Acta Veterinaria (Beograd), Vol. 56, No. 2-3, 157-170, 2006.

DOI: 10.2298/AVB0603157T UDK 619:616-005.1

L-ARGININE AND PHYSOSTIGMINE IN HEMORRHAGIC SHOCK: LACK OF EVIDENCE FOR SYNERGISM

TODOROVIĆ Z, PROSTRAN MILICA, VUČKOVIĆ SONJA, STOJANOVIĆ R, NEŠIĆ ZORICA, LASICA R and MIRKOVIĆ LJILJANA

School of Medicine, Belgrade

(Received 19. December 2005)

We have previously shown the protective effects of both L-arginine and phystigmine in an experimental model of severe hemorrhagic shock, when these substances were used as monotherapy. It was of interest to investigate whether the combination of L-arginine (300 mg/kg, i.v. bolus) and physostigmine (0.07 mg/kg, i.v. bolus) could produce further beneficial cardiovascular and/or metabolic effects in anaesthetized hemorrhaged rabbits (intermittent bleeding; 40% of the estimated blood volume for 15 min). Selected cardiovascular and biochemical parameters were assessed before bleeding and at several points up to 60 min after the end of bleeding. Drugs were injected 1-2 min after the end of bleeding (Phy-group) or 10 min later (L-Arg+Phy-group). Control rabbits received the corresponding volumes of saline only (0.6-2.0 ml; S-group). Physostigmine (0.07 mg/kg) produced a rapid and sustained increase in mean arterial pressure, the effect being attenuated in L-arginine-pretreated rabbits (Phy- and L-Arg+Phy-group, respectively). The beneficial effecs of L-arginine on heart rate and hemoglobin oxygen saturation in venous blood were not completely lost in rabbits of the L-Arg+Phy group, and such a combination partially improved acid-base status (decrease in $PaCO_2$ in arterial blood) and produced further hemodilution (decresase in hematocrit) 15-60 min after the end of bleeding. However, the combination of L-arginine and physostigmine does not offer any significant advantage over the monotherapy with these drugs in hemorrhagic shock.

Key words: L-arginine, physostigmine, hemorrhagic shock; rabbit

INTRODUCTION

Physostigmine has been extensively used in various models of hemorrhagic shock with remarkable success (Guarini *et al.*, 1989; Savić *et al.*, 1991, 1992; Žunić *et al.*, 1995; Todorović *et al.*, 1996, 1997). As a lipid-soluble anticholinestrase, it is distributed in the central nervous system and may increase blood pressure *via* central cholinergically mediated activation of the peripheral

adrenergic system (Varagić, 1955; Prostran *et al.*, 1994, 1996, 1997). However, the beneficial effects of physostigmine (0.07 mg/kg, i.v. bolus) on arterial pressure and survival in hemorrhaged rats and rabbits could not be attributed only to the increased peripheral vasoconstriction, and several other hypothesis have arisen (Savić *et al.*, 1991, 1992; Žunić *et al.*, 1995). The presumed anti-shock effects of physostigmine have been explained by the stimulation of adrenaline release from the adrenal medulla since (a) nicotinic, but not muscarinic antagonists abolished the protective effects of physostigmine in hemorrhaged rabbits (Guarini *et al.*, 1989), and (b) nicotinic agonist dimethylphenylpiperazinium (DMPP) (0.5 μg/kg) produced a rapid and sustained reversal of hemorrhagic shock in rats *via* stimulation of adrenaline release from the adrenal medulla (Bazzani *et al.*, 1996). Also, physostigmine caused a significant hemodilution in hemorrhaged rabbits (Savić *et al.*, 1991, 1992). In addition, the same authors surmised that this anticholinesterase may antagonize the action of endogenous substances (e.g. opioides) known to aggravate hemorrhagic hypovolemia state.

Endogenous L-arginine, a semi-essential amino acid, is involved in L-arginine-nitric oxide (NO) pathway of blood pressure control, contributing to vasorelaxation of blood vessels and negative inotropic action in the cardiac muscle, and playing an important role in pathophysiological response to hemorrhagic hypovolemia and shock (Szabo and Thiemermann, 1994; Thiemermann, 1995; Cylwik *et al.*, 2005). Nitric oxide is well known to contribute to organ damage in hemorrhagic shock (Szabo and Billiar, 1999). However, the protective effects of L-arginine in various models of hemorrhagic shock have also been observed (Daughters *et al.*, 1996; Mellander *et al.*, 1997; Sato, 1998; Angele *et al.*, 1999; Todorović *et al.*, 2001). It was assumed that the beneficial actions of L-arginine in such models could be attributed to the improvement of vascular endothelial function and tissue oxygenation (NO-related, *stereoselective* effects) (Todorović *et al.*, 2001). In addition, certain *non-specific* effects of L-arginine have also been assumed (e.g. weak antioxidant and direct cardioprotective action; increase in tissue oxygen extraction). Also, it should be taken into account that higher doses of L-arginine were predominantly protective in models of hemorrhagic shock and a significant percentage of the injected L-arginine is metabolized *via* arginase, but not nitric oxide synthase. The role of such a metabolic pathway in this model remains to be assessed.

The interaction between L-arginine and physostigmine was extensively investigated in normotensive and spontaneously hypertensive non-hemorrhaged rats (Prostran *et al.*, 1994, 1997). Such investigations may be of a great importance in the elucidation of the role of peripheral and central mechanisms of beneficial actions of both physostigmine and L-arginine in hemorrhagic shock. Accordingly, we have evaluated the effects of i.v. bolus injections of physostigmine in L-arginine pretreated and untreated anaesthetized hemorrhaged rabbits.

MATERIAL AND METHODS

Chemicals

The following substances were used: L-arginine hydrochloride (Sigma, St. Louis, USA), physostigmine salicylate (Serva, Heidelberg, Germany) and thiopentone-sodium (Trapanal®, Byk Gulden, Konstanz, Germany). All substances were dissolved in distilled water, and diluted in saline solution (0.9% NaCl) immediately before injection.

Animals

The experiments were performed on rabbits, bred and kept under standard laboratory conditions. The investigation conforms with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Experimental protocols

Rabbits were anaesthetized with thiopentone-sodium (5% solution in 0.9% NaCl, 0.4 ml/kg, i.v.). Both the right femoral artery and vein and the left femoral artery were cannulated and blood was heparinized (250 IU/kg b.w. in 0.05 mL volume). An intravenous cannula was placed in the right femoral vein to reach the middle part of the iliac vein, while an intraarterial cannula was placed in the right femoral artery. Intermittent bleeding (1 min of bleeding + 4 min of pause) through the cannulated right femoral blood vessels, lasting 15 min, was used to remove 40% of the estimated blood volume (approx. 5% of body mass). The obtained samples of arterial and venous blood were immediately analyzed. The 15th minute blood sample (5.5 ml) was immediately replaced with an identical volume of whole blood, taken from the same animal (Todorović *et al.*, 1998; 2001).

Hemorrhaged rabbits were treated with physostigmine (0.07 mg/kg) or the combination of L-arginine (300 mg/kg) and physostigmine (0.07 mg/kg). The experimental groups of rabbits were as follows:

– Phy, treated with i.v. bolus of physostigmine in a dose of 0.07 mg/kg 1-2 min after the end of bleeding (N = 5; b.w.: 3262 ± 142 g);

– L-Arg+Phy, treated with i.v. bolus of L-arginine in a dose of 300 mg/kg 1-2 min after the end of bleeding followed by i.v. bolus of Phy in a dose of 0.07 mg/kg 10 min later (N = 6; b.w.: 2958 ± 40 g);

– S, the control group of rabbits, received a corresponding volume of saline (0.6 - 2.0 ml) 1-2 min after the bleeding was stopped (N = 6; b.w.: 2978 ± 110 g).

Both the systolic and diastolic arterial blood pressures were continuously recorded from the left femoral artery by means of a pressure transducer (Burdon type, Physiograph "SIX", Huston). The mean arterial pressure (MAP) was calculated according to the following formula: MAP = DP + (SP - DP):3. The heart rate was also monitored *via* precordial electrodes (Cardiac Preamplifier Physiograph MK IV) and expressed in beats per minute (b.p.m.). Cardiovascular parameters were measured: 1) before bleeding (PB values); 2) immediately after the end of bleeding and before the addition of a drug or saline (AB values); 3) 5,

10, 15, 30 and 60 min after the end of bleeding (5, 10, 15, 30 and 60 min values, respectively).

Selected biochemical parameters, including serum sodium, potassium, chloride and protein concentrations (Astra 8, Beckman) and hematocrit (micromethod) were measured before bleeding, immediately after bleeding and 15 and 60 minutes after bleeding was stopped. The acid--base balance parameters: arterial and venous pH, actual bicarbonate (ActB), excess base (EB), partial pressure of carbon dioxide ($PaCO_2$) and hemoglobin saturation with oxygen (sO_2) were determined according to Astrup and Siggaard-Andersen method at 37°C (Siggaard-Andersen, 1963), employing ABL-3 system (Radiometer, Copenhagen). Biochemical parameters were measured: 1) before bleeding (PB values); 2) immediately after the end of bleeding and before the addition of any drug or saline (AB values); 3) 15 and 60 min after the end of bleeding (15 and 60 min values, respectively).

Statistics

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Values are expressed as the mean ± standard error of the mean (S.E.M.) of N determinations. Mean values before and after bleeding, as well as differences between experimental and control groups, were compared. The statistical significance of drug effects and comparison of the different time-response curves was performed by analysis of variance (ANOVA) and Dunnet's post hoc test. Linear regression model, the coefficient of the linear correlation and test for parallelism of the time-response curves were also used in analysis when appropriate. Values of $P<0.05$ were taken as statistically significant.

RESULTS

Cardiovascular responses

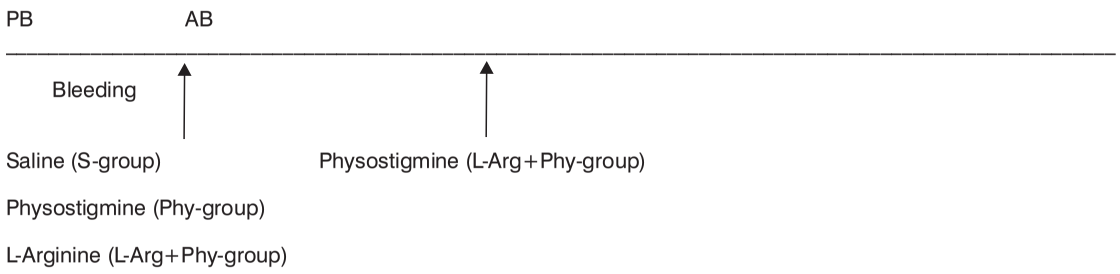
The mean arterial pressure (MAP) and heart rate (HR) of anaesthetized rabbits (S-, Phy, L-Arg+Phy-group, respectively) were statistically similar under basal conditions (i.e., before bleeding and injection of saline or any other drug). Severe blood loss (40% of the estimated blood volume) caused a profound fall in MAP and HR in all groups of rabbits. After-bleeding values of MAP and HR were significantly lower than the corresponding pre-bleeding values (PB vs. AB, 0 min: $P<0.05$) (Table 1).

In saline-treated rabbits, insignificant changes in MAP and HR were observed 0-60 min after the end of bleeding (Table 1). During the same 60 min experimental period, i.v. bolus of Phy (0.07 mg/kg) caused a rapid and sustained raise in MAP from approx. 40 to approx. 80 mm Hg, the effect being most pronounced 5 min after the injection of the drug (S vs. Phy, 5 min: $P<0.05$). Such an effect of Phy on the MAP was abolished with L-Arg (300 mg/kg) in L-Arg+Phy group, i.e. the MAP even decreased from approx. 68 to approx. 66 mm Hg 5 min after the i.v. bolus injection of Phy in rabbits pretreated with L-Arg (values recorded 10 and 15 min after the end of bleeding, respectively) (Table 1). (Please note that

Table 1. Mean arterial pressure and heart rate of anaesthetized rabbits before and 0-60 min after the cessation of bleeding (S-, Phy-, and Phy+L-Arg-group, respectively) (mean ± SEM)

Group		PB	AB					
			0 min	5 min	10 min	15 min	30 min	60 min
S	N MAP HR	6 115 ± 9 330 ± 17	6 39 ± 2 269 ± 14	6 51 ± 6 252 ± 14	6 57 ± 9 255 ± 12	6 61 ± 6 271 ± 9	5 60 ± 8 266 ± 9	4 60 ± 9 255 ± 23
Phy	N MAP HR	5 122 ± 4 330 ± 6	5 43 ± 5 270 ± 25	5 82 ± 11 * 253 ± 31	5 77 ± 5 267 ± 21	5 75 ± 3 282 ± 17	5 73 ± 7 292 ± 11	5 80 ± 7 289 + 12
L-Arg+Phy	N MAP HR	6 113 ± 6 299 ± 10	6 27 ± 2 † 244 + 15	6 65 ± 5 264 ± 13	6 68 ± 7 275 ± 11	6 66 ± 9 266 ± 9	6 64 ± 5 277 ± 9	6 69 ± 10 276 ± 10

S – S-group; Phy – Phy-group; L-Arg+ Phy - L-Arg+ Phy-group; N – number of animals survived; MAP – mean arterial pressure (mm Hg); HR – heart rate (b.p.m.); PB – pre-bleeding values; AB – after-bleeding values (measurements were performed 0, 5, 10, 15, 30 and 60 min after the cessation of bleeding, respectively); * - P <0.05 in comparison with the S group; † - P< 0.05 in comparison with the Phy-group.
Experimental design is shown below.



the MAP values were somewhat lower immediately after the end of bleeding in L-Arg+Phy- than in Phy-group; 0 min: $P<0.05$.). Neither Phy itself nor the combination of L-Arg and Phy significantly changed the HR 0-60 min after the end of bleeding, although an insignificant raise of the heart rate was observed in both groups (0 min vs. 60 min: ΔHR of 19 and 32 b.p.m., respectively).

Acid-base balance parameters

a) Partial pressure of carbon dioxide

Basal values of partial pressure of carbon dioxide in arterial and venous blood ($PaCO_2a$ and $PaCO_2v$, respectively) were similar in all rabbits. A sustained decrease in $PaCO_2a$ values was observed in S-, Phy and L-Arg+Phy-group, 0-60 min after the end of bleeding (0 min vs. 60 min: $P<0.01$, $P<0.05$, and $P<0.001$, respectively). The most profound fall in $PaCO_2a$ values was observed in rabbits treated with the combination of L-Arg and Phy, 60 min after the end of bleeding (L-Arg+Phy vs. Phy, 60 min: $P<0.05$) (Table 2).

Values of $PaCO_2v$ significantly increased in S- and Phy-treated rabbits 15 and 60 min after the end of bleeding, while such an increase was markedly attenuated in L-Arg+Phy-group (L-Arg+Phy vs. S, 15 min: $P<0.05$) (L-Arg+Phy vs. Phy, 15 min: $P<0.01$; 60 min: $P<0.05$).

b) Actual bicarbonate, excess base, pH and hemoglobin saturation with oxygen

Basal values for actual bicarbonate, excess base, pH and hemoglobin saturation with oxygen in arterial and venous blood (ActBa, ActBv, EBa, EBv, pHa, pHv, sO_2a and sO_2v, respectively) displayed no difference between any of the groups studied (Table 2).

Hematocrit, serum protein and electrolyte levels

Basal values of hematocrite (Ht) were similar in all groups studied. The only exception was a small but significant difference between S- and L-Arg+Phy-group before bleeding (PB) ($P<0.05$; Fig. 1, A). On the other hand, Ht values were statistically similar in all groups studied immediately after the end of bleeding (0 min; Fig. 1, A).

Ht values decreased during 60 min after the bleeding period in all groups, reaching the plateau 15-60 min after the end of bleeding. Such plateau was significantly shifted to the right in L-Arg+Phy-group in comparison with the S-group, 15 and 60 min after the end of bleeding ($P<0.05$, both; Fig. 1, A).

At the same time, total serum protein levels (TPr) decreased in a similar manner in all groups 0-60 min after the end of bleeding (Fig. 1, B). However, a significant correlation was found between the 60 min values of Ht and TPr in L-Arg+Phy-group only ($r = 0.9377$; $P<0.05$) (Fig. 1, C).

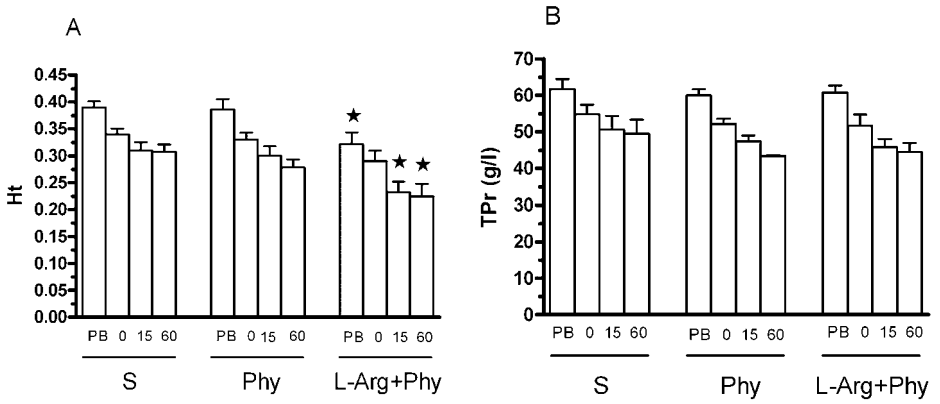
There were no differences between basal serum electrolyte values in any of the groups studied (not shown). Neither hemorrhage itself nor the bolus injections of saline, Phy or the combination of L-Arg and Phy significantly changed these parameters 0-60 min after the cessation of bleeding (data not shown). The only exception was a small but significant increase in serum potassium levels in L-

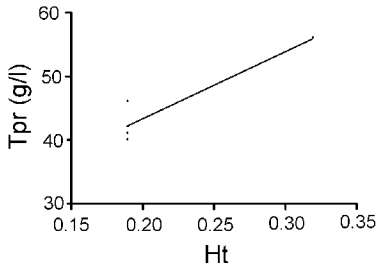
Table 2. Acid-base balance parameters of anaesthetized rabbits before and 0-60 min after the cessation of bleeding (S-, Phy-, and Phy+L-Arg-group, respectively) (mean ± SEM)

Group		PB		AB					
				0 min		15 min		60 min	
		a	v	a	v	a	v	a	v
S	ActB	21.87±1.48	24.57±1.40	20.62±1.13	23.92±1.11	15.53±0.77	19.08±1.04	11.78±0.75	16.53±1.21
	EB	-2.33±1.67	-1.13±1.62	-2.95±1.04	-2.10±1.11	-8.42±0.83	-9.65±1.45	-12.93±1.07	-12.85±1.89
	pH	7.39±0.03	7.34±0.02	7.42±0.01	7.31±0.01	7.37±0.02	7.15±0.02	7.31±0.03	7.10±0.04
	sO_2	96.78±0.83	73.98±1.75	98.55±0.17	58.33±5.49	96.98±1.38	34.53±5.77	98.17±0.38	40.23±5.60
	$PaCO_2$	5.10±0.30	6.14±0.14	4.31±0.23	6.48±0.30	3.68±0.18	7.57±0.26	3.19±0.15	7.48±0.27
Phy	ActB	23.64±1.14	27.06±1.06	23.00±1.58	26.36±1.07	15.82±0.95	20.78±0.57	13.80±2.14	17.65±2.54
	EB	0.00±1.08	2.02±1.15	-0.70±1.67	0.46±1.35	-8.36±1.11	-7.32±1.02	-11.35±2.91	-10.20±3.12
	pH	7.43±0.01	7.40±0.01	7.43±0.02	7.34±0.03	7.36±0.02	7.18±0.03	7.29±0.06	7.13±0.09
	sO_2	98.10±0.44	81.34±4.73	98.26±0.49	52.08±8.37	98.08±0.35	39.14±7.90	98.60±0.17	43.43±7.76
	$PaCO_2$	4.74±0.20	5.90±0.18	4.68±0.15	6.65±0.43	3.78±0.18	7.75±0.38	3.78±0.13	7.62±0.74
Phy+ L-Arg	ActB	21.82±0.70	23.55±0.78	21.25±1.13	24.72±1.12	15.65±1.05	19.80±1.45	12.92±2.34	16.25±2.26
	EB	-2.43±0.72	-1.97±0.82	-2.32±1.15	-0.77±1.26	-8.17±1.09	-6.73±1.73	-12.22±2.90	-11.00±2.89
	pH	7.39±0.01	7.34±0.01	7.43±0.01	7.34±0.02	7.39±0.02	7.23±0.03	7.33±0.04	7.18±0.05
	sO_2	96.32±0.52	75.93±3.38	97.67±0.54	40.18±3.46	97.97±0.40	36.78±5.46	98.05±0.26	36.78±10.98
	$PaCO_2$	4.74±0.17	5.68±0.20	4.13±0.17	6.02±0.12	3.39±0.23	6.27±0.13 †	2.72±0.20 ††*	5.76±0.29 †

S – S-group; Phy – Phy-group; Phy+L-Arg - Phy+L-Arg-group; a, v – values in arterial and venous blood, respectively;
ActB – actual bicarbonate (mM); EB – excess base (mM); pH – values of pH; sO_2 – hemoglobin saturation with oxygen (%);
$PaCO_2$ – partial pressure of carbon dioxide (kPa); PB - pre-bleeding values; AB – after-bleeding values (measurements were performed 0, 15 and 60 min after the cessation of bleeding, respectively); * – $P < 0.05$ in comparison with the S group; †, †† – $P < 0.05$, $P < 0.01$, respectively, in comparison with the Phy-group.

Arg+Phy-group in comparison with Phy-group, 60 min after the end of bleeding (4.68±0.22 mM vs. 3.50±0.21 mM; P±0.05).





L-Arg+Phy, 60 min

Fig. 1. Effects of i.v. bolus injections of physostigmine (0.07 mg/kg) (Phy), combination of L-arginine (300 mg/kg) and physostigmine (0.07 mg/kg) (L-Arg+Phy), and the corresponding volumes of saline (S) on hematocrit (Ht) (panel A) and total serum protein levels (TPr, g/l) (panel B) of hemorrhaged rabbits. Measurements were performed before bleeding (PB), and 0, 15 and 60 min after the end of bleeding (0, 15 and 60, respectively). Each bar represents mean + S.E.M. (N = 5-6, each group, before bleeding). * - P<0.05 in comparison with S group. Inserted graph (panel C) represents the correlation between Ht and TPr in L-Arg+Phy-group, 60 min after the end of bleeding

Mortality

The number of rabbits in each group, which survived during the course of the experiment, is shown in Table 1. In saline-treated animals, severe hemorrhage resulted in a mortality rate of 33% following 60 min of the after-bleeding period. In contrast, none of rabbits treated with i.v. bolus injection of Phy or the combination of L-Arg and Phy died during the course of the experiment.

DISCUSSION

The complex interplay between nitric oxide and cholinergic system in cardiovascular control is still a metter of debate (Sartori *et al.*, 2005). Such interaction is even less elucidated in various models of shock (Todorović *et al.*,

1997). Since both physostigmine and nitric oxide system modulators have been successfully used as monotherapy of hemorrhaged animals, it is of great importance to assess the therapeutic potential of their combination. In the present experiments, physostigmine was administered in hemorrhaged rabbits pretreated with L-arginine but not with a nitric oxide synthase inhibitor because the latter drugs could aggravate ischemia and metabolic acidosis during early phases of hemorrhagic shock (Todorović *et al.*, 1998; Szabo and Billiar, 1999). A preliminary series of experiments with an early i.v. bolus injection of physostigmine (0.07 mg/kg) in hemorrhaged rabbits pretreated with L-NAME (30 mg/kg) immediately after the end of bleeding, resulted in severe bradycardia, metabolic acidosis and high mortality during the first 15 min of the after-bleeding period (unpublished results from our laboratory). On the other hand, we have previously indicated that physostigmine and L-arginine might produce a sinergistic action in this model of shock (Savić *et al.*, 1991, 1992; Žunić *et al.*, 1995).

Physostigmine-induced a raise of the mean arterial pressure (MAP) in rabbits subjected to severe hypovolemia (approx. 40% of the estimated blood volume for 15 min) (Table 1) could be explained by several mechanisms (Varagić *et al.*, 1991; Savić *et al.*, 1991, 1992; Žunić *et al.*, 1995; Prostran *et al.*, 1994, 1996, 1997). First, its central cholinergically-mediated stimulation of the peripheral sympathetic tonus and the release of vasopressin could contribute to defence mechanisms in shock. However, the central actions of physostigmine, at least in non-hemorrhaged rats, may involve both muscarinic M_1 and M_2 receptors (Prostran *et al.*, 1997; Prostran and Varagić, 1990; Lazartigues *et al.*, 1999), but muscarinic antagonists did not modulate the anti-shock effects of the same substance in hemorrhaged rats (Guarini *et al.*, 1989). Nevertheless, the nicotinic antagonists abolished the protective effects of physostigmine in rats subjected to severe hemorrhage, and nicotinic agonists produced a reversal of hemorrhagic shock in rats *via* stimulation of adrenaline release from the adrenal medulla (Bazzani *et al.*, 1996). It should be noted that the increase in MAP after injection of physostigmine in the present experiments (as already shown by Varagić and Prostran, 1991) was not accompanied by bradycardia, which may support the hypothesis of the increased release of adrenaline as mentioned above. On the other hand, additional peripheral vasoconstriction caused by postulated physostigmine-induced increase in both sympathetic tone and vasopressine release, does not seem to play a significant role in the present model: the acid-base balance parameters in the venous blood does not indicate the increased vasoconstriction (Table 2).

Several authors indicated that physostigmine may increase the volume of circulating blood in hemorrhaged animals, but they were in disagreement about the nature of the phenomenon: mobilization of peripheral pooling vs. increased tissue fluid extraction (Guarini *et al.*, 1989; Savić *et al.*, 1991). Our present results support the former hypothesis, since hematocrit values were not significantly different between physostigmine- and saline-treated animals 0-60 min after the end of bleeding (Fig. 1, A).

The physostigmine-induced increase in MAP seems to be blocked in rabbits pretreated with i.v. bolus injection of L-arginine when compared to L-Arg-untreated animals (Table 1). There are several possible explanations of this phenomenon.

First, we may assume that L-arginine *antagonized* the pressor effects of physostigmine. It was recently shown that i.p. injection of L-arginine (400 mg/kg) to anaesthetized rats did not significantly influence the central mechanisms of blood pressure control (Tassorelli *et al.*, 2005), while physostigmine is well known to exert its central cardiovascular actions rapidly, any possible interaction between those substances could occur only at the level of peripheral circulation. Animal and human studies suggest that nitric oxide attenuates responses to endogenous vasoconstrictors (van der Linde *et al.*, 2005). However, as mentioned above, physostigmine does not seem to induce significant additional peripheral vasoconstriction in the immediate after-bleeding period due to early vascular hyporeactivity to vasoconstrictors in the first period of hemorrhagic shock (Szabo and Thiemermann, 1994). Lack of difference in acid-base balance parameters between the physostigmine- and saline-treated animals in the present experiment supports this point of view (Table 2). In addition, the injected L-arginine could only partially restore the impaired endothelial function in such a condition, but could not induce a significant vasodilation, at least not before the activation of the inducible isoform of nitric oxide synthase in blood vessels. On the other hand, it has recently been shown that endogenous nitric oxide inhibits the evoked release of adrenaline and noradrenaline from the adrenal medulla in dogs (Barnes *et al.*, 2001). Consequently, L-arginine could probably interfere with the postulated physostigmine-induced release of adrenaline from the adrenal medulla, but such a possibility remains to be tested in the appropriate experimental model.

The second hypothesis *rejects the antagonism* between L-arginine and physostigmine. In the currrent model of hemorrhagic shock (without replacement of the shedded blood), defence mechanisms of hemorrhaged rabbits, supported by the injection of L-arginine immediately after the end of bleeding, have probably induced a maximal possible increase in MAP during the first 10 min of the after-bleeding period. The observed increase in MAP 10 min after the injection of L-arginine or saline was more pronounced in L-Arg+Phy- than in S-group (0 min vs. 10 min: ΔMAP of 41 vs. 18 mm Hg, respectively), and even somewhat higher than the maximal pressor effect of physostigmine in Phy-group (0 min vs. 5 min: ΔMAP of 39 mm Hg) (Table 1). Thus, it would not be possible to produce a further increase in MAP with the i.v. bolus injection of physostigmine in L-Arg+Phy-group of rabbits previously treated with L-arginine (10 min vs. 15 min: ΔMAP of -2 mm Hg). Such an explanation is additionally supported by several observations: (a) heart rate increased 0-60 min after the end of bleeding in L-Arg+Phy-group and decreased in S-group (ΔHR of 32 and -14 b.p.m., respectively), i.e. physostigmine did not antagonize previously reported beneficial effects of the same dose of L-arginine on heart rate (Todorović *et al.*, 2001); (b) mortality was 0% in L-Arg+Phy-group 60 min after the end of bleeding, which would not be possible if the drugs used have mutually antagonized their anti-shock effects (note that the number of animals was insufficient for a reliable analysis of the mortality rate); (c) additional beneficial effects of the combination of L-arginine and physostigmine on

hematocrit and partial pressure of carbon dioxide in arterial blood ($PaCO_2a$) were observed (Fig. 1, A and B, and Table 2, respectively). The possible hemodilution caused by the combination of L-arginine and physostigmine is in agreement with the previous results from our laboratory when those substances were used as monotherapy in the same model of shock (Savić *et al.*, 1991, 1992; Todorović *et al.*, 2001). Significant differences in hematocrit between S- and L-Arg+Phy-group 15 and 60 min after the end of bleeding was not accompanied with the corresponding difference in total serum protein level (Fig. 1, B), but Ht and TPr values significantly correlated 60 min after the end of bleeding in the L-Arg+Phy-group only. On the other hand, the effects of the drug combination on $PaCO_2a$ could be related to the results of Fineman *et al.* (1991), who have shown that L-arginine produced pulmonary vasodilation in non-hemorrhaged lambs. However, the combination of L-arginine and physostigmine did not improve the oxygenation of the arterial blood (sO2 values, Table 2). The observed decrease in $PaCO_2a$ 60 min after the end of bleeding in L-Arg+Phy-group in comparison with S- and Phy-group could be explained by the improved respiratory compensation of metabolic acidosis in rabbits treated with L-arginine and physostigmine. (It should be noted that the corresponding pH values in both arterial and venous blood were somewhat higher in L-Arg+Phy group than in saline-treated animals). A possible explanation of the significantly lower values of $PaCO_2$ in venous blood ($PaCO_2v$) in L-Arg+Phy group than in Phy-group 15 and 60 min after the cessation of bleeding could be related to the decreased oxygen consumption in peripheral tissues (Žunić *et al.*, 1995). Such differences in $PaCO_2v$ were not accompanied with a similar change in pH in venous blood (Phy- vs L-Arg+Phy-group: NS; Table 2).

We have previously shown that L-arginine in the same doses used as in the present experiment (300 mg/kg, i.v. bolus, 1-2 min after the end of bleeding) increases tissue oxygen extraction and attenuates late bradycardia in hemorrhaged rabbits (Todorović *et al.*, 2001). As shown in Table 2, hemoglobin saturation with oxygen in the venous blood of rabbits treated with a combination of L-arginine and physostigmine was still lower than in the other two groups 60 min after the end of bleeding, but there was no significant difference (AB, 60 min; Table 2). Accordingly, it seems that physostigmine does not completely block the beneficial effects of L-arginine on tissue oxygen extraction. Also, beneficial effects of L-arginine on the heart rate of hemorrhaged rabbits was not lost in the presence of physostigmine. High doses of L-arginine used in the present model do not seem to exert stereospecific effects because D-arginine produced similar changes (Todorović *et al.*, 2001). Even if we assume that L-arginine exerts direct cardioprotective action and possesses free radical scavenging capacity in hemorrhaged rabbits, further investigation is needed to explain the mechanisms of the interaction between this arginine isomer and physostigmine.

In conclusion, physostigmine could not produce an increase in MAP in hemorrhaged rabbits pretreated with i.v. bolus injection of L-arginine immediately after the end of bleeding. On the other hand, beneficial effects of L-arginine on heart rate and tissue oxygen uptake (i.e. decrease in hemoglobin oxygen saturation in venous blood) in such a model were not completely lost in the presence of physostigmine. Despite certain beneficial effects on the mortality rate,

hematocrit and partial pressure of carbon dioxide in arterial blood of hemorrhaged rabbits, their combination does not seem to offer advantage over monotherapy with these drugs.

Address for correspondence:
Prof. M. Prostran
Department of Clinical Pharmacology, Pharmacology and Toxicology
School of Medicine, University of Belgrade
P.O. Box 840
11129 Belgrade
Serbia&Montenegro
E-mail: mprostran@doctor.com

REFERENCES

1. *Angele MK, Smail N, Ayala A, Cioffi WG, Bland KI, Chaudry IH*, 1999, L-arginine: a unique amino acid for restoring the depressed macrophage functions after trauma-hemorrhage, *J Trauma*, 46, 1, 34-41.
2. *Barnes RD, Ward LE, Frank KP, Tyce GM, Hunter LW, Rorie DK*, 2001, Nitric oxide modulates evoked catecholamine release from canine adrenal medulla, *Neurosci*, 104, 4, 1165-73.
3. *Bazzani C, Bertolini A, Ricigliano GM, Cainazzo MM, Balugani A, Guarini S*, 1996, The reversal of experimental hemorrhagic shock induced by nicotine and dimethylphenylpiperazinium is adrenal-dependent, *Resuscitation*, 31, 2, 145-50.
4. *Cylwik D, Mogielnicki A, Buczko W*, 2005, L-arginine and cardiovascular system, *Pharmacol Rep*, 57, 1, 14-22.
5. *Daughters K, Waxman K, Nguyen H*, 1996, Increasing nitric oxide production improves survival in experimental hemorrhagic shock, *Resuscitation*, 31, 2, 141-4.
6. *Fineman JR, Chang R, Soifer SJ*, 1991, L-Arginine, a precursor of EDRF in vitro, produces pulmonary vasodilation in lambs, *Am J Physiol*, 261(5 Pt 2), H1563-9.
7. *Guarini S, Tagliavini S, Ferrari W, Bertolini A*, 1989, Reversal of haemorrhagic shock in rats by cholinomimetic drugs, *Br J Pharmacol*, 98, 1, 218-24.
8. *Lazartigues E, Brefel-Courbon C, Tran MA, Montastruc JL, Rascol O*, 1999, Spontaneously hypertensive rats cholinergic hyper-responsiveness: central and peripheral pharmacological mechanisms, *Br J Pharmacol*, 127, 1657-65.
9. *Mellander S, Bjornberg J, Ekelund U, Alm P*, 1997, Cardiovascular regulation by endogenous nitric oxide is essential for survival after acute haemorrhage, *Acta Physiol Scand*, 160, 1, 57-65.
10. *Prostran M, Todorović Z, Varagić VM, Savić JD, Žunić G, Vujnov S*, 1996, The effects of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on serum glucose level of hemorrhaged rabbits, *Iugoslav Physiol Pharmacol Acta*, 32, 139-45.
11. *Prostran M, Varagić VM*, 1990, Modulatory influences of two specific agonists and antagonists of adenosine receptors on the blood pressure response to physostigmine, *Eur J Pharmacol*, 183, 2003-4.
12. *Prostran M, Varagić VM, Todorović Z, Jezdimirović M*, 1994, The effects of physostigmine, L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on the mean arterial pressure of the rat, *J Basic Clin Physiol Pharmacol*, 5, 2, 51-66.
13. *Prostran MŠ, Todorović Z, Varagić VM*, 1997, Physostigmine and modulators of nitric oxide system on the mean arterial pressure of the spontaneously hypertensive rat, *Gen Pharmacol*, 28, 1, 105-12.
14. *Sartori C, Lepori M, Scherrer U*, 2005, Interaction between nitric oxide and the cholinergic and sympathetic nervous system in cardiovascular control in humans, *Pharmacol Ther*, 106, 2, 209-20.
15. *Sato S*, 1998, The role of nitric oxide in hemorrhagic shock (Abstr in English). *Masui*, 47, 4, 392-403.

16. *Savić J, Varagić VM, Prokić D, Vujnov S, Prostran M, Žunić G et al.*, 1991, The life-saving effect of physostigmine in haemorrhagic shock, *Resuscitation*, 21, 1, 57-60.
17. *Savić J, Varagić VM, Prostran MŠ, Vujnov S, Mihajlović M, Djurdjević D*, 1992, The effect of physostigmine on the haemorrhagic hypovolemia in anaesthetized rabbits, *Gen Pharmacol*, 23, 2, 221-4.
18. *Siggaard-Andersen O*, 1963, The acid-base status of the blood, *Scand J Clin Lab Invest*, 15, Suppl 70, 1-134.
19. *Szabo C, Billiar TR*, 1999, Novel roles of nitric oxide in hemorrhagic shock, Shock, 12,, 1, 1-9.
20. *Szabo C, Thiemermann C*, 1994, Invited opinion: role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury, *Shock*, 2, 2, 145-55.
21. *Tassorelli C, Greco R, Cappelletti D, Sandrini G, Nappi G*, 2005, Comparative analysis of the neuronal activation and cardiovascular effects of nitroglycerin, sodium nitroprusside and L-arginine, *Brain Res*, 1051, 1-2, 17-24.
22. *Thiemermann C*, 1997, Nitric oxide and septic shock, *Gen* Pharmacol, 29, 2, 159-66.
23. *Todorović Z, Prostran M, Varagić VM, Savić JD, Žunić G, Vujnov S*, 1996, The effects of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on some cardiovascular parameters of hemorrhaged rabbits, *Iugoslav Physiol Pharmacol Acta*, 32, 159-68.
24. *Todorović Z, Prostran M, Vučković S*, 2001, The influence of L-arginine on heart rate and tissue oxygen extraction in haemorrhaged rabbits. *Pharmacol Res,* 43, 4, 321-7.
25. *Todorović Z, Prostran M, Žunić G, Varagić VM, Savić J*, 1997, The role of nitric oxide in the pathogenesis and therapy of hemorrhagic shock, *Vojnosanit Pregl*, 54, 2, 133-8.
26. *Todorović Z, Prostran MŠ, Varagić V, Žunić G, Savić J, Vujnov S*, 1998, The cardiovascular effects of the administration of L-NAME during the early posthemorrhagic period, *Gen Pharmacol*, 30, 5, 763-9.
27. *van der Linde NA, Boomsma F, van den Meiracker AH*, 2005, Role of nitric oxide in modulating systemic pressor responses to different vasoconstrictors in man, *J Hypertens*, 23, 5, 1009-15.
28. *Varagić VM*, 1955, The action of eserine on the blood pressure of the rat, *Br J Pharmacol*, 10, 349-53.
29. *Varagić VM, Prostran MŠ*, 1991, Modulating effect of adenosine on the hypertensive response to physostigmine, *Arch Int PharmacodynTher*, 311, 144-54.
30. *Varagić VM, Prostran MŠ, Stepanović S, Savić J, Vujnov S*, 1991, Transmitter interactions in the central cholinergic control of blood pressure regulation, *Drug Metabol Drug Interact*, 9, 1, 49-76.
31. *Žunić G, Savić J, Prostran M, Varagić V, Vujnov S, Todorović Z*, 1995, The effect of physostigmine on acid-base status in arterial and venous blood of anaesthetized rabbits following hypovolemic shock, *Gen Pharmacol*, 26, 2, 291-5.

L-ARGININ I FIZOSTIGMIN U HEMORAGIJSKOM ŠOKU: NEMA DOKAZA ZA SINERGIZAM

TODOROVIĆ Z, PROSTRAN MILICA, VUČKOVIĆ SONJA, STOJANOVIĆ R,
NEŠIĆ ZORICA, LASICA R i MIRKOVIĆ LJILJANA

SADRŽAJ

U našoj laboratoriji, već je pokazano protektivno dejstvo monoterapije L-argininom i fizostigminom u eksperimentalnom modelu teškog hemoragijskog šoka. Stoga, ispitali smo da li i kombinacija L-arginina (300 mg/kg, i.v. bolus) i fizostigmina (0,07 mg/kg, i.v. bolus) može imati dodatne povoljne kardiovaskularne

i/ili metaboličke efekte kod anestetiziranih iskrvarenih kunića (intermitentno krvarenje; 40% od procenjene zapremine cirkulišuće krvi za 15 min). Odabrani kardiovaskularni i biohemijski parametri praćeni su pre iskrvarenja i više puta u periodu do 60 min posle iskrvarenja. Lekovi su ubrizgavani 1-2 min posle iskrvarenja (Phy-grupa) ili 10 min kasnije (L-Arg+Phy-grupa). Kunići kontrolne grupe dobijali su samo odgovarajuću zapreminu fiziološkog rastvora NaCl (0,6-2,0 ml; S-grupa). Fizostigmin (0,07 mg/kg) doveo je do brzog i trajnog porasta srednjeg arterijskog pritiska (Phy-grupa), što se moglo poništiti pretretmanom L-argininom (L-Arg+Phy-grupa). Povoljni efekti L-arginina na srčanu frekvenciju i saturaciju hemoglobina kiseonikom u venskoj krvi nisu bili u potpunosti poništeni kod kunića L-Arg+Phy-grupe, a ta kombinacija delimično je poboljšala acido-bazni status (smanjila $PaCO_2$ u arterijskog krvi) i izazvala dodatnu hemodiluciju (smanjenje hematokrita) 15-60 min posle iskrvarenja. Međutim, kombinacija L-arginina i fizostigmina nije imala značajne prednosti u odnosu na monoterapiju istim lekovima u hemoragijskom šoku.