25).

It is described that, in vitro, NO donors can inhibit NOS activity (26) and NO derived from the endothelial isoforms of NOS modulates the expression of iNOS in macrophages (27) in a NF-κB-dependent way (28). Moreover, Duma and colleagues showed that nNOS knockout animals or wild-type animals treated with a nNOS inhibitor, when subjected to CLP model, exhibited reduction of the expression of iNOS and lower NO production than CLP control animals (12). Also, nNOS-derived NO is shown to act as signalling agent for iNOS expression in vascular smooth muscle cells (29). With this in mind, we have also investigated the role of nNOS inhibition on iNOS expression in HS-rats. Our data clearly show that nNOS-derived NO is able to modulate iNOS expression in HS in a NF-κB-dependent manner. According to our data, both nNOS inhibitors attenuated the phosphorylation of IκBα and NF-κB activation. Indeed, the inhibition of NF-κB has been shown to be benefit in HS, attenuating the organ failure and dysfunction (30). The reduction of NF-κB activation led to the attenuation of iNOS expression, while the expression of nNOS (as expected) was unchanged in any of the animal groups investigated. The systemic levels of NOx, the stable metabolites of NO, were increased in HS-rats and were attenuated in rats treated with both nNOS inhibitors. It is unclear whether the reduction of NOx is due to inhibition of nNOS activity or prevention of iNOS expression by the nNOS inhibitors used.

Indeed, NO can react with superoxide anion to form peroxynitrite, which may nitrate proteins causing cytotoxicity and interfering with signal transduction mechanisms. The detection of nitrotyrosine represents a reliable marker of nitrosative stress and tissue injury (31). Not surprisingly, nitration of proteins by peroxynitrite is linked with organ injury and dysfunction in critical conditions associated with high production of NO, such as sepsis and HS (5, 7). Also, peroxynitrite scavengers decrease protein nitration and improve renal function in sepsis (32). Our data confirm that the formation of peroxynitrite in HS-rats is increased, causing protein nitration and consequently organ injury and dysfunction. We show that the attenuation of nNOS activity and iNOS expression (and the subsequent decreased NO formation) attenuated peroxynitrite and consequently the nitration of

proteins in liver and kidney from HS-rats. We do believe that the reduction of nitrotyrosine in the tissues may importantly contribute to the organ protection observed in HS-rats treated with nNOS inhibitors.

The attenuation of NF-κB activation also led to the reduction of pro-inflammatory cytokines formation (TNF-α and IL-6) in HS-rats, which may also account for the beneficial effect on organ injury and dysfunction. Elevated serum IL-6 concentrations predict the development of organ failure in trauma-haemorrhage patients and increases in TNF-α level are associated with development of organ injury and death followed trauma (30, 33, 34). This is an interesting finding, as the beneficial effects of nNOS inhibition in HS were clearly beyond iNOS modulation.

Our study has some limitations that need to be considered. The HS model that we used in this study is a very acute model of severe HS, which leads to MOF and systemic inflammation within a few hours of the onset of resuscitation. Although acute, at this time point (4 h after resuscitation) we have observed organ dysfunction, a substantial activation of the NF-κB pathway, enhanced expression of iNOS and formation of pro-inflammatory cytokines. However, although effective in this acute setting, we cannot conclude that nNOS inhibition will have the same beneficial effect in an animal model with long-term resuscitation periods and/or affect mortality.

Moreover, it could be argued that the observed effects of nNOS inhibitors are due to non-specific effects. We cannot exclude the possibility that the inhibitors used in this study have exerted their beneficial effects through off-target effects not related to nNOS-inhibition, such as eNOS inhibition, especially with the use of 7NI. We regard this as the main limitation of our work, as we were not able to perform this study in knockout animals. In vitro, 7NI is equipotent as an inhibitor of both nNOS and eNOS. On the other hand, in vivo, evidences suggest 7NI is more selective for nNOS (17, 35). However, there are some controversies (36), and we cannot exclude that 7NI may (at the dose used in vivo) also inhibits eNOS activity, which would in turn result in an increase in blood

pressure. This could explain the more pronounced effect of 7NI on mean arterial pressure. However, we have used 2 different nNOS inhibitors in order to reinforce our findings. The fact that 7NI may cause a weak inhibition of eNOS does not decrease the importance of our data, as i) ARL 17477 is a 17-times more selective inhibitor of nNOS than eNOS (IC_{50} are 1 and 17 μM, respectively); and ii) at the dose that we have chosen for our studies (1 mg/kg), the estimated plasma levels of ARL 17477 are well below the IC_{50} for eNOS inhibition (16). Other non-specific effects are unlikely, as (a) we have used two potent and chemically distinct nNOS inhibitors, (b) both reduce nNOS activity in vivo at the doses used in our study (15, 18, 19), (c) both nNOS inhibitors show qualitatively and quantitatively similar beneficial effects in HS on a large number of parameters, (d) similar beneficial data with nNOS inhibitors in sepsis have been already confirmed with nNOS knockout animals and similar results were found (13).

The fact that we have administered the nNOS inhibitors at the onset of resuscitation, and not prior to haemorrhage as performed by previous studies evaluating the iNOS role in HS (8), increases the clinical relevance of our findings. We believe that nNOS inhibitors could be employed as an adjunct therapy before the administration of fluids and blood in HS patients in order to avoid the MOF associated with reperfusion (during resuscitation).

In conclusion, we show that the NO derived from nNOS has a harmful role, acting as a trigger for the initiation of the inflammatory response in HS. An enhanced formation of NO by nNOS is involved in the development of vascular dysfunction and MOF associated with HS. The organ protection observed in HS-rats treated with nNOS inhibitors was associated with reduction of neutrophil migration, attenuation of NF-κB activation and consequently reduction of systemic pro-inflammatory cytokines TNF-α and IL-6, iNOS expression and nitrosylation of proteins by NO-derived species.

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N/A.

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FIGURE LEGENDS

Figure 1. nNOS inhibition protects against multiple organ failure and dysfunction associated with HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Four hours later, blood was obtained and serum creatinine (A), estimated creatinine clearance (B), serum aspartate aminotransferase (AST; C), serum alanine aminotransferase (ALT; D), serum creatine kinase (CK; E) and blood lactate (F) were determined. Sham animals were used as control. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p<0.05$ vs sham group and #$p<0.05$ vs HS group.

Figure 2. nNOS inhibition attenuates cardiovascular dysfunction associated with HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were used as control. Panel A: The mean arterial pressure (MAP) of the animals was recorded during the whole experiment. Panel B: A dose-response curve of norepinephrine (1, 3 and 10 nmol/kg; i.v.) was performed two hours after resuscitation. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p<0.05$ vs sham group and #$p<0.05$ vs HS group.

Figure 3. nNOS inhibition attenuates neutrophils migration to kidney and liver in HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation.

Sham animals were used as control. Immunostaining for myeloperoxidase (MPO) as a neutrophils marker was performed in kidney (Panel A) and liver (Panel B) tissue and the quantitative analysis of the number of cells / mm^2 are shown. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

Figure 4. nNOS inhibition attenuates NF-κB activation associated with HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were used as control. The phosphorylation of IκBα on $Ser^{32/36}$ (A and B) and the nuclear translocation of the p65 NF-κB subunit (C and D) on the kidney (A and C) and liver (B and D) were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related mean value of sham-operated band. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

Figure 5. nNOS inhibition attenuates the expression of inducible isoform of nitric oxide synthase (iNOS) in HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were used as control. The iNOS (A and B) and nNOS (C and D) expression in the kidney (A and C) and liver (B and D) were determined by western blotting. Protein expression is measured as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related mean value of sham-operated band. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

Figure 6. nNOS inhibition attenuates serum nitrite + nitrate (NOx) in HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were

used as control. Serum NOx was determined through Griess reaction. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

Figure 7. nNOS inhibition attenuates nitrotyrosine staining in kidney and liver in HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were used as control. Tyrosine nitration as an index of nitrosylation of proteins by peroxynitrite was determined by immunohistochemistry. The relative quantification of nitrotyrosine immunostaining was achieved through densitometry analysis and it is expressed as arbitrary units. Representative figures of kidney (A-F) and liver (Panel H-M) tissue and the quantitative analysis are shown (G and N). Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

Figure 8. nNOS inhibition attenuates serum inflammatory cytokines in HS. Rats were subjected to HS and received ARL 17477 (ARL), 7-nitroindazol (7NI) or vehicle at resuscitation. Sham animals were used as control. Serum concentrations of TNF-α (A) and IL-6 (B) were determined. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05 vs sham group and #p<0.05 vs HS group.

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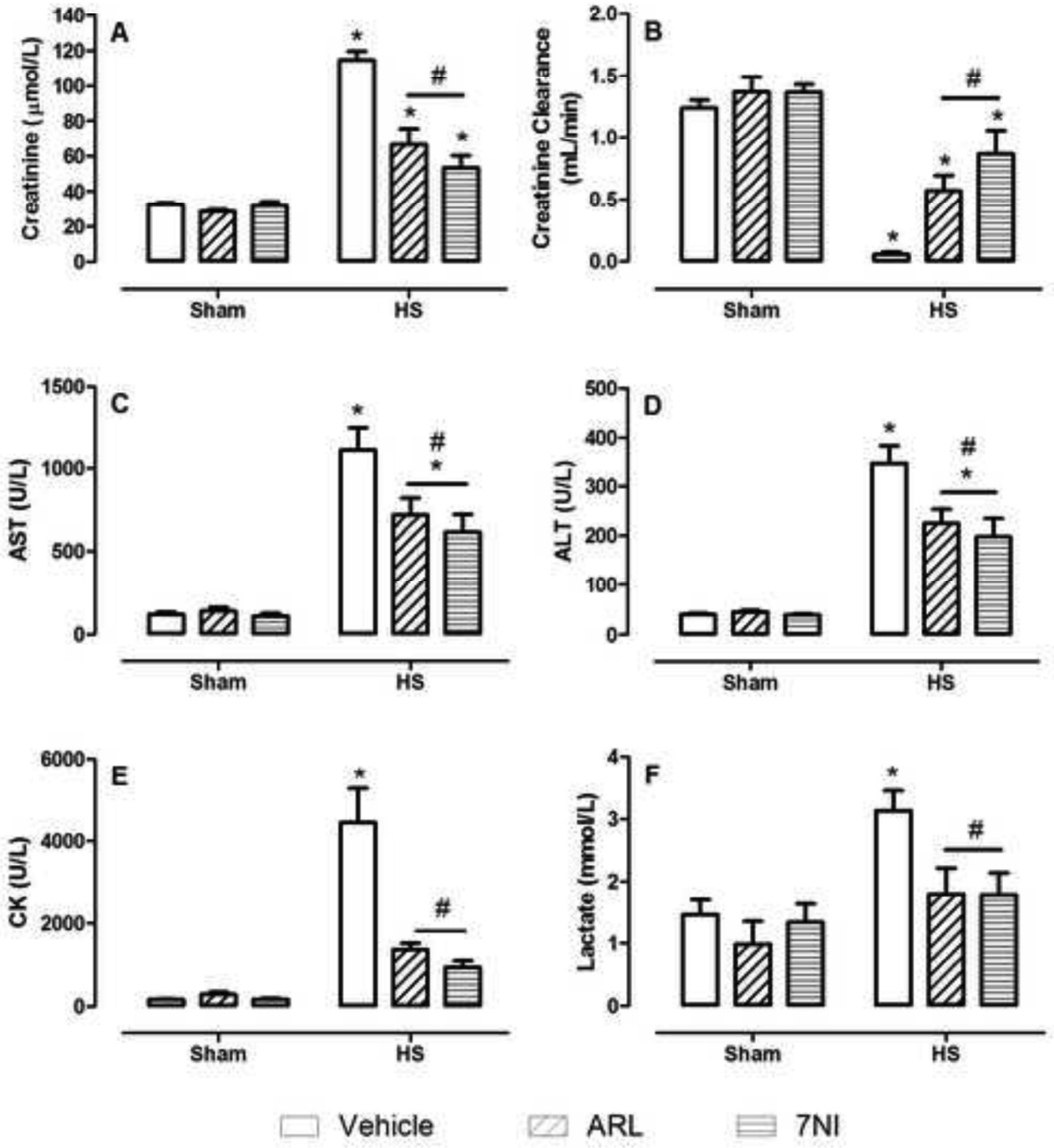

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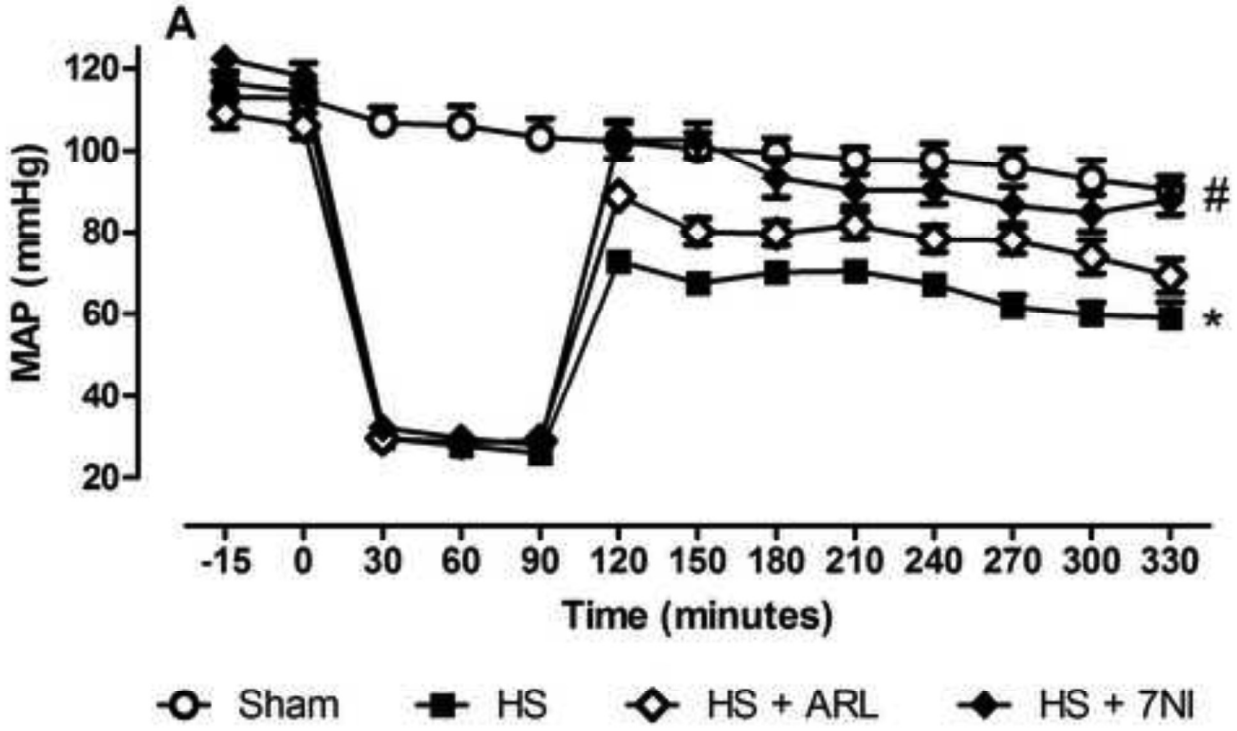

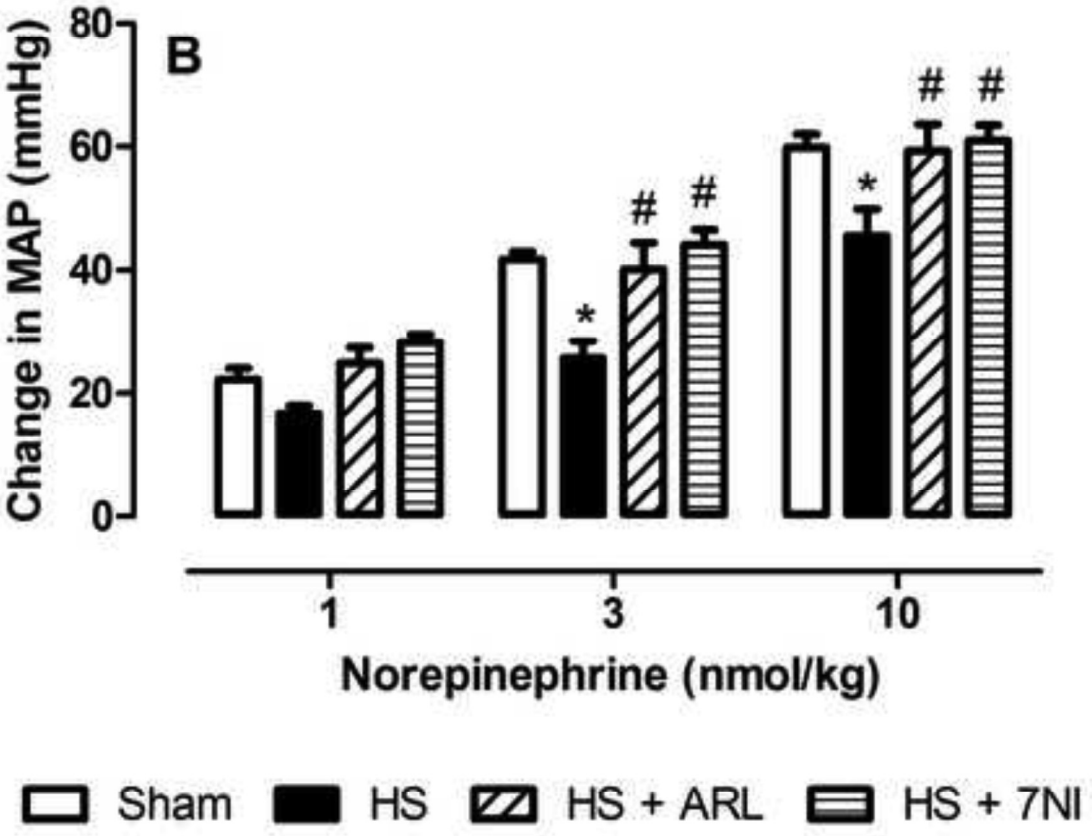

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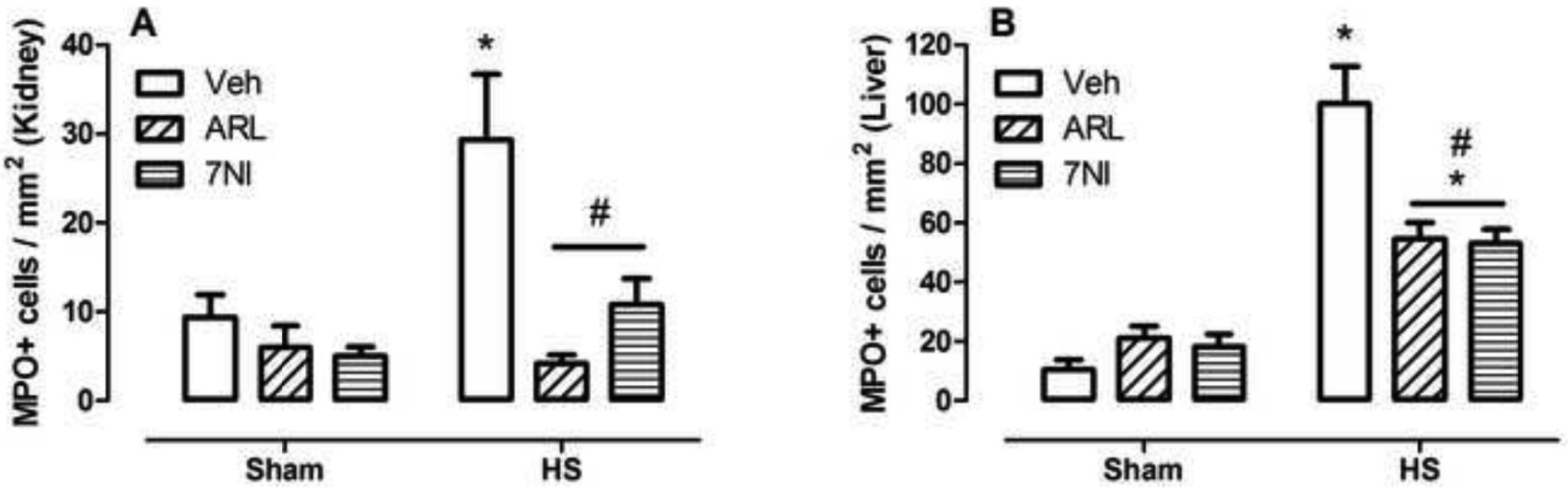

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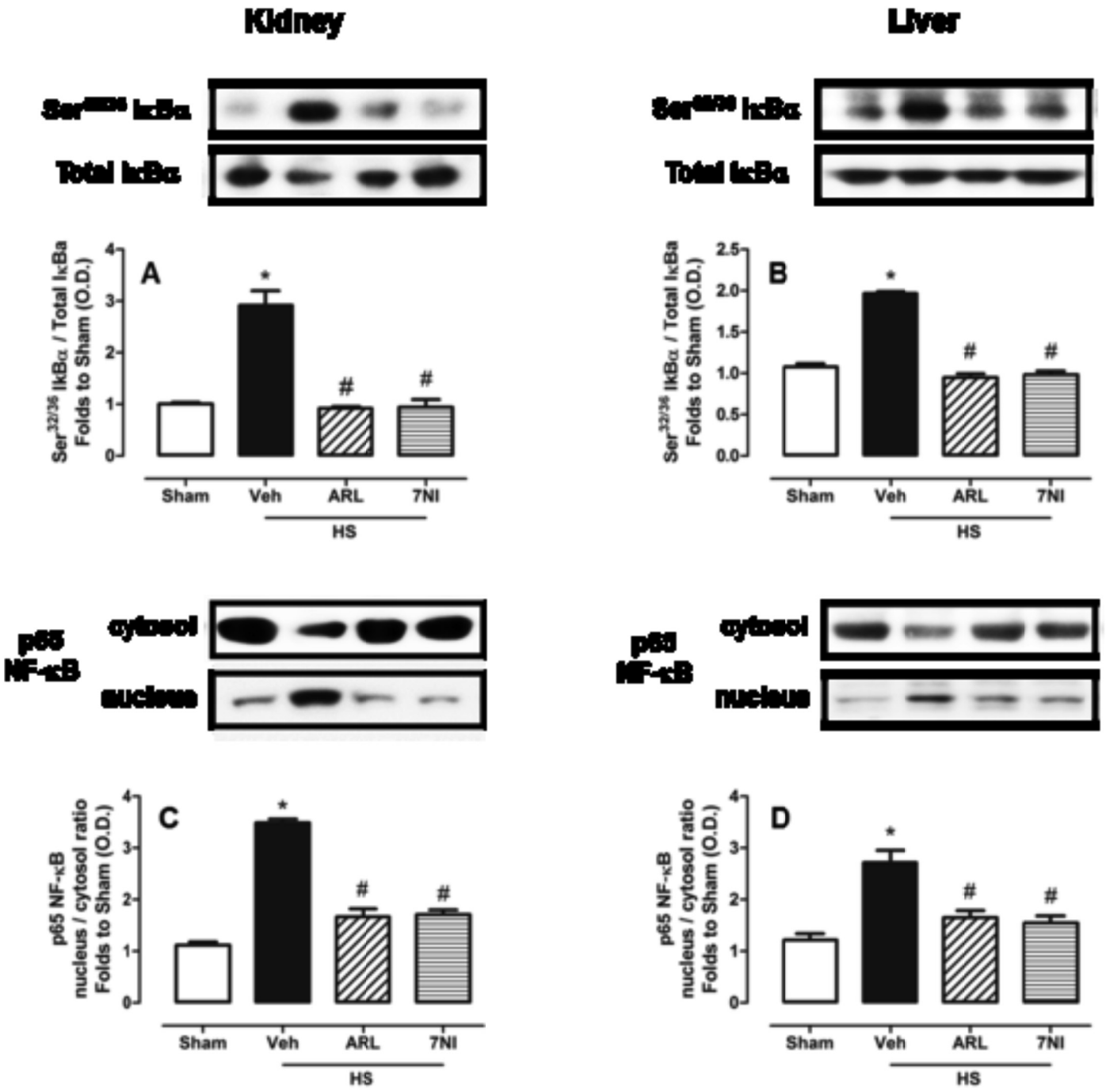

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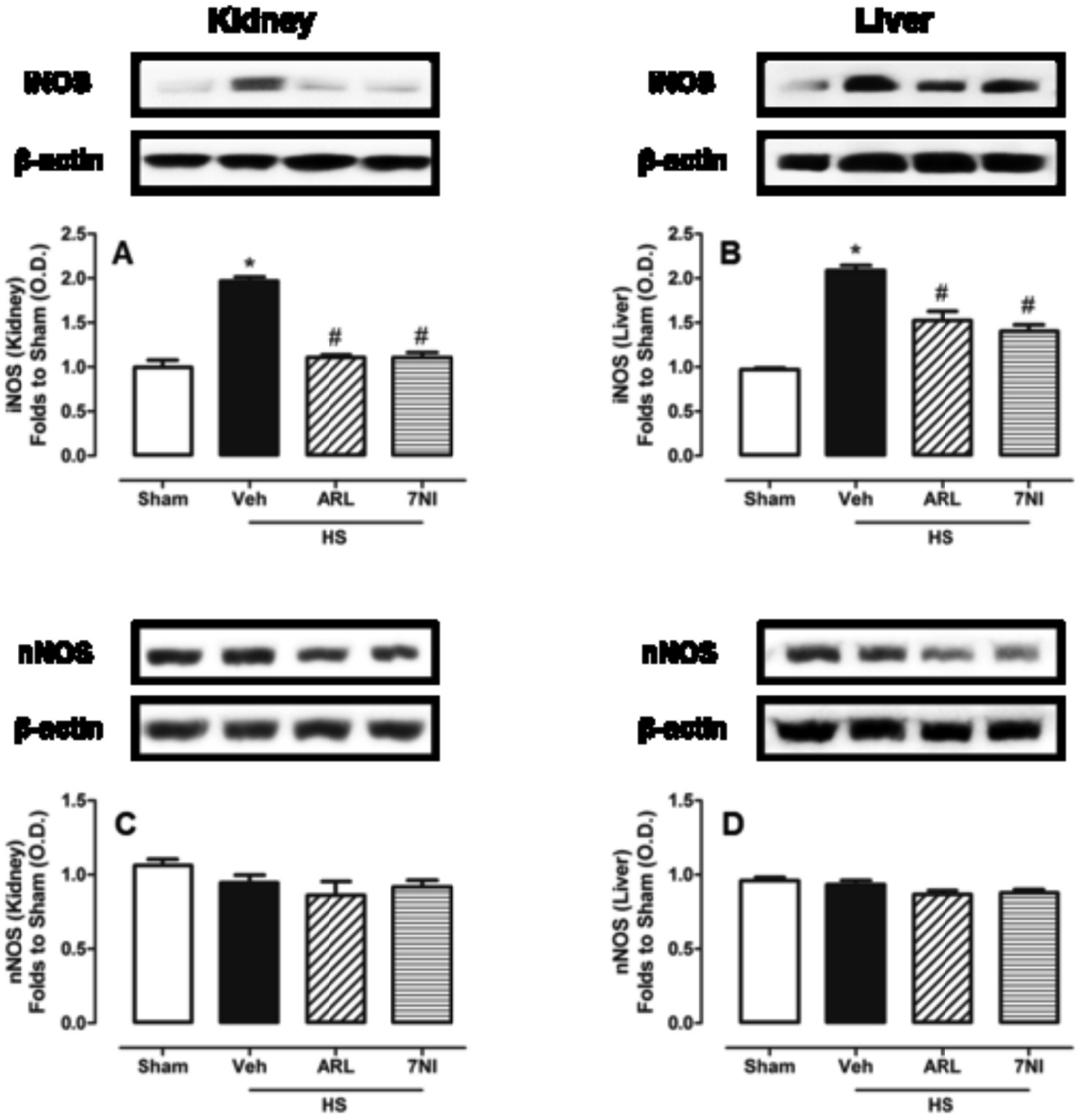

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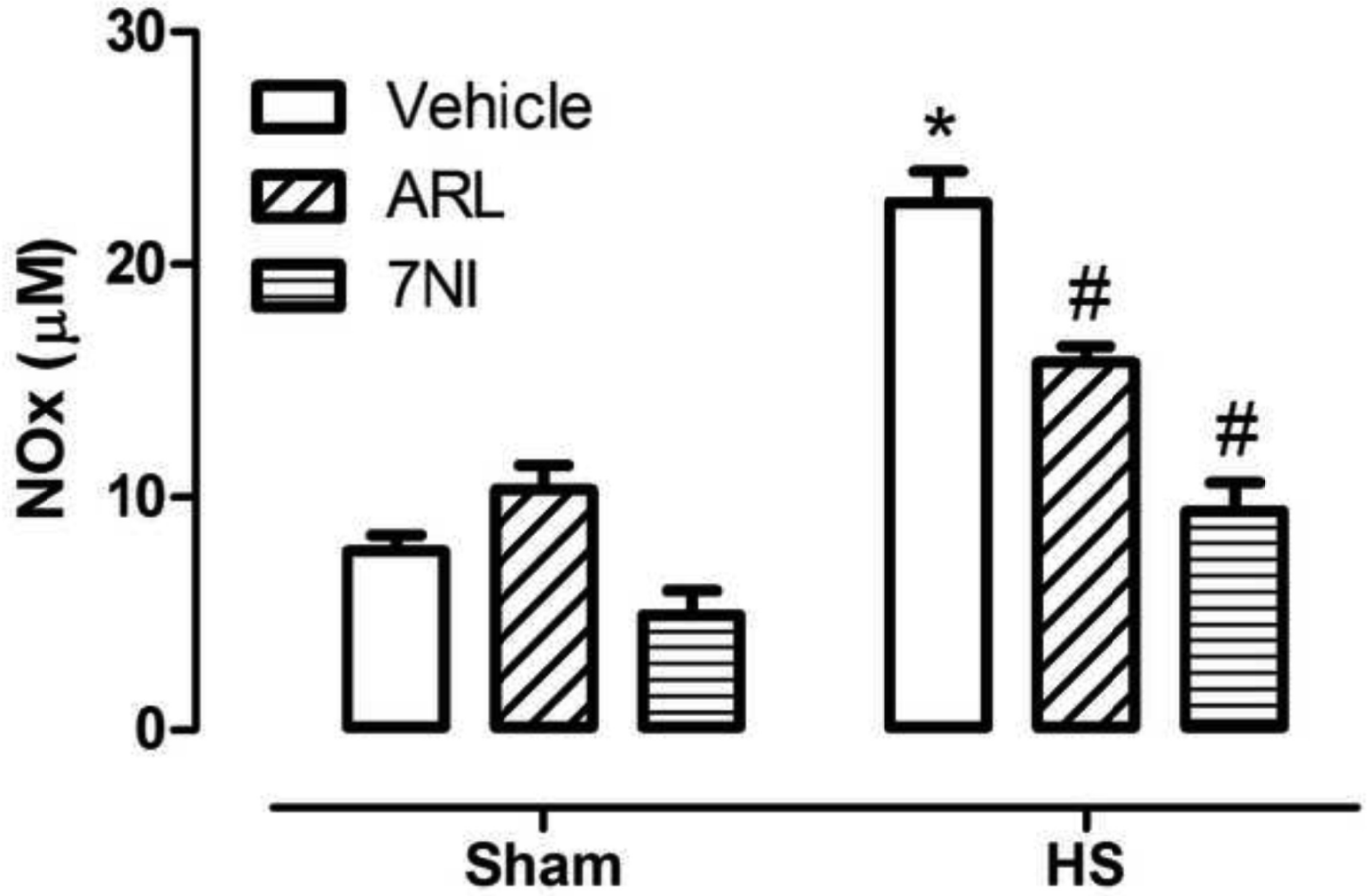

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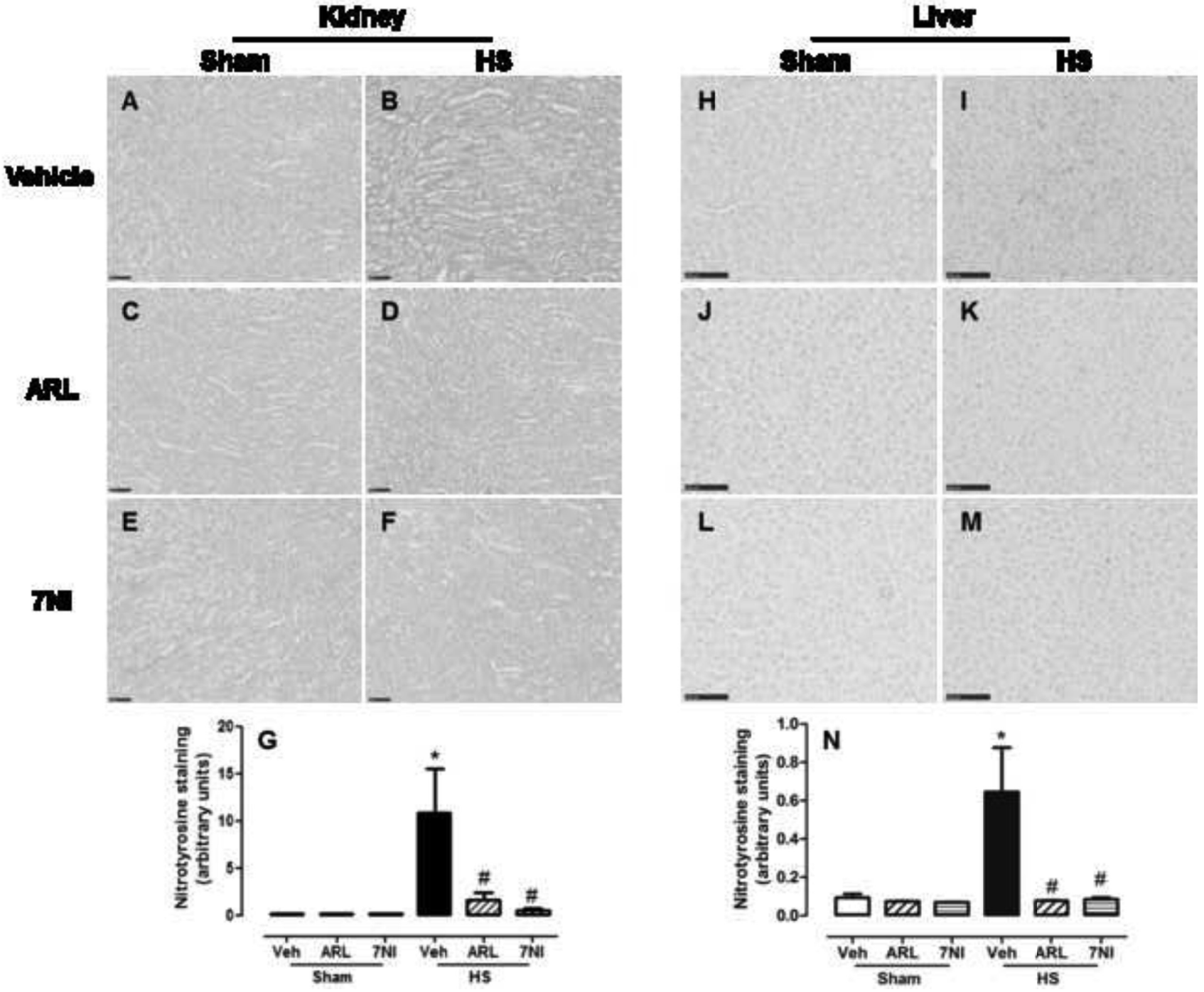
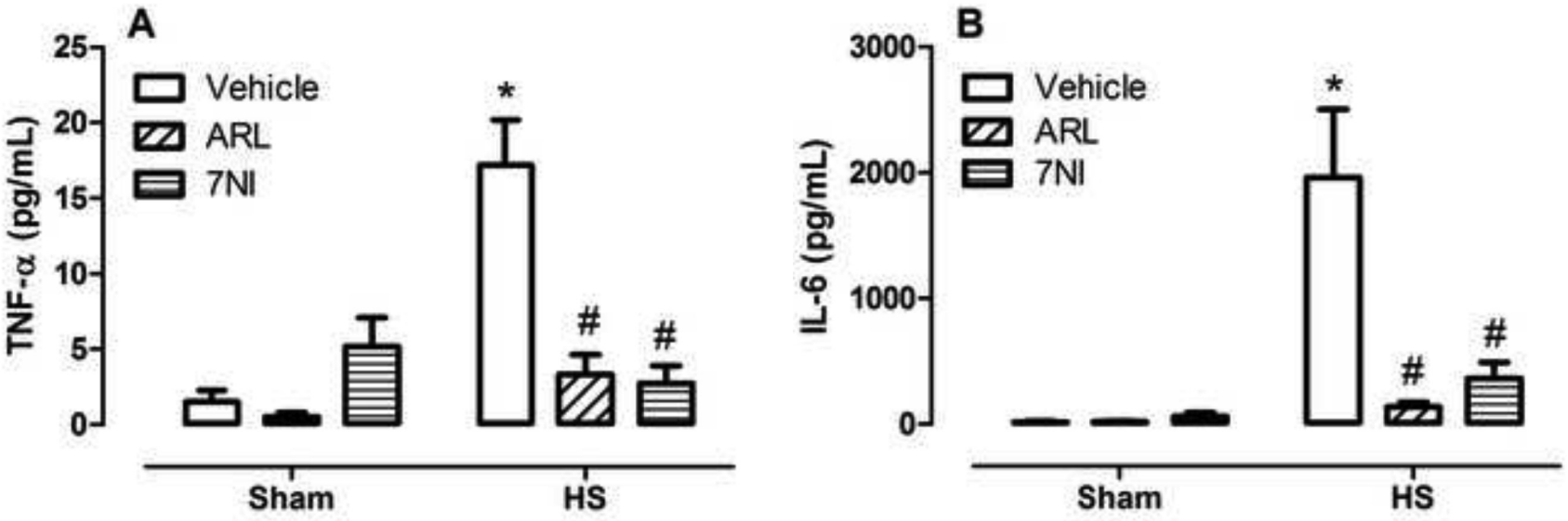
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