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# Diastolic function during hemorrhagic shock in rabbits

**Verónica D'Annunzio · Martín Donato · Andrea Fellet · Bruno Buchholz · Valeria G. Antico Arciuch · María C. Carreras · Laura B. Valdez · Tamara Zaobornyj · Celina Morales · Alberto Boveris · Juan J. Poderoso · Ana M. Balaszczuk · Ricardo J. Gelpi**

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**Abstract** Hemorrhage (H) is associated with a left ventricular (LV) dysfunction. However, the diastolic function has not been studied in detail. The main goal was to assess the diastolic function both during and 120 min after bleeding, in the absence and in the presence of L-NAME. Also, the changes in mRNA and protein expression of nitric oxide synthase (NOS) isoforms were determined. New Zealand rabbits were divided into three groups: Sham group, H group (hemorrhage 20% blood volume), and H L-NAME group (hemorrhage treated with L-NAME). We evaluated systolic and diastolic ventricular functions in vivo and in vitro (Langendorff technique). Hemodynamic parameters and LV function were measured before, during, and at 120 min after bleeding. We analyzed the isovolumic relaxation using t ½ in vivo (closed chest). After that, hearts were excised and perfused in vitro to measure myocardial stiffness. Samples were frozen to measure NOS mRNA and protein expression. The t½ increased during bleeding and returned to basal values 120 min after bleeding. L-NAME blunted this effect. Data from the H group revealed a shift to the left in the LV end diastolic pressure–volume curve at 120 min after bleeding, which was blocked by L-NAME. iNOS and nNOS protein expression and mRNA levels increased at 120 min after the hemorrhage. Acute hemorrhage induces early and transient isovolumic relaxation impairment and an increase in myocardial stiffness 120 min after bleeding. L-NAME blunted the LV dysfunction, suggesting that NO modulates ventricular function through iNOS and nNOS isoforms.

**Keywords** Hemorrhagic shock · Nitric oxide · Ventricular function · Nitric oxide synthase · Rabbit

Martín Donato, Andrea Fellet, María C. Carreras, Laura B. Valdez, Tamara Zaobornyj, Alberto Boveris, Juan J. Poderoso and Ricardo J. Gelpi: Member of the National Council of Scientific and Technological Research of Argentina (CONICET).

Verónica D'Annunzio and Martín Donato contributed equally to this manuscript.

V. D'Annunzio · M. Donato · B. Buchholz · C. Morales · R. J. Gelpi (✉)
School of Medicine, Institute of Cardiovascular Physiopathology and Department of Pathology, University of Buenos Aires, JE Uriburu 950—2nd floor, 1114 Buenos Aires, Argentina
e-mail: rgelpi@fmed.uba.ar

A. Fellet · A. M. Balaszczuk
Department of Physiology, School of Pharmacy and Biochemistry, IQUIMEFA-CONICET, University of Buenos Aires, Buenos Aires, Argentina

V. G. A. Arciuch · M. C. Carreras · J. J. Poderoso
Laboratory of Oxygen Metabolism, University Hospital, University of Buenos Aires, Buenos Aires, Argentina

L. B. Valdez · T. Zaobornyj · A. Boveris
Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

## Introduction

Hemorrhage constitutes a stress on the cardiovascular system that results in not only decreased cardiac loading conditions but also decreased blood pressure and thus decreased tissue perfusion pressure [1–3]. The heart's response to hemorrhage is governed by both an increase in the sympathetic nervous system activation of the heart and decreased preload and afterload [4]. Although myocardial

contractile dysfunction after hemorrhagic shock has been confirmed by numerous experimental approaches and in a variety of animal models [3, 5–7], only a few studies have considered the diastolic function during hypovolemic shock. Suzuki et al. demonstrated that a hypovolemic state alters the diastolic function in hemorrhaged animals, but they used a burn model [8]. In agreement, Walley et al. [9] showed that pigs subjected to a shock protocol present an increase in myocardial stiffness. Alyono et al. [10] showed impairment in diastolic function in dogs with severe hemorrhage (2 h of hemorrhagic shock) and a reversal of these changes in surviving dogs. However, to our knowledge, the two components of diastolic function (i.e., stiffness and relaxation) have not been studied in detail during a moderate and acute hemorrhagic shock.

Our first objective was to evaluate isovolumic relaxation during and after acute hemorrhage (early and late stages), and diastolic stiffness only at late stages, in anesthetized rabbits. Since acute hemorrhage is also associated with an increase in nitric oxide (NO) production [11–14], a second goal was to study the effect of NG-nitro-L-arginine methyl ester (L-NAME) both during and after the induction of hemorrhage in ventricular function behavior. A third objective was to determine the changes in mRNA and protein expression of nitric oxide synthase (NOS) isoforms.

## Materials and methods

The experiments were performed in male "New Zealand" rabbits. The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires. Rabbits weighing 1.8–2.0 kg were anesthetized with ketamine (75 mg/kg) and xylazine (0.75 mg/kg).

### Experimental protocols

The animals were randomized into three experimental groups (Fig. 1):

(1) Sham group (S group) ($n = 4$): left ventricular and femoral artery catheterization was performed in the rabbits and the hemodynamic variables were monitored for 120 min.
(2) Hemorrhagic rabbits (H group) ($n = 7$): after a 20-min stabilization period, the rabbits were subjected to acute blood bleeding equivalent to 20% of the circulating blood volume (1.4 ml/100 g body weight) for 2 min followed by 120 min of recovery.
(3) L-NAME hemorrhagic rabbits (H-L-NAME group) ($n = 7$): after a 20-min stabilization period, an L-NAME bolus (1 mg/kg) was administered, followed by an L-NAME infusion (0.5 mg/kg/h) during bleeding and recovery.

The hemodynamic variables were monitored before and every 15 s during the two-min bleeding, as well as every 5 min as from the end of hemorrhage to complete the recovery period (120 min). The blood was not reinfused, in any of the protocols.

Additional experiments were performed using the same three experimental groups in order to obtain ventricular tissue samples at 2 and 120 min after the bleeding ($n = 4$ each time and group).

### Ventricular function in vivo assays

Left ventricular pressure (LVP) was measured with fluid-filled catheters (3-Fr) inserted in the left ventricle through the right carotid artery and connected to a pressure transducer. Second and third catheters were placed into the left and right femoral arteries to measure arterial pressure and

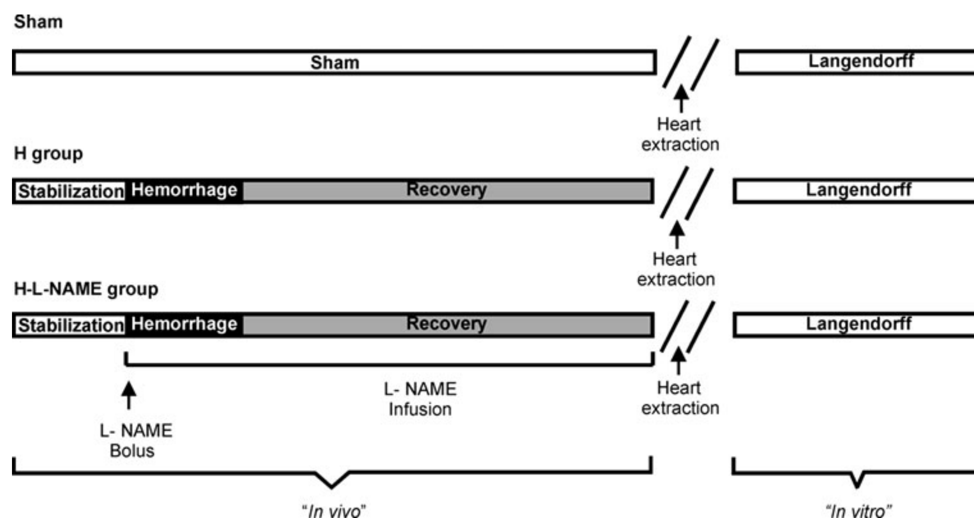


**Fig. 1** Experimental protocol scheme

to perform blood bleeding, respectively. A fourth catheter was placed in the femoral vein for L-NAME intravenous administration. Heart rate (HR, beats/min) and mean arterial pressure (MAP, mmHg) were calculated in all experimental protocols. The systolic function was also studied through the left ventricular systolic pressure (LVSP) and the maximal rate of rise in left ventricular pressure LV + $dP/dt_{max}$. The isovolumic relaxation was analyzed using $T_{½}$, defined as the time required for the left ventricular pressure to fall to 50% from LV $dP/dt_{min}$. The left ventricular end diastolic pressure (LVEDP) was also measured.

### Ventricular function in vitro assays

After the in vivo assays, all rabbits were killed at the end of the recovery period (120 min after hemorrhage) with sodium thiopental (35 mg/kg), and the hearts were excised and retrogradely perfused according to the Langendorff technique with Krebs-Henseleit solution as previously described [15]. Briefly, the solution was maintained at a constant temperature of 37°C and bubbled with a gas solution containing 95% $O_2$+5% $CO_2$ to obtain a pH value between 7.35 and 7.45. A latex balloon, linked to a polyethylene tube connected to a pressure transducer, was placed into the left ventricular chamber to allow the measurement of the LVP (mmHg). The HR was maintained constant throughout the experiment using a pacemaker (QRS electronic) at 175 beats/min. After a 20-min stabilization period, active pressure–volume curves were generated, gradually filling the latex balloon with water (0.20 ml each time) until the LVEDP reached an approximate value of 40 mmHg, as described previously [16].

### Western blot analysis

In all experimental protocols, ventricular tissue samples were obtained in the Sham group at 120-min follow-up, whereas in the H group, samples were obtained at 2 and 120 min after the bleeding to determine NOS expression. Samples were homogenized using a Tissue Tearor (Biospec Products Inc) in homogenization buffer (50 mmol/l Tris, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1% Triton, 1 mmol/l PMSF, 1 μmol/l pepstatin, 2 μmol/l leupeptin, and 1× protease inhibitor cocktail from Roche Diagnostics). The protein concentration was determined using the Lowry method. Also, 50 μg of protein underwent electrophoresis with 7.5% SDS–polyacrylamide gel, electro-transferred to polyvinylidene difluoride membranes, incubated with anti-nNOS, anti-eNOS, and anti-iNOS antibodies (Santa Cruz Inc.), and detected using the Amersham Western Blotting System (ECL system). Equal loading was controlled with the appropriate subcellular markers.

### Reverse transcriptase PCR

Total left ventricle RNA was extracted with TRIzol, and RT-PCR was performed using 4 μg RNA. Nested PCR for nNOS and iNOS was carried out with 0.5 μl of PCR product in 25-μl final volume. Reactions included 0.4 mM dNTPs, 1 μM specific primers, 4 mM MgCl2, and 2.5 units of Taq DNA polymerase and consisted of an initial denaturing step (94°C for 4 min), followed by 35 cycles (each of 94°C for 1 min, 55°C for 40 s, 72°C for 1 min). Sample quantification was normalized to endogenous $\beta$-actin. Each experiment included a DNA minus control.

### Data analysis

Data are expressed as the mean values ±SEM. Analysis of variance (ANOVA) for paired and unpaired samples followed by post hoc Bonferroni test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

## Results

Table 1 shows the MAP, HR, LVSP, LVEDP, and LV + $dP/dt_{max}$ of the S, H and H-L-NAME groups, in basal conditions, 2 and 120 min after the bleeding. Hemorrhage induced a significant decrease in MAP in the H and H-L-NAME groups, reaching a value of 26.4 ± 2.5 and 29.8 ± 2.3 mmHg 2 min after the bleeding, respectively ($P < 0.05$ vs. basal values). MAP values were recovered at 120 min in both groups. Hemorrhage did not change the HR compared with basal values. Pretreatment with L-NAME did not alter this parameter in the H-L-NAME group. Hemorrhage decreased LVSP and LV + $dP/dt_{max}$ during bleeding in the H and H-L-NAME groups. During recovery, LV + $dP/dt_{max}$ reached similar basal values. Pretreatment with L-NAME did not affect the behavior of these variables induced by bleeding. There were no differences in LVEDP during bleeding and recovery in the H and H-L-NAME groups.

Figure 2 shows significant changes in t½, a ventricular isovolumic relaxation index. Before hemorrhage, t½ was 9.9 ± 0.7 ms. During bleeding, it reached 19.2 ± 1.1 ms ($P < 0.05$ vs. Sham group) and then returned to similar basal values at 120 min of recovery. Pretreatment with L-NAME blunted the increase in t½ induced by hemorrhage during the bleeding (11.2 ± 1.1 ms at 90 s of bleeding, $P < 0.05$ vs. H group).

**Table 1** Hemodynamic variables and ventricular function

| Parameter | Groups | Basal | Basal L-NAME | 2 min | 120 min |
|---|---|---|---|---|---|
| MAP (mmHg) | S | 69.6 ± 6.2 | | 65.9 ± 6.1 | 63.3 ± 4.9 |
| | H | 69.6 ± 2.9 | | 26.4 ± 2.5* | 60.1 ± 5.3 |
| | H-L-NAME | 70.5 ± 2.6 | 74.1 ± 4.4 | 29.8 ± 2.3* | 60.9 ± 3.8 |
| HR (beats/min) | S | 195.5 ± 21.3 | | 199.8 ± 29.8 | 195.6 ± 10.8 |
| | H | 196.8 ± 10.1 | | 234.1 ± 37.7 | 223.3 ± 12.4 |
| | H-L-NAME | 191.8 ± 19.9 | 186.1 ± 11.1 | 200.7 ± 7.9 | 209.8 ± 4.8 |
| LVSP (mmHg) | S | 84.2 ± 3.9 | | 89.1 ± 3.2 | 85.3 ± 4.1 |
| | H | 84.3 ± 3.2 | | 39.2 ± 2.9* | 75.5 ± 4.5 |
| | H-L-NAME | 83.8 ± 3.9 | 93.5 ± 7.9 | 41.3 ± 3.4* | 75.5 ± 5.2 |
| LVEDP (mmHg) | S | 1.8 ± 0.2 | | 1.4 ± 0.3 | 2.2 ± 0.4 |
| | H | 2.2 ± 0.3 | | 1.5 ± 0.3 | 3.6 ± 0.5 |
| | H-L-NAME | 1.9 ± 0.3 | 1.7 ± 0.2 | 1.3 ± 0.1 | 2.1 ± 0.9 |
| $+dP/dt_{max}$(mmHg/sec) | S | 1,536 ± 502 | | 1,352 ± 325 | 1,458 ± 265 |
| | H | 1,245 ± 94 | | 450 ± 107* | 1,010 ± 161 |
| | H-L-NAME | 1,404 ± 76 | 1,422 ± 95 | 590 ± 66* | 1,121.5 ± 153 |

*MAP* mean arterial pressure, *HR* heart rate, *LVSP* left ventricular systolic pressure, *LVEDP* left ventricular end diastolic pressure, $+dP/dt_{max}$ maximal rate of rise in left ventricular pressure

* $P < 0.05$ versus Sham group (S)

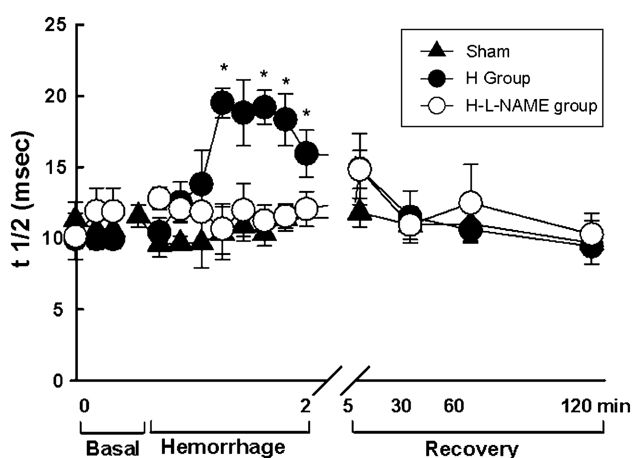


**Fig. 2** The LV pressure half-time (t½), an isovolumic relaxation index, increased early during bleeding in the H group and normalized during recovery. The administration of L-NAME blunted the diastolic alteration. In the Sham group, t½ did not change during the follow-up. *$P < 0.05$ versus H and Sham groups

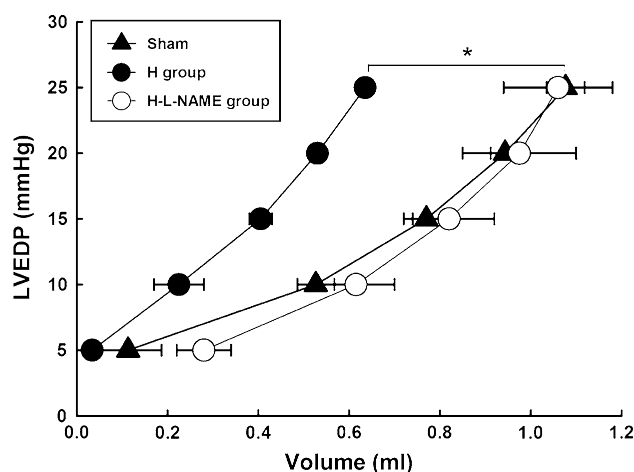


**Fig. 3** Left ventricular end diastolic pressure–volume relationship in the Sham, H, and H-L-NAME groups 120 min after bleeding. With acute bleeding, there is a left shift of the pressure–volume curve showing an increase in diastolic stiffness. L-NAME administration to the H group induced a behavior similar to that of the Sham group, abolishing the increase in stiffness. *$P < 0.05$ H group versus Sham and H-L-NAME groups. *LVEDP* left ventricular end diastolic pressure

Figure 3 shows the relationship between LVEDP and LV volume in isolated rabbit hearts, in the Sham, H, and H-L-NAME groups. A significant left shift of the LVEDP-LV volume curve was observed in the H group compared with the Sham group, suggesting an increase in myocardial stiffness. Pretreatment with L-NAME blunted the changes in diastolic ventricular function induced by bleeding.

### Determination of protein expression and mRNA levels of the NOS isoforms in cardiac tissue

The bar graphs (Fig. 4) show the mean values of nNOS, iNOS, and eNOS protein expression for the S and H groups 2 and 120 min after bleeding. iNOS and nNOS significantly increased at 120 min of recovery ($P < 0.05$ vs. S). In agreement with the increase in nNOS and iNOS, results of RT-PCR showed that mRNA levels increased ($P < 0.05$) 120 min after hemorrhage (Fig. 5). Because eNOS levels did not show any early or late change after hemorrhage, we did not evaluate the mRNA expression of this isoform (Fig. 4).

## Discussion

The main finding of the present study is that acute, brief, and moderate hemorrhage alters not only systolic but also

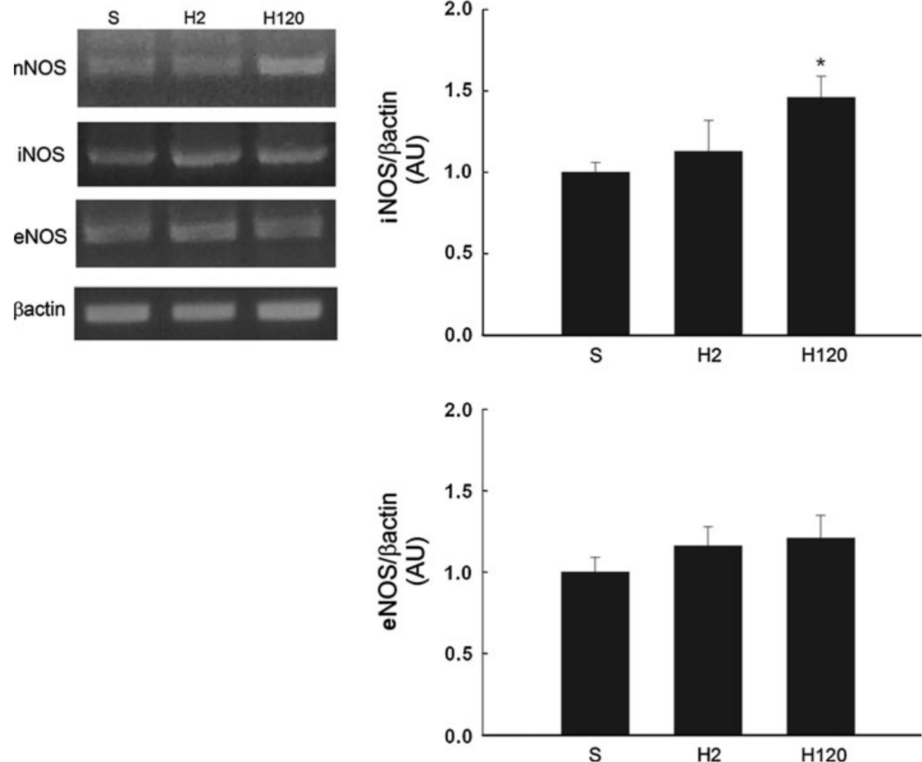


**Fig. 4** Representative western blots showing the expression of nNOS, iNOS, and eNOS in left ventricular tissue samples from the S and H groups 2 and 120 min after bleeding. nNOS and iNOS expression significantly increased 120 min after bleeding. $*P < 0.05$ versus Sham. *AU* arbitrary units, *S* Sham, *H2* 2 min after bleeding, *H120* 120 min after bleeding

diastolic ventricular function in anesthetized rabbit hearts. The isovolumic relaxation was impaired during the bleeding (early stage) and then normalized during the recovery period (late stage). In addition, we observed an increase in myocardial stiffness at the end of the recovery period. On the other hand, L-NAME administration significantly attenuated the isovolumic relaxation and myocardial stiffness impairment, evidencing the involvement of NO in the modulation of the ventricular function.

Myocardial contractile dysfunction after hemorrhagic shock has been confirmed by numerous experimental approaches and in a variety of animal models [3–7]. In our study, we also detected a myocardial contractile dysfunction, using LV $+ dP/dt_{max}$ as an index of contractility, which is also a preload-dependent index. The fact that we did not detect significant changes in LVEDP in the "in vivo" experiments allows us to conclude that the decrease in LV $+ dP/dt_{max}$ was not influenced by loading conditions.

Only few studies have considered the diastolic function during a hypovolemic shock, and most of these studies were performed in severe hemorrhage models when the ventricular function is clearly depressed. In the present work, we found, as expected, decreased systolic function associated with the impairment of the isovolumic relaxation during early bleeding. Interestingly, the inhibition of the NO system blunted these bleeding-induced changes. These findings suggest that NO could be involved in myocardial diastolic dysfunction during hemorrhagic states. It is important to mention that L-NAME administration did not modify blood pressure in our experiments. However, it is known that L-NAME effect on blood

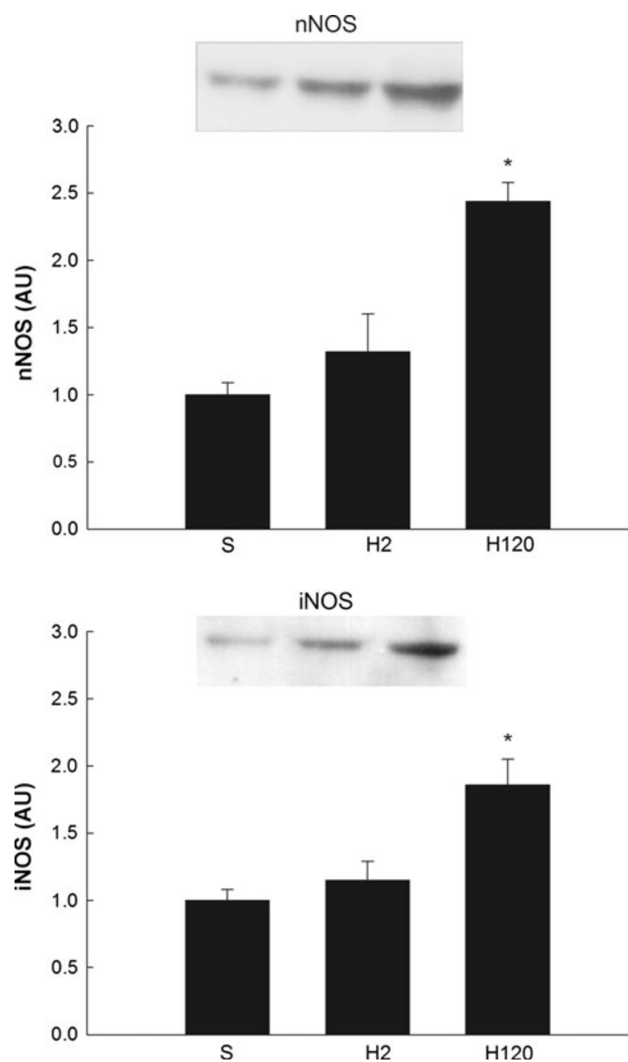


**Fig. 5** Transcriptase reverse PCR analyses of nNOS, and iNOS mRNA in the left ventricular tissue samples. nNOS and iNOS significantly increased at 120 min of recovery. $*P < 0.05$ versus Sham. *AU* arbitrary units, *S* Sham, *H2* 2 min after bleeding, *H120* 120 min after bleeding

pressure is dose dependent [17]: high doses increase blood pressure, whereas small or medium doses do not modify this parameter. In our study, we used a dose of 0.5 (infusion) and 1 mg/Kg (bolus) of L-NAME which could explain the lack of effect on blood pressure.

The decrease in diastolic blood pressure to less than 40 mmHg during bleeding could cause subendocardial ischemia [18], a phenomenon that could explain, at least in part, the slower relaxation rate. Interestingly, isovolumic relaxation was impaired during the bleeding but return to basal values at the end of recovery period. Furthermore, the experiments performed in isolated hearts showed an increase in myocardial stiffness late after hemorrhage, showing a clear dissociation between relaxation and stiffness in the late stage of the hemorrhage. These findings are interesting because they resemble the diastolic alterations in myocardial stunning where, at the beginning of reperfusion, there is a slower relaxation with normal stiffness and then a normal relaxation with an increase in stiffness [19]. Similarly, in our experiments, we found that the relaxation was normalized during the recovery period and that there was a late increase in myocardial stiffness associated with a normal ventricular relaxation. Although in L-NAME group, the diastolic blood pressure remains lesser than 40 mmHg, we observed an attenuation of relaxation rate and myocardial stiffness. These data suggest that the L-NAME ameliorate the diastolic dysfunction independently of changes in blood pressure.

It is highly possible that in our study, the hypovolemia could be responsible for the decrease in blood pressure, and at least in part, some recovery of the arterial pressure values could be due to the movement of fluid from the interstitial/intracellular to the intravascular space. On the other hand, since hematopoiesis is a process that takes several days after bleeding [20], it is difficult to think that in our study it could explain the changes in the ventricular function. It has also been shown that acute anemia occurs in massive hemorrhages with fluid reposition [21], and it is also known that oxygen supply is adequate even at low hemoglobin levels (e.g., 5 g/dl) [22]. For all the above, it is difficult to think that anemia might induce changes in myocardial stiffness secondary to the resulting hypoxia in our study.

In the present work, the systolic ventricular function assessed through LV $+ dP/dt_{max}$ was recovered earlier than myocardial stiffness, at the end of the recovery period. In this regard, a diastolic dysfunction in the presence of preserved systolic function has been described in several models of stunned myocardium [23, 24]. On the other hand, several studies showed the dissociation between the systolic and diastolic functions in different experimental or clinical situations. For example, it is known that arterial hypertension can evolve with diastolic dysfunction and normal ejection fraction [25]. It has also been observed that approximately 40% of patients with heart failure show diastolic dysfunction and preserved systolic function [26]. Thus, in accordance with our study, the observed dissociation between systolic function and myocardial stiffness is a frequent finding in different diseases including the stunned myocardium.

In the present study, hemorrhage should have induced an increase in the adrenergic activity, which would have caused an increase in heart rate and an improvement in the isovolumic relaxation rate. However, these changes did not occur, perhaps due to an inhibitory effect of anesthesia on the autonomic response. Several papers that used similar protocols with anesthetized animals did not find increased

heart rate either [27, 28]. In our study, we used ketamine and xylazine, and it is known that this combination of anesthetics decreases the heart rate [28, 29]. Finally, the probable existence of subendocardial ischemia during hemorrhage could have also masked the positive lusitropic effect of adrenergic stimulation.

No changes were observed in the mRNA of iNOS and nNOS isoforms during the early hemorrhage stage but increased at 120 min of recovery. However, we showed that L-NAME treatment restored the changes induced by bleeding in the earlier stages of the hemorrhagic shock, suggesting a role of NO as a modulator of ventricular relaxation in the hypovolemic state. The lack of changes in mRNA and protein levels could be related to the fact that is too early for mRNA and protein expression. Even though we are not able to detect changes in NOS mRNA or protein at 2 min after bleeding, hemorrhage confers some degree of post-translational modification of the protein. It is well known that in the first minutes of reperfusion, after ischemia, increase in NO and reactive oxygen species (ROS) levels was found [30–32]. Since in our hemorrhagic model, there could be certain degree of subendocardial ischemia, the increase in ROS production could be responsible for the impairment of ventricular relaxation without changes in the NOS mRNA expression or protein. On the other hand, it was shown that L-NAME administration attenuates ischemia/reperfusion injury [33, 34]. Overall, it is possible that these mechanisms could be involved in ventricular relaxation impairment.

Interestingly, our in vitro experiments showed a left shift of the pressure–volume relationship in the H group, indicating an increase in myocardial stiffness. In accordance, Alyono et al. [10] demonstrated an increase in the stiffness of hemorrhaged animals. Furthermore, Walley and Cooper demonstrated an increase in diastolic stiffness as compared to control values at the end of the hemorrhagic shock (120 min) [9]. However, these authors did not study the intrinsic mechanisms. We showed that L-NAME administration abolished the alterations in myocardial stiffness at 120 min of recovery. In this sense, Jessup et al. [35] suggest that a possible mechanism for the increase in myocardial stiffness may have resulted from a deficiency in available tetrahydrobiopterin and subsequent increase in nNOS-derived superoxide and reduction in nitric oxide synthase metabolites within the heart [35]. These authors used a different animal model but related nNOS with myocardial stiffness.

In summary, we demonstrated that an acute hemorrhagic shock resembles the myocardial stunning phenotype, inducing early and transient isovolumic relaxation impairment and a later increased myocardial stiffness, which persists 120 min after bleeding. The inhibition of NO blunted these effects, suggesting that NOS isoforms may modulate both isovolumic relaxation and diastolic stiffness during hypovolemia.

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