Inhibition of Nitric Oxide Synthase with Amino-guanidine Decrease the Systemic Inflammatory Response Following Hemorrhagic Shock in Rats

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ABSTRACT

Background: Hemorrhagic shock and resuscitation activates inflammatory cascade that involve the up regulation of cytokine synthesis. This process is associated with organ damage and death. Inducible nitric oxide synthase (iNOS) is increased during hemorrhagic shock and participate in pro-inflammatory signaling. Objectives: The aim of the present study was to examine the protective effects of inhibition of inducible nitric oxide synthase (iNOS) using Amino-guanidine against systemic inflammatory response in a rat model of hemorrhagic shock. Methods: Male Sprague- Dawley rats were assigned to 3 experimental groups (n = 6 per group): Normotensive rats (N); Hemorrhagic shock rats (HS); and Hemorrhagic shock rats treated with AG (HS-AG). After 60 min hemorrhagic shock, rats were treated or not by injection of 1ml of 60 mg/Kg Amino-guanidine (AG) intra-arterially. Rats were then resuscitated *in vivo* by reinfusion of the shed blood to restore norm tension. The mean arterial blood pressure was monitored. Blood was collected following 60 min hemorrhage and 30 min *in vivo* treatment and resuscitation for TNF-α measurement. Results: The present study showed that inhibition of inducible nitric oxide synthase (iNOS) using Amino-guanidine decreased the levels of tumor necrosis factor α (TNF-α) in the plasma after one hour of hemorrhagic shock and resuscitation in rats. The results showed that hemorrhagic shock and resuscitation significantly increased the levels of TNF-α. Conclusion: Inducible nitric oxide synthase (iNOS is involved in the up regulation of the inflammatory response in resuscitated hemorrhagic shock. Inhibition of inducible nitric oxide synthase (iNOS) using Amino-guanidine (AG) reduced the inflammatory response to hemorrhagic.

منع النيتريك أوكسايد سينثيز باستحدام الأمينوقوانيدين يقلل من الاستجابة الالتهابية الشاملة بعد الصدمة النزيفية في الجرذان

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المُستلخص

تعد الصدمة النزيفية والانعاش من محفزات سلسلة الاستجابة الالتهابية والتي تشمل زيادة تصنيع السيتوكاينز. مما يؤدي إلى فشل الأعضاء والوفاة. تزيد الصدمة النزيفية من مادة النيتريك أوكسايد سينثيز والتي تتسبب في بدء الاستجابة الالتهابية. الهدف من الدراسة الحالية هو دراسة التأثيرات الوقائية لمنع النيترك أوكسايد سينثيز باستحدام الأمينوقوانيدين ضد الاستجابة الالتهابية في نموذج الصدمة النزيفية بالجرذان. طرق البحث: تم استخدام فئران سبراج دولي (350-300جم) والتي تم توزيعها على 3 مجموعات تجارب (عدد 6/ كل مجموعة): مجموعة ضغط الدم الطبيعي، ومجموعة الصدمة النزيفية، ومجموعة الصدمة النزيفية المعالجة بمادة الأبلين. تم نزف الفئران بسحب الدم على مدى 60 دقيقة للوصول إلى ضغط دم 40 مم زئبق. تم علاج الفئران باستخدام 1ملل من مادة الأمينوقوانيدين (60 مغم/كغم) وذلك بحقنها داخل الشريان بعد 60 دقيقة من الصدمة النزيفية. تم إنعاش الفئران بعد ذلك وذلك بإعادة حقن الدم المسجوب على مدى 30 دقيقة لاستعاد الضغط الطبيعي. تم مراقبة وتسجيل ضغط الدم الشرياني. تم سحب عينات الدم بعد 60 دقيقة من الصدمة النزيفية، ومن ثم بعد 30 دقيقة من الإنعاش وذلك لقياس معدل حموضة الدم وكذلك لقياس معدل مادة عامل نخرالورم. النتائج: أوضحت النتائج أن منع مادة النيترك أوكسايد باستخدام الأمينوقوانيدين قللت من معدلات مادة عامل نخر الورم في الدم والتي تستخدم كدلالة على حدوث الاستجابة الالتهابية . كما أوضحت النتائج أن الصدمة النزيفية والإنعاش سببت زيادة ملحوظة في معدلات عامل نخر الورم. خلاصة البحث هي أن العلاج باستخدام مادة الأمينوقوانيدين والتي تمنع النيترك أوكسايد سينثيزقبل الإنعاش من الصدمة النزيفية قللت من الاستجابة الالتهابية بعد الصدمة النزيفية والإنعاش في الجرذان.

Introduction

Hemorrhagic shock induces an inflammatory response that involved the up regulation of cytokine production (Chaudry and Ayala 1993; Ulloa and Tracey 2005; Akkose, Ozgurer, *et al.* 2007). This inflammatory response result in multiple organ damage following hemorrhagic shock and resuscitation. The exact mechanism by which hemorrhagic shock and resuscitation initiates inflammatory responses not fully understood.

Hemorrhagic shock increased the levels of nitric oxide (NO) (Chaudry and Ayala 1993). NO is synthesized from L-arginine (Kelly, Balligand, *et al.* 1996). Under physiological conditions, NO maintains vascular tone, inhibits platelet aggregation and prevents neutrophil adherence (Palmer, Ferrige, *et al.* 1987; Moncada 1993; Moncada and Martin 1993). In pathological conditions, excessive NO production has a critical role in tissue damage (Tatsumi, Matoba, *et al*. 2000). Following hemorrhagic shock, enhanced nitric oxide (NO) production due to increased levels of inducible nitric oxide synthase (iNOS) contribute to the activation of the inflammatory pathways (Shi, Wu, *et al*. 2001; Liu, Ward, *et al*. 2003).

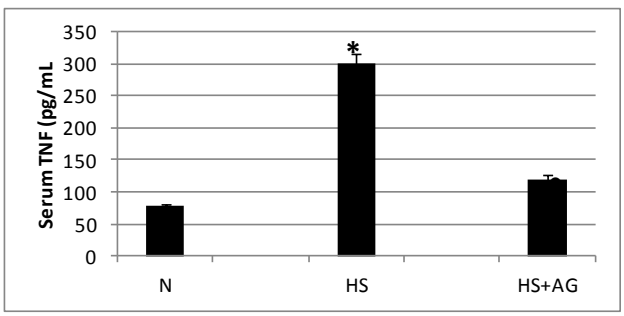
Elevated serum TNF-α levels are found during systemic inflammation (Cavriani, Domingos, *et al*. 2007). TNF-α is one of the inflammatory mediators in hemorrhagic shock and a cardio-depressant factor (Ulloa and Tracey 2005; Soliman 2011). Furthermore, TNF-α neutralization attenuates cardiovascular shock and inhibits production of inflammatory cytokines during resuscitation. TNF-α inhibition protects against the local and distant inflammatory responses following ischemia-reperfusion (Koksoy, Kuzu, *et al.* 2001). Previous study showed that inhibition of nitric oxide synthase (NOS) using Amino-guanidine (AG) before resuscitation following hemorrhagic shock protect the myocardium against post-resuscitation myocardial dysfunction(Soliman 2013; Barmaki and Khazaei 2015). Amino-guanidine (AG) belongs to the family of relatively specific inhibitors of inducible nitric oxide synthase (iNOS) activity(Southan and Szabo 1996).

The aim of the present study was to examine the protective effects of inhibition of nitric oxide synthase using Amino-guanidine (AG) against systemic inflammatory response in a rat model of hemorrhagic shock.

Material and Methods

1. Animal Preparation

The National Plan for Sciences and Technologies, King Saud University, approved this study. Male Sprague- Dawley rats weighing 300-350g were used. Rats were injected intra-peritonealy (i.p.) with 2000 I.U of heparin sodium 15 minutes prior to anesthesia. The rats were anesthetized using 125mg/kg urethane intra-peritonealy. The left carotid artery was annulated, and a three- way stopcock was attached in-line for monitoring the mean arterial blood pressure (MABP) using a blood pressure transducer. The animals were allowed to stabilize for a period of 30 minutes. The animals were assigned to the following 3 experimental groups (n = 6 per group): 1) Normotensive rats (N), 2) Hemorrhagic shock rats (HS), and 3) Hemorrhagic shock rats treated with AG (HS-AG) (figure 1). After 60 min hemorrhagic shock, rats were treated or not by injection of 1ml of 60 mg/Kg AG intra-arterially.



(* *represents P<0.05 versus sham group (n=6 per group), and • represents P<0.05 versus hemorrhage group "n=6 per group*").

Figure 1: Hemorrhage Increased TNF-α levels.

2. Hemorrhagic Shock

After a stabilization period of 30 min, hemorrhagic shock was induced. The rats were hemorrhaged using a reservoir (a 10 ml syringe) that was connected to the arterial (carotid artery) three way stopcocks. Opening the stopcock and

aspirating gently and gradually with the syringe induced the hemorrhage. Blood was aspirated at a rate of 1 ml/min. Blood was continuously withdrawn or re-infused to maintain a mean arterial blood pressure (MABP) of approximately 35-40 mmHg. With the exception of inducing the hemorrhage, the surgical procedure was the same for the sham hemorrhage group.

The study was approved by the ethics committee at King Abdulaziz City for Science and Technology (The National Plan for Science, Technology and Innovation (08-MED560-02) at King Saud University, Riyadh, Saudi Arabia).The rats were resuscitated *in vivo* by reinfusion of the shed blood to restore normotension, and the MABP was monitored for 30 min.

Amino-guanidine was obtained from Sigma (Sigma, St Louis, MO). The drug was dissolved in a 0.9% sodium chloride solution (Sigma).

3. Experimental Protocols

Three experimental groups (n = 6) were assigned for the study:

3.1. Normotensive Rats (N): The rats underwent the same surgical preparation and continuous blood pressure measurements were obtained for the 120- min experimental period.

3.2. Hemorrhagic Shock Rats (HS): After a 30- min stabilization period, the rats were hemorrhaged to 40 mmHg for 60 min. The rats were then resuscitated and monitored for 30 min.

3.3. Effect of Amino-guanidine (AG) during Hemorrhagic Shock (HS-AG): After a 30 min stabilization period, the rats were hemorrhaged to 40 mmHg for 60 min. AG was injected intra-arterially. The rats were then resuscitated and monitored for 30 min.

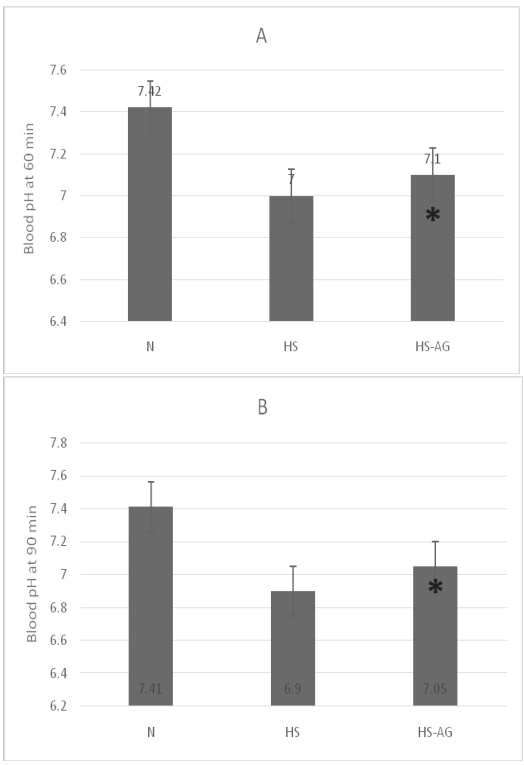
4. Blood Samples for Enzyme-linked Immunosorbent Assay and Plasma pH

Blood (0.5 ml) was collected from the left carotid artery cannula before hemorrhage, before resuscitation and 30 min after resuscitation and centrifuged at 2500 g for 10 min and plasma was stored at -80˚C until analysis for TNF-∞ measurement. Serum samples were analyzed by ELISA (R&D systems) according to the manufacturer's instructions. Blood samples were analyzed for plasma pH.

5. Statistical Analysis

The data were analyzed using the SPSS program. Statistical analysis was performed using a paired samples *t*-test. All data was initially analyzed using Bartlett's test for homogeneity. The data were analyzed using analysis of variance (ANOVA). The means were analyzed using Duncan's test and was considered significant when the "p" value was less than or equal to 0.05. The data was expressed as the means ± SD.

Results and Discussion



(* *represents P<0.05 versus normotensive group "n=6 per group*").

Figure 2 A & B: Figure 2A: Arterial pH in Two Blood Samples: A: At 60 min
Following Hemorrhagic Shock, and Figure 2B. After 60 min Hemorrhagic Shockand 30 min Resuscitation in the 3 Experimental Groups: Normotensive group (N), Hemorrhagic Shock Group (HS), and Hemorrhagic Shock Treated with AG (HS-AG)

1. Results

1.1. The animals were subjected to hemorrhagic shock to lower the MABP to the desired level

of hypotension (35-40 mmHg). The total volume of blood withdrawn was 15 ± 2.1 mL/kg body weight. There was no significant difference in the amount of blood withdrawn among the groups of animals subjected to hemorrhagic shock.

1.2. AG decreased TNF-α levels: Figure 1 showed that hemorrhagic shock caused a significant increase of serum TNF- α levels compared to normotensive group (see figure 1). The levels of TNF-α were significantly decreased with treatment with AG before resuscitation following hemorrhagic shock.

1.3. Blood pH: After 60 min of hemorrhagic shock, blood pH was significantly decreased (P>0.05) compared to the normotensive group (figure 2 A*)*. After 30 min of in vivo resuscitation and treatment, blood pH was significantly lower in the hemorrhagic shock and hemorrhagic shock treated group as compared to the normotensive group (Figure 2 B).

2. Discussion

This study was conducted to examine the protective effects of Amino-guanidine in preventing the inflammatory response associated with hemorrhagic shock and resuscitation by inhibition of inducible nitric oxide synthase (iNOS) production. Resuscitation following hemorrhagic shock result in systemic inflammatory response (Cai, Deitch, *et al*. 2009; Soliman 2011). Inflammatory cytokines such as TNF cause lethal cardiovascular shock (Ulloa and Tracey 2005). Previous studies have shown that inducible nitric oxide synthase (iNOS) is up-regulated following hemorrhagic shock and resuscitation (Thiemermann, Szabo, *et al*. 1993; Hierholzer, Kelly, *et al*. 1997). Previous study from our laboratory demonstrated that treating the animals with AG before in vivo resuscitation following one hour of hemorrhagic shock ,protected the myocardium against post-resuscitation myocardial dysfunction (Soliman 2013).The present study demonstrated that hemorrhagic shock and resuscitation activated an inflammatory response by up regulation of TNF-μ. Inhibition of inducible nitric oxide synthase (iNOS) using Amino-guanidine prevents the up regulation of TNF-μ. These data provide evidence that inducible nitric oxide synthase (iNOS) is in part responsible for the organ damage and myocardial dysfunction following hemorrhagic shock and resuscitation, and that the induction of nitric oxide synthase (iNOS) is a key event in the activation of the inflammatory pathway after resuscitation following hemorrhagic shock.

Hemorrhagic shock results in up regulation of inducible nitric oxide synthase (iNOS) levels (Szabo and Salzman 1996). Studies have shown that Inducible Nitric Oxide Synthase (iNOS) expression increases in parallel with the duration of shock (Kelly, Shah, *et al*. 1997). The present study support the idea that Nitric Oxide (NO) can activate an inflammatory response as demonstrated by the increase in the levels of TNF- α. Further studies are needed to examine the effects of inhibition of nitric oxide synthase (NOS) on other inflammatory markers.

Previous studies have shown that inhibition of inducible nitric oxide synthase (iNOS) increase organ injury in hemorrhagic shock (Harbrecht, Wu, *et al*. 1995; Soliman 2013). We previously demonstrated that lowering the levels of TNF-α by using Na-H exchange blocker, amiloride, protect against myocardial contractile dysfunction following hemorrhagic shock and resuscitation (Soliman 2011). Results of the present study suggest that myocardial contractile dysfunction in hemorrhagic shock is due, at least in part, to the proinflammatory action of Nitric Oxide (NO). Nitric oxide (NO) in combination with superoxide forms peroxynitrite, which could exert direct tissue toxicity.

Lowering the levels of nitric oxide (NO) in hemorrhagic shock could be of therapeutic benefit(Md, Moochhala, *et al*. 2005). Trauma patients with hemorrhagic shock or patients with other causes of bleeding can develop multiple organ injury and dysfunction. The results of the present study indicate that inhibition of inducible nitric oxide synthase (iNOS) may open the field for new therapeutic approaches that target the inflammatory response following hemorrhagic shock.

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