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Experimental Hemorrhagic Shock Protocol in Swine Models: The Effects of 21-Aminosteroid on the Small Intestine

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ABSTRACT

Background: The protective potential of lazaroids has been reported in previous studies on ischemia/reperfusion and induced hemorrhagic shock protocols.
Objectives: The present study is the first experimental protocol on the effects of the antioxidant factor U-74389G on the small intestine of swine models in a hemorrhagic shock protocol and resuscitation with 3 different types of fluids.
Methods: The study included 49 Landrace breed swine that were divided into groups of 7 each. Hemorrhage was provoked 45 minutes after starting the surgical protocol (0 minutes), followed by resuscitation starting 30 minutes after haemorrhage ceased by using 3 different fluids. Three groups (Group A, resuscitation using blood; Group B, resuscitation with Ringer's lactate solution; and Group C, resuscitation with hypertonic saline solution) underwent resuscitation with fluid alone, and another 3 groups (named A', B,' and C') were administered lazaroid U-74389G in addition to fluid. Control Group S underwent all the surgical procedures without hemorrhagic shock. Vital signs, complete blood count, and biochemical markers were analyzed, and tissue samples of the small intestine were collected from all animals. Further, malondialdehyde, tumor necrosis factor-α, and levels of inflammation in the tissue sample were measured.
Results: In Group S-A-A' and Group S-C-C', the analysis did not show statistically significant differences in the percentage changes of histopathology, malondialdehyde, and tumor necrosis factor-α through time. In Group S-B-B', the malondialdehyde levels in the small intestine were reduced in both the B and B' groups, without lazaroid (Group B) ($P = 0.038$) and lazaroid (Group B') ($P = 0.011$), compared with Group S (control), but the group without lazaroid (Group B) had greater reduction in malondialdehyde levels than the group treated with lazaroid (Group B'). With regard to the biochemistry results, 24% reduction was observed for alkaline phosphatase ($P = 0.022$) in Group A' treated with lazaroid compared with that in the untreated group. Lastly, for the complete blood count parameters, a 14% reduction in white blood cells was observed in Group B', which was treated with lazaroid in all phases ($P = 0.015$) (absolute value = 6.23) compared with Group B (absolute value = 13.74).
Conclusions: Despite few initial findings of this study suggesting that administration of lazaroid U-74389G may have some potential in attenuation of the effects of hemorrhagic shock in the small intestine of swine models, no differences remained after correction for multiple comparisons was made. Therefore, further research is required to investigate this result thoroughly.

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Introduction

In 1862, Samuel Gross described shock as the "rude unhinging" of the cycle of life. Adequate oxygen delivery and metabolism are essential for the maintenance of cellular energy stores. Failure of adequate oxygen delivery to and use by tissues during shock can lead to organ dysfunction and death.[1]

Hemorrhagic shock is an ischemic insult against an entire organism, and the restoration of the intravascular volume with fluid administration may cause a subsequent systematic ischemia-reperfusion injury.[2,3] Ischemia-reperfusion injury following major trauma can induce some inflammatory sequelae.[4,5] The multiorgan failure caused by hemorrhagic shock may lead to morbidity and mortality after severe trauma-related injury.[6,7] The inflammatory response to hemorrhagic shock causes the expression of cytokines[8] and the accumulation of neutrophils[9] in a variety of tissues.[10]

Hemorrhagic shock induces bacterial translocation in the small intestine[11] and increases mucosal permeability, membrane disintegration, and lipid peroxidation due to the action of oxygen free radicals targeting the cell membranes of the small intestine. The result of this increased mucosal permeability is the activation and adhesion of polymorphonuclear neutrophils and the release of proinflammatory factors.[12,13]

Numerous methods of resuscitation have been reported to manage hemorrhagic shock, such as blood volume replacement (ie, blood transfusion)[14] and fluid replacement with Ringer's lactate (RL)[15] or normal saline (NS) 7.5%.[16] However, replacing the current blood volume and fluid seems insufficient, and pharmacologic support is often needed.[17] The 21-aminosteroids, or lazaroids, belong to the group of lipid peroxidation inhibitors and their potential therapeutic effect has been widely studied. Lazaroids are a family of glucocorticoids without adverse side effects, and they have been found to scavenge lipid peroxyl and phenoxyl radicals, to inhibit iron-dependent lipid peroxidation, and to protect against peroxynitrite-induced cell toxicity.[18]

The present experimental protocol aimed to determine the effect of lazaroids on the small intestine postresuscitation with 3 different types of fluids in a hemorrhagic shock model. The study also investigated the effects of lazaroids on other organs (ie, liver, brain, heart, and stomach); however, these results will be presented separately in the future. The current protocol is the first experimental model of hemorrhagic shock in swine models that investigated the effect of the 21-aminosteroid U-74389G on the small intestine.

Methods

The experimental protocol of hemorrhagic shock in swine models was conducted in our Experimental Research Centre. A total of 49 laboratory animals, divided into groups of 7 each, were used. They were Landrace breed swine with a mean (SD) weight of 30 (2) kg. The animals were fed with standard laboratory food and kept adequately hydrated with free access to water. Twelve hours before the operative procedures, the animals were deprived of solid food, but their access to water was not limited. The experimental protocol was acute (all animals were euthanized immediately after the operative procedure and resuscitation, firmly applying all domestic and international laws for treating animals in experimental model protocols). Each animal was placed in the supine position on the operating table, which was covered by a heated mattress and blanket to maintain the core temperature of each animal at 39°C (±0.5°C).

This experiment was performed by a single qualified, experienced veterinarian.

Preanesthesia

Midazolam 0.5 mg/kg animal body weight was used (Dormicum 50 mg/10 mL → ~4 mL).

In addition, ketamine 15 mg/kg animal body weight and atropine 0.045 mg/kg animal body weight (1 mg/1 mL→ ~1 amp) was administered 10 minutes before intubation.

Introduction

The animals were intubated with a 6- or 7-mm endotracheal tube. They were infused with a solution of propofol 3 mg/kg (1% 10 mg/mL → 2–6 mL), fentanyl 0.012 mg/kg (0.05 mg/mL → 6 mL) and cisatracurium besylate 0.5 mg/kg (2 mg/mL → 6 mL) by bolus intravenously.

Maintenance

Initial ventilator settings: Fraction of inspired oxygen (FiO2) 40%, 20 breaths per minute.

Propofol 1% (6–8 mg/kg/h), fentanyl (2 mg = 4 amp in 500 mL NS), and cisatracurium besylate (200 mg = 10 amp Nimbex in 500 mL NS) were administered by infusion 60 to 80 mL/h intravenously.

Within 10 minutes, FiO_2 increased up to 60%, with 14 breaths per minute and inspiration volume of 15 mL/kg. Then, 750 mg intravenous cefuroxime was administered. The settings of the ventilator were continuously changed with the goal of maintaining the partial pressure of carbon dioxide in the arterial blood in the 35 to 45 mm Hg range; FiO_2 was kept as low as possible while still maintaining oxygen saturation of 98%. The ventilator was a Siare Alpha Delta Lung Ventilator, Italy (SN: MJ0097MM). An Irma Blood Gas Analyzer, Italy (SN: PIN406700) was used to measure blood gas.

Surgical protocol

The laboratory animals, sedated under general anesthesia and mechanically ventilated, underwent an open surgical dissection to initially expose the left common carotid artery in the left cervical area and then the left external jugular vein. The left common carotid artery was catheterized with a 20G probe for continuous monitoring of the arterial pressure and sampling for blood gas analysis. The left external jugular vein was catheterised with a probe of larger diameter (7Fr) for the administration of fluids. Subsequently, through an open surgical technique, the right external jugular vein in the right cervical area was prepared and catheterized with a 7.5Fr pulmonary artery probe.

Subsequently, a central abdominal incision (laparotomy) and splenectomy were performed. The spleen was weighed, and RL solution was rapidly delivered intravenously in a volume equal to 3 times the weight of the removed spleen. Subsequently, the inferior vena cava was catheterized through direct venepuncture with a 14G catheter and fixed in place with a 5-0 nylon suture. An extension line was placed, exiting the abdomen without any tension. The extension was fixed at the lower point of the abdominal surgical wound to control the hemorrhage after closure of the midline laparotomy. Finally, a urinary catheter (Foley 16Fr) was placed in the urinary bladder through open cystotomy and was connected to a urinary bag collector by exiting the abdomen through the lower part of the laparotomy surgical wound. Subsequently, a quick closure of the laparotomy wound was performed.

At the end of the surgical procedure and after a stabilization period of 30 minutes, the mean arterial pressure (MAP) was recorded, and this was considered the reference MAP (ie, baseline) for the animal. Subsequently, the phase of provoked hemorrhage occurred. Blood was withdrawn from the inferior vena cava, which was catheterized as mentioned previously to achieve a mean (SD)

MAP of 35 (5) mm Hg[19] within a 10-minute mark, whereas the MAP level was kept within this range for 45 minutes. Any unexpected MAP increase above this level during the 45-minute period was directly managed by provoking further controlled bleeding. A MAP decrease below this level during the experimental 45-minute period was managed by administrating the necessary blood volume required to do so, which had already been collected in a heparinized container.

After 45 minutes of controlled hemorrhagic shock, each laboratory animal was randomized with regard to the reperfusion method it would be subjected to:

- Group A: Readministration of the blood withdrawn,
- Group B: Administration of RL solution in a volume 3 times the blood volume that was withdrawn, and
- Group C: Administration of hypertonic solution of sodium chloride (7.5%) at a volume of 10 mL/kg test weight.

In groups A', B' and C', 5 minutes after the reperfusion was started, the molecule U-74389G was also administered intravenously at a dose of 10 mg/kg[20] immediately after the beginning of the resuscitation through systemic venous access from the left external jugular vein.

Last, Group S was the control group, in which all the above surgical procedures were performed; however, there was no hemorrhagic shock and therefore no resuscitation was performed (Table 1).

Key stages

Phase 0: Immediately before provoking the hemorrhage (0 minutes),

Phase I: 45 minutes after the completion of the controlled hemorrhage (just before the initiation of resuscitation) (Baseline),

Phase II: 30 minutes after the completion of resuscitation,

Phase III: 60 minutes after the completion of resuscitation,

Phase IV: 90 minutes after the completion of resuscitation, and

Phase V: 120 minutes after the completion of resuscitation (165 minutes since initiation of haemorrhage).

In the above phases, the following hemodynamic parameters were recorded: systolic, diastolic, and mean blood pressure; blood gases; heart rate; and central venous pressure. In all of the above phases, blood sampling was also performed for the measurement of complete blood count (ie, white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, Mean Corpuscular Hemoglobin Concentration (MCHC), red cell distribution, and platelet count) as well as biochemical parameters (ie, prothrombin time, activate partial thromboplastin time, international normalized ratio, sodium, potassium, calcium, glucose, albumin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, gamma-glutamyl transferase, alkaline phosphatase, total cholesterol, creatine, and urea).

Lastly, measurements of the small intestine tissue were performed (ie, tumor necrosis factor-α [TNF-α] and malondialdehyde [MDA]-lipid peroxidation) in each phase, together with a pathologic examination of the tissue itself.

All the procedures regarding randomization and drug preparation were performed by laboratory technicians who were blinded from the researchers. The groups to which the tissues belonged to were masked from the surgeon, pathologist, and statistician.

Materials

The 21-aminosteroid U-74389G (CAS No. 153190-29-5; pregna-1,4,9(11)-triene-3,20-dione,21-[4-(4,6-di-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-2-butenedioate; Sigma-Aldrich Company, United Kingdom, catalog No. 098K1540) was administered intravenously by bolus in a dose of 10 mg/kg animal body weight. The dose of the lazaroid was based on our previous experience in the same laboratory with the use of U-74389G.[21] Studies on the mechanism of action of 21-aminosteroid in the liver revealed this dosage to be beneficial.[22] The carrier solution was prepared by adding 4.2 mg/mL citric acid monohydrate, 0.5 mg/mL sodium citrate decahydrate, and 1.8 mg/mL sodium chloride to 480 mL water for injection. The solution was then placed in an ultrasonic bath until completely dissolved. The lazaroid solution was prepared by adding 10 mg/kg animal body weight of U-74389G to the carrier solution.

Animal model

Forty-nine Landrace breed swine, weighing 30 to 32 kg (initial body weight) were used in this study, after a period of acclimatization. The animals were purchased from Ekefe Dimocritos (Ag. Paraskevi, Athens, Greece). The animals had free access to laboratory feed and tap water. They were maintained on a 12:12 hour day/night cycle and kept in a room with strict control of temperature (22°C–24°C) and humidity (65%–75%). The pigs were acclimatized to laboratory conditions for 7 days before their use in experiments.

All appropriate ethical standards and regulations for animal experiments were followed, and approval was obtained from the Veterinary Authorities of East Attica County under Greek Law No. 160, A-64, May 1991, which incorporates the European Union regulations and the principles of the Declaration of Helsinki (approval code 2738/22-10-2010, Athens, Greece). The experimental procedure fully complied with the guidelines for animal studies and animal welfare regulations, conforming to the institutional standards as approved by the Ethics Committee (code of approval 9320/02-05-2011) as well as by the Scientific Committee (code of approval Ε.Σ. 22-06-2011, 11665/01-06-2011) of Hippokration General University Hospital, Athens, Greece).

Pathologic examination

In every phase, tissue specimens (open biopsy) were obtained from the small and large intestine, liver, pancreas, kidney, and stomach, and at the end of the experiment, from the heart and brain.

A biopsy of the small intestine was taken from the antimesenteric segment and separated into 2 pieces: the first was embedded in 10% paraformaldehyde and the second was frozen at –80°C for tissue MDA and TNF-α measurements. The Chiu score was used for histopathology assessment.[23,24] Thus, the injury was classified

Table 1
Experimental groups.

Group S	All the listed surgical procedures took place; however, without any blood loss		
Group A	Administration of the blood withdrawn	Group A'	Administration of blood withdrawn plus lazaroid
Group B	Administration of Ringer's lactate solution	Group B'	Administration of Ringer's lactate solution plus lazaroid
Group C	Administration of hypertonic sodium chloride solution (7.5%)	Group C'	Administration of hypertonic sodium chloride solution (7.5%) plus lazaroid

using a semiquantitative grading system with a numerical score based on the mucosal and submucosal damage. The histologic grading scheme was as follows:

- 0: Healthy mucosal villi;
- 1: Subepithelial Gruenhagen's space, capillary congestion;
- 2: Extension of subepithelial space with moderate lifting of the epithelial layer from lamina propria;
- 3: Massive epithelial lifting downsides of villa, few tips denuded;
- 4: Denuded villi with lamina propria and dilated capillaries exposed; or
- 5: Digestion and disintegration of lamina propria, hemorrhage, and ulceration.

MDA and TNF-α measurements

The MDA reagent pack was purchased from OxisResearch (Portland, OR). Each tissue piece from the small intestine was weighed and homogenized in phosphate-buffered saline to measure MDA and TNF-α levels, as described by Boada et al.[25] (modified from Esterbauer and Cheeseman[26]), on a Beckman DU7400 spectrophotometer (Beckman Instruments Inc, CA, USA). The ELISA kits for the quantification of TNF-α were purchased from R&D Systems (Abington, United Kingdom). All solvents were of HPLC grade and were obtained from Merck Company (Darmstadt, Germany). The homogenate tissues were enriched with 0.5 M acetonitrile to avoid further oxidation of the samples. These samples were then centrifuged at 3000 g for 15 minutes at 4°C, aliquoted, and kept at 80°C. During the period of homogenization, the total protein content was measured following Bradford's assay. The assessment was based on quantitative ELISA kits according to the manufacturer's instructions, and the results were expressed as pictograms per milliliter of total protein. To quantify MDA content, a commercial kit was used following the manufacturer's instructions. The measurement was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. The stable chromophore product exhibits maximal absorbance at 586 nm; these results were expressed as picomoles per milligram of total protein.

The MDA and TNF-α analyses were performed at the Laboratory of Chemistry, Biochemistry, Physical Chemistry of Foods, Department of Dietetics and Nutrition, School of Health Science and Education, Harokopio University, Kallithea, Athens, Greece.

Statistical analysis

Data are expressed as mean (SD) or median (in cases of violation of normality) for continuous variables and as percentages for categorical data. The Kolmogorov-Smirnov test was used for normality analysis of the parameters.

The comparison of absolute values of variables at each time point was performed using Kruskal-Wallis, and Mann-Whitney tests were used in cases of violation of normality. To examine changes in variables during the observation period of treatments, the median percentage changes from baseline to Time 1 and from Time 1 to any other time point were calculated. The comparison of percentage changes in variables during the observation period of the 3 groups were then analyzed using the 1-way ANOVA model. Pairwise comparisons were performed using the Bonferroni test, and in cases of violation of normality, the Kruskal-Wallis and Mann-Whitney tests were used.

All tests were 2 sided and statistical significance was set at $P < 0.05$. Each analysis was carried out using the statistical package SPSS version 17.00 (IBM-SPSS Inc, Armonk, NY).

Results

The results are presented as 3 separate groups of resuscitations, as described in the experimental procedure.

Table 2
Comparison of absolute values and percentage change from Phase I to other phases between Group S versus Group A versus Group A' for histopathologic results, malondialdehyde, and tumor necrosis factor-α (TNF-α) at each time point.

		Absolute value						Absolute change from Phase I		Percent change from Phase I			
		Histopathologic results*	*P* value	Malondialdehyde*	*P* value	TNF-α*	*P* value	Histopathologic results	*P* value	Malondialdehyde	*P* value	TNF-α	*P* value
Baseline	S	2.0 (2.0)	0.49	171.64 (113.21)	0.047	20.48 (27.86)	0.58						
	A	2.0 (2.0)		122.06 (84.44)†		10.00 (37.20)							
	A'	1.0 (3.0)		107.85 (17.89)†		21.57 (54.33)							
Phase I	S	4.0 (1.0)	***0.004***	175.40 (113.21)	***0.009***	17.48 (28.63)	0.953						
	A	2.0 (3.0)†		115.64 (56.47)†		26.75 (80.60)							
	A'	1.0 (2.0)†		135.01(91.34)		25.75 (26.06)							
Phase II	S	3.0 (2.0)	0.245	167.68 (39.77)	***0.035***	21.08 (22.45)	0.872	−1.0	***0.045***	3.57	0.942	−12.27	0.698
	A	1.0 (2.0)		67.10 (28.07)		18.45 (109.25)		0		−8.46		−16.44	
	A'	1.0 (2.0)		144.84 (87.07)		19.18 (22.69)		1.0†		−16.86		6.32	
Phase III	S	4.0(4.0)	0.322	149.75 (71.10)	0.213	21.08 (37.19)	0.631	0	0.161	−17.78	0.274	24.84	0.272
	A	1.0 (2.0)		120.79 (93.68)		11.65 (102.31)		−1.0		16.55		−7.83	
	A'	1.0 (1.0)		171.94 (233.02)		26.71 (27.34)		1		30.46		3.77	
Phase IV	S	4.0 (1.0)	***0.027***	179.92 (152.89)	***0.022***	23.60 (42.24)	0.974	0	0.203	−2.27	0.297	11.6	0.725
	A	1.0 (3.0)†		100.06 (70.34)†		51.42 (69.82)		0		−16.78		57.11	
	A'	2.0 (3.0)†		106.69 (100.86)†		25.44 (31.43)		1		−25.51		43.36	
Phase V		5.0 (5.0)	0.258	179.92 (148.49)	***0.037***	23.60 (19.62)	0.865	1	0.423	−2.27	0.28	13.39	0.953
	A	2.0 (1.0)		132.28 (60.61)†		30.00 (49.43)		0		42.02		12.15	
	A'	2.0 (4.0)		114.47 (75.38)†		27.09 (11.46)		0		−15.21		37.87	

Histopathologic results we have statistical significant result in Phase I and IV for Group with lazaroid in comparison with Group S. For the MDA we have statistical significant result in Phase I, II, IV, and V for lazaroid Group in comparion with Group S.

* Values are presented as median (interquartile range).
† $P < 0.05$ versus Group S.

Table 3
Comparison of percent change and absolute values from Phase I to other phases between Group S versus Group A versus Group A' for alkaline phosphatase.*

% Change from Phase I			*P* value	Mean (SD)	*P* value
Phase I	S			181.71 (69.13)	0.786
	A			159.86 (57.46)	
	A'			167.29 (50.70)	
Phase II	S	0	0.585	183.29 (67.12)	0.942
	A	5.26		181.57 (27.59)	
	A'	5.41		174.14 (4.33)	
Phase III	S	0	***0.066***	179.43 (73.38)	0.458
	A	4.82		178.57 (35.91)	
	A'	−8.00		145.57 (53.91)	
Phase IV	S	−1.60	**0.047**	179.29 (68.41)	0.395
	A	3.51		180.00 (32.48)	
	A'	−13.13†‡		145.57 (50.78)	
Phase V	S	−1.60	***0.022***	178.00 (72.72)	0.168
	A	1.08		178.43 (37.42)	
	A'	−24.55†‡		127.57 (49.13)	

In Phase III, IV and V we have statistical significant reduce in ALP in Group with Lazaroid and Blood.

* Absolute value at each time point and percent change from Phase I are presented as mean (SD), median, respectively.
† $P < 0.05$ versus Group S.
‡ $P < 0.05$ versus Group A.

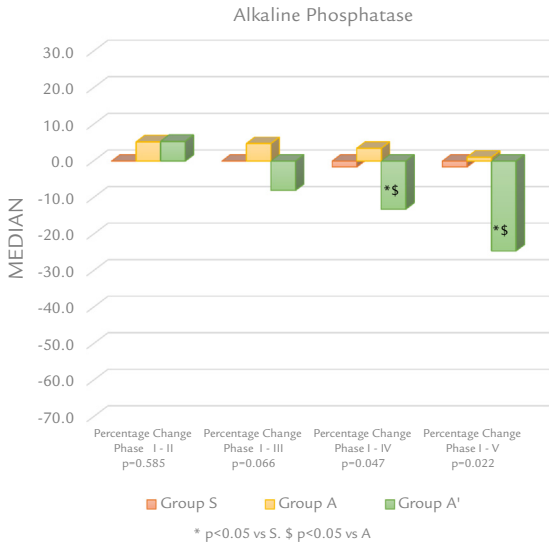

Figure 1. Percent change over time period for alkaline phosphatase in Group S versus Group A versus Group A'. *$P < 0.05$ versus Group S. †$P < 0.05$ versus Group A.

Baseline

All procedures regarding randomization and drug preparation were done by lab technicians who were blinded from the researchers.

Further, the histopathology examination and the analyses of MDA and TNF-α as well as the statistical analysis were also blinded from the researchers in all stages.

For statistical analysis, the phase just after the hemorrhage (Phase/Time 1) was considered as the baseline, and all subsequent measurements were compared to this baseline. The groups with resuscitation and without administration of the agent (Group A, Group B, and Group C) were considered control groups.

Results of Group S-A-A' (resuscitation with blood and lazaroid)

The analysis did not show statistically significant differences in the percentage changes of histopathology, MDA, and TNF-α in the last phase of the experiment over time periods in the group resuscitated with blood and lazaroid (Table 2). With regard to the absolute values of the same group, the histopathologic score was statistically significantly lower for Group A' (with the drug) ($P = 0.053$) and Group A (without the drug) ($P = 0.017$) than that for the sham group in Phase IV. The MDA level was statistically significantly reduced ($P = 0.017$) in Phase V (end of experiment) in both groups (with the drug [median, 114.47] and without the drug [median, 132.28]), with a significant reduction in the group administered with lazaroid. However, no similar difference was found for the TNF-α levels in the same groups (Table 2). With regard to the biochemistry results, a statistically significant result ($P = 0.022$) was observed for alkaline phosphatase (ALP) with 24% reduction in the group treated with lazaroid compared with the untreated group. There was a statistically significant difference with improvement in Phase V among the 3 groups treated with lazaroid (last phase of the experiment) (Table 3 and Figure 1).

Results of Group S-B-B' (resuscitation with RL and lazaroid)

Statistical analysis did not show any difference in the percentage changes over time in the group treated with RL (Table 4). The absolute value analysis showed a statistically significant reduction in the histopathologic scores for Phases IV and V, with improvement in the treated group. The MDA levels in the small intestine were reduced in both the B and B' groups, without lazaroid (Group B) ($P = 0.038$) and with lazaroid (Group B') ($P = 0.011$), compared with Group S (control), but the group without lazaroid (Group B) had greater reduction in MDA levels than the group treated with lazaroid (Group B') (Table 4). Lastly, for the complete blood count parameters, a reduction in white blood cell count was observed in the groups with lazaroid among all phases ($P = 0.015$) with 14% reduction in the group with lazaroid (Group B') (absolute value = 6.23) compared with Group B (absolute value = 13.74), (Table 5 and Figure 2).

Results of Group S-C-C' (NS 7.5% and lazaroid)

In Group C', in which resuscitation was performed with NS 7.5% and lazaroid, a statistically significant difference ($P = 0.001$) was found in the histopathologic score for Group C (without drug) in comparison with the Group S. In the MDA and TNF-α analysis (percent change and absolute value), there was no statistically significant difference in the last phase (Table 6).

Discussion

Swine models have been used in many experimental protocols studying the effects of lazaroids on hemorrhagic shock. Pigs and humans have fairly similar blood clotting mechanisms as well as cardiovascular and hemodynamic responses to hemorrhagic shock.[27] Hemorrhagic shock is a serious complication in surgical patients that may cause death (eg, postoperative or postpartum). The study of the effects of lazaroids and of the different methods of resuscitation is also important. Many experimental models have served this purpose. Previous research has shown that the production of free radical species because of ischemia and reperfusion plays a significant role in lipid peroxidation, a process that can cause the instability of cell membranes. In the small intestine, this may destroy the mucosa barrier and cause bacterial translocation or activation of inflammatory responses.[28] Previous studies have shown that lazaroids may play a significant role in hemorrhagic shock, especially in ischemia/reperfusion, as a scavenger of reactive oxygen species that can protect organs from oxidative stress.[29]

Table 4
Comparison of absolute values and percentage change from Phase I to other phases between Group S versus Group B versus Group B' for histopathologic results, malondialdehyde, and tumor necrosis factor-α (TNF-α) at each time point.

		Absolute value						Absolute change from Phase I		Percent change from Phase I			
		Histopathologic results*	*P* value	Malondialdehyde*	*P* value	TNF-α*	*P* value	Histopathologic results	*P* value	Malondialdehyde	*P* value	TNF-α	*P* value
Baseline	S	2.0 (2.0)	0.878	171.64 (113.21)	***0.015***	20.48 (27.86)	0.264						
	B	1.0 (2.0)		91.15 (25.75)†		27.16 (46.97)							
	B'	1.0 (3.0)		120.82 (50.82)†		53.47 (68.54)							
Phase I	S	4.0 (1.0)†	***0.001***	175.40 (113.21)	***0.054***	17.48 (28.63)	0.244						
	B	2.0 (1.0)†		81.04 (74.03)†		34.56 (64.92)							
	B'	1.0 (2.0)		165.86 (197.09)†		56.63 (101.45)							
Phase II	S	3.0 (2.0)	0.716	167.68 (39.77)	***0.048***	21.08 (22.45)	0.202	−1.0	***0.003***	2.4	0.925	21.08	0.86
	B	2.0 (2.0)		84.08 (47.56)†		37.82 (59.88)		0.0†		2.58		22.47	
	B'	1.0 (2.0)		136.43 (73.45)		42.65 (38.05)		0.0†		−5.52		−13.67	
Phase III	S	4.0 (4.0)	0.392	149.75 (71.10)	0.106	21.08 (37.19)	0.284	0	0.214	−8.28	0.4	50.89	0.488
	B	2.0 (2.0)		101.20 (36.56)		31.21 (17.44)		1		6.27		36.43	
	B'	1.0 (2.0)		118.30 (79.85)		42.92 (46.57)		0		−23.80		−2.93	
Phase IV	S	4.0 (1.0)	***0.005***	179.92 (152.89)	***0.054***	23.60 (42.24)	0.508	0	0.346	17.67	0.732	92.98	0.797
	B	2.0 (1.0)†		94.86 (59.70)†		30.05 (17.31)		1		14.68		12.77	
	B'	1.0 (2.0)†		120.11 (136.66)		52.87 (76.67)		0		14.4		2.23	
Phase V	S	5.0 (5.0)	***0.059***	179.92 (148.49)	***0.023***	23.60 (19.62)	0.652	1	0.453	20.08	0.561	58.51	0.557
	B	3.0 (4.0)†		98.78 (111.33)†		18.02 (39.14)		2		16.32		−3.43	
	B'	1.0 (2.0)†		139.64 (37.07)†		50.09 (51.14)		0		−8.86		−17.27	

The absolute value analysis showed a statistically significant reduction in the histopathologic scores for Phases IV and V, with improvement in the treated group. For the MDA we have significant statistical reduce both of Groups with R/L and R/L with Lazaroid.

* Values are presented as median (interquartile range).
† $P < 0.05$ versus Group S.

A literature review revealed many similar experimental protocols that have investigated lazaroid U-74389G, but only a few of them are hemorrhagic shock experimental protocols. Small animals such as mice, rats, and cats and large animals like sheep have been used in experiments on hemorrhagic shock protocols. In 1996, Sullivan studied the positive effect of the same type of lazaroid on recovery of mice in hemorrhagic shock. The resuscitation fluid was RL, and the author showed that the group administered the drug required a lower volume of fluid for adequate resuscitation.[18] In the same year, another research team studied hemorrhagic shock protocol in sheep. Their animal model survived for 1 week. In this study, the fluid of resuscitation was crystalloid plus lazaroid for the control group.[30] Many hemorrhagic shock experimental protocols with resuscitation also have been conducted with different subtypes of lazaroid and various experimental animals, such as U-83836E in rats,[31] U-74006F in cats,[32] and U-74006F in rats.[33] These protocols have some differences and some similarities to the present study protocol. The duration of the observation in each protocol varied, ranging from a couple of hours to up to 5 days (the 1 that studied sheep). In most of these previous protocols, RL was used. Overall, the results for using lazaroid are favorable, but further research on hemorrhagic shock is needed, specifically to overcome the limitations of the current studies.

Table 5
Comparison of percent change and absolute values from Phase I to other phases between Group S versus Group B versus Group B' for white blood cell count.*

% Change from Phase I			*P* value	Mean (SD)	*P* value
Phase I	S			12.27 (5.70)	***0.057***
	B			10.10 (1.890	
	B'			7.11 (2.91)	
Phase II	S	4.11	***0.001***	13.91 (7.06)	***0.008***
	B	37.04†		13.11 (2.85)	
	B'	−17.95†,‡		5.97 (2.23)	
Phase III	S	0.88	***0.058***	13.37 (6.15)	***0.053***
	B	37.12		11.90 (6.75)	
	B'	−25.64‡		6.21 (3.03)	
Phase IV	S	12.2	***0.002***	12.13 (4.14)	***0.0005***
	B	42.59†		14.66 (2.08)	
	B'	−20.00‡		6.30 (3.29)	
Phase V	S	11.38	***0.015***	13.01 (5.81)	***0.007***
	B	29.89		13.74 (3.12)	
	B'	−14.29‡		6.23 (3.24)	

The value of the first column is in median.
In all Phase of the Group with lazaroid and R/L, we have statistical significant reduce in WBC.

* Absolute value at each time point and percent change from Phase I are presented as mean (SD), median, respectively.
† $P < 0.05$ versus Group S.
‡ $P < 0.05$ versus Group B.

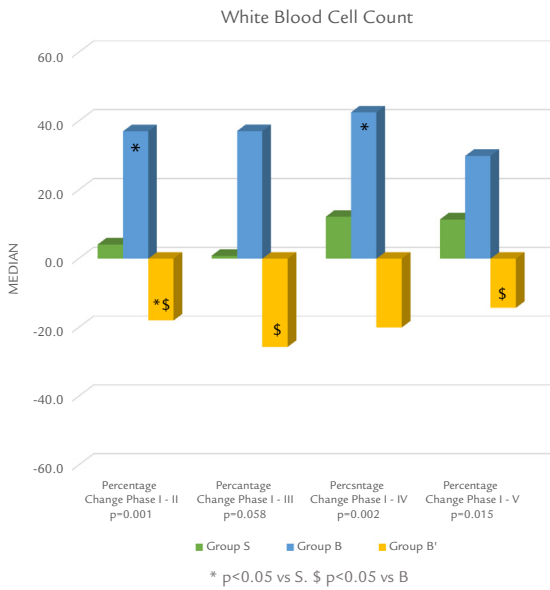

Figure 2. Percent change over time for white blood cell count in Group S versus Group B versus Group B'. *$P < 0.05$ versus Group S. †$P < 0.05$ versus Group B.

Table 6
Comparison of absolute values and percent change from Phase I to other phases between Group S versus Group C versus Group C' for histopathologic results, malondialdehyde, and tumor necrosis factor-α (TNF-α) at each time point.

		Absolute value						Absolute change from Phase I		Percent change from Phase I			
		Histopathologic results*	*P* value	Malondialdehyde*	*P* value	TNF-a*	*P* value	Histopathologic results	*P* value	Malondialdehyde	*P* value	TNF-α	*P* value
Baseline	S	2.0 (2.0)	0.946	171.64 (113.21)	0.563	20.48 (27.86)	***0.033***						
	C	1.0 (2.0)		225.09 (92.03)		40.18 (36.52)							
	C'	1.0 (1.0)		246.85 (306.62)		9.69 (9.77)							
Phase I	S	4.0 (1.0)	***0.013***	175.40 (113.21)	0.246	17.48 (28.63)	0.961						
	C	2.0 (2.0)†		171.37 (75.60)		23.83 (22.29)							
	C'	2.0 (3.0)†		228.77 (64.48)		27.10 (39.71)							
Phase II	S	3.0 (2.0)	0.239	167.68 (39.77)	0.337	21.08 (22.45)	0.603	−1.0	0.11	3.57	0.3	−12.27	0.975
	C	2.0 (2.0)		211.47 (96.91)		41.54 (49.44)		0		15.1		15.16	
	C'	1.0 (1.0)		253.37 (104.66)		27.98 (29.11)		0		6.11		−10.96	
Phase III	S	4.0 (4.0)	0.209	149.75 (71.10)	***0.043***	21.08 (37.19)	0.628	0	0.262	−17.78	0.136	24.84	0.803
	C	3.0 (1.0)		154.61 (167.72)†		51.71 (50.12)		1		−6.98		31.28	
	C'	2.0 (2.0)		232.39 (100.54)†,‡		22.71 (6.32)		0		0.44		−11.55	
Phase IV	S	4.0 (1.0)	0.686	179.92 (152.89)	0.263	23.60 (42.24)	0.204	0	0.215	−2.27	***0.058***	11.6	***0.052***
	C	3.0 (3.0)		161.52 (42.60)		27.04 (18.65)		2		−24.97		−25.03	
	C'	4.0 (3.0)		163.21 (106.06)		41.50 (7.92)		0		−28.66†		53.14$	
Phase V	S	5.0 (5.0)	0.688	179.92 (148.49)	0.381	23.60 (19.62)	0.246	1	***0.02***	−2.27	0.499	13.39	0.27
	C	4.0 (2.0)		138.85 (169.43)		16.93 (21.59)		2.0†		−18.98		−8.54	
	C'	3.0 (4.0)		224.44 (191.18)		17.28 (12.63)		0		−1.89		−63.00	

* Values are presented as median (interquartile range).
† $P < 0.05$ versus Group S.
‡ $P < 0.05$ versus Group C.

The present study is the first study of 21-aminosteroid U-74389G used in hemorrhagic shock in swine models with the use of 3 different types of fluids for resuscitation: hypertonic NS, blood, and RL.

MDA is generally used as a marker of the peroxidation of n-3 and n-6 fatty acids. MDA reacts in vivo with primary amines to form Nε-(2-propenal) lysine and forms lysine-lysine cross-links with 1-amino-3-iminopropene and pyridyledihydropyridine type bridges. These reaction products have been discovered in apolipoprotein B (APO B) fractions of oxidized lipoproteins and are involved in the impaired interaction of modified lipoproteins with macrophages.[34]

In the present study with different types of fluid resuscitation, MDA and TNF-α did not show a statistically significant difference between groups except for the group resuscitated with lazaroid and blood (Group A') where it showed a reduction in MDA levels. Further, in the same group, there was a significant reduction in ALP levels. However, in the TNF-α and histopathologic score analysis, there was no statistically significant difference between the treated and control groups.

Moreover, from the analysis of the complete blood count, a statistically significant reduction in white blood cell count was observed for Group B'.

From all the above statistical analyses, there were observed statistically significant results (except for white blood cell count and ALP) in activate partial thromboplastin time in the first experimental group (blood and lazaroid), international normalized ratio, and hematocrit in the second experimental group (RL and lazaroid) and serum glutamic pyruvic transaminase in the first and third (sodium chloride 7.5% and lazaroid) experimental groups.

The TNF-α analysis did not show any statistical significant difference between all the experimental groups.

On the basis of the data from the haemodynamic parameters and oxygen measurement among the 3 different types of resuscitation fluids, there were no statistically significant improvements or differences.

In this study, resuscitation with the administration of blood showed a favorable effect in the reduction of ALP in treated animals. ALP (E.C.3.1.3.1.) is a membrane-bound glycoprotein responsible for the hydrolysis of phosphate at basic pH. ALP comprises 4 related isozymes depending upon the site of tissue expression: intestinal ALP, placental ALP, germ cell ALP or tissue-nonspecific ALP, or liver/bone/kidney ALP. Intestinal ALP is expressed and secreted by intestinal epithelial cells and remains active within the mucosal membrane and the intestinal lumen. Intestinal ALP is also secreted into serum, where it remains biologically active; the levels of this isoenzyme in the serum seem to be a function of input from the gut and elimination by the liver.[35,36] Studies have shown that ALP is significantly higher in intestinal ischemia.[37] Because intestinal enzymes are released during intestinal ischemia, the measurement of serum levels of these enzymes have been investigated both clinically and experimentally for detecting early intestinal ischemia. Change occurs in the intestinal mucosa within minutes of ischemic injury and progresses to necrosis of the villi and inflammation of the underlying submucosa and muscular layers. The intestinal mucosa is most sensitive to the ischemic insult.[38]

The present study had the key limitation of only a limited period of 120 minutes of observation after resuscitation. The experiment was acute; at the end of the surgical procedures and before the start of the resuscitation, a single dose of the lazaroid was administered to the treated animals. This may be another limitation of our experimental protocol. Further, biomarkers like ALP probably need a more extended time of observation, such as >6 hours, to show potential improvement in study results.[39] Studies have shown that there are more accurate biomarkers for the investigation of small intestine ischemia. Intestinal fatty acid-binding protein (I-FABP) and glutathione S-transferase, enzymes of the mucosa of the small intestine, can be used in research on intestinal ischemia with an addition of D-dimer, which has a high sensitivity of approximately 100%.[40]

Although the approach of performing a splenectomy in experimental hemorrhagic shock models is still debated, this approach creates a more stressful environment for the laboratory animals during the shock and mimicked trauma scenarios. Further, the spleen retains up to 25% of their blood volume and leads to the

activation of a complex autotransfusion mechanism in severe hemorrhagic shock.

Further investigations are therefore needed to assess the possible medium- and long-term effects of the lazaroid U-74389G on the small intestine by using large animal models and a longer time of observation, as already has been done in other studies,[30] or different dosages, before a clinical trial on human patients could be considered. The above biomarkers may also be used.

Conclusions

The administration of 21-aminosteroid U-74389G in swine models during resuscitation from hemorrhagic shock with 3 different types of fluids showed some evidence of improvements in Groups A' and B' (use of blood or RL, respectively). These results are related to the white blood cell count, ALP level, and MDA level. However, no differences remained after correction for multiple comparisons was made. Therefore, further research is required to investigate this result—which is contradictory to part of the available literature—thoroughly.

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2. Dr. Vasileios G. Bonatsos; Experimental protocol execution, team leading, data collection, data analysis, literature review, main synthesis of current article - Co-author

3. Dr. Ageliki I. Katsarou; - Measurement of MDA and TNF-a.

4. Dr. Andreas Karameris; – Pathology examination

5. Mr. Antonis Galanos; – Statistical analysis (professional bio statistician)

6. Miss Argyro Zacharioudaki; – Animal sedation and welfare (professional veterinary surgeon).

7. As. Professor George Theodoropoulos; - References and manuscript correction

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9. As. Professor Apostolos E. Papalois - Study design and Head of Elpen Experimental Centre

10. As. Professor Konstantinos Toutouzas; - Study design, Supervision, support, corrections and overall guidance of experimental procedure and manuscript building.

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Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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