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"Effects of Circadian Rhythms on Decompression Outcome"

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This Final Report describes our work conducted under the terms of NASA NAG 9-1040

INTRODUCTION

Overall Objectives: Astronauts undertaking extravehicular activities were subjected to decompression. In this project we examined the effects of the body's endogenous circadian rhythms on the physiological consequences of decompression-induced venous bubble formation. Specific indices of inflammation and of lung injury were previously reported with decompression sickness, even with mild to moderate levels of decompression. Based on these observations, we studied the effect of the circadian rhythms on the tolerance to decompression exposure and the underlying 24 hour bioperiodic dependencies. Our overall objectives included the quantitative evaluation of the physiological stress of decompression at different circadian times and to relate these to rhythms in cellular and biochemical variations over the 24 hr. span. Measurement of these responses may elucidate some of the less understood risks associated with decompression illness, and may lead directly to operationally useful procedures.

Critical Path Roadmap: This study addresses the Critical Question contained in the July 30, 2003 Bioastronautics Critical Path Baseline Roadmap Document. Under section 7.11 of Clinical Capabilities, and Risk No. 47, the Critical Question 11.33 states, "What are the strategies to mitigate the risk of Decompression Sickness during all NASA operations (i.e., extravehicular activities from the International Space Station and future habitats beyond LEO, during training activities in underwater facilities, or altitude chambers, during flight using NASA aircraft, and in research designed to prevent decompression sickness?"

Specific Hypotheses and Objectives:

1. Hypothesis: Changes in decompression-induced neutrophil and platelet counts are circadian rhythm dependent. Objective: Differential cell analyses were performed on samples of systemic arterial blood, bronchoalveolar lavage (BAL) and pleural fluid collected from different but comparable groups of rats exposed to conditions which induce decompression sickness at 6 different times, i.e., at 4 hr. intervals throughout a 12-hr. light/12-hr. dark cycle. Result: The time of day differences were found to be significant.

2. Hypothesis: Severity of decompression-induced changes in inflammatory mediators (leukotrienes and thromboxanes) correlates with specific stages of known circadian rhythms. Objective: Enzyme immunoassay of post decompression/air infusion arterial plasma, bronchoalveolar lavage (BAL) and urine for mediators of the inflammatory response and correlated as to the circadian staging above. Result: There were clear circadian rhythm dependencies in the inflammatory mediator levels post decompression.

3. Hypothesis: Severity of decompression sickness symptoms and lung injury correlate with circadian rhythms. Objective: Hemodynamic and post mortem edema assessment correlated as to the circadian staging above. Result: There were clear circadian rhythm dependencies in the extravascular lung water ratio levels post decompression.

4. Hypothesis: Decompression-induced variables in Aims 1-3 above are the result of venous bubble formation and blood interactions. Objective: Venous gas bubble infusions alone without decompression, at pathophysiological minimum and maximum with reference to circadian rhythm dependent responses. Result: There were clear circadian rhythm dependencies in measured variables comparing the HALO 2 and 12 time points.

5. Hypothesis: Pharmacological inhibition of decompression-induced symptoms and inflammatory mediators is dose-responsive as a result of circadian rhythm dependencies. Objective: Inhibition of cyclo-oxygenase and 5-lipooxygenase-induced arachidonic acid metabolites (thromboxanes and leukotrienes following decompression with circadian rhythmic variations. There were clear circadian rhythm dependencies in measured variables comparing the HALO 2 and 12 time points.

Summary of Background Material

The biology of animals and human beings is precisely organized in time as rhythms of specific period duration -- 24 hours, week, month and year, for example (Reinberg & Smolensky, 1983; Touitou & Haus, 1992). The body's circadian (*circa*=about; *dies*=day) time structure has been the most researched. Nearly every process and function of the body exhibits 24-hr. rhythmicity. The efficiency of mental and physical performance, adjustment to alterations in the rest-activity routine resulting from travel across time zones and shift work plus tolerance to stress, among others, all vary in a systematic manner during the 24 hours (Scott, 1990; Smolensky et al., 1985; Halbert, 1975).

NASA has long recognized that the circadian time structure of astronauts is important operationally for successful missions. During the 1980's, NASA-JSC convened the Circadian Shifting Committee (M. Smolensky member) to explore interventions to rapidly pre-adjust the circadian time structure of shuttle astronaut crews to early morning launch times and night shift schedules. Prescribed bright light exposure is now used to accomplish this goal. Special facilities at NASA-JSC and the Cape can be utilized to achieve the circadian shifting of the crew members within a few days prior to launch. Ongoing shuttle experiments were exploring circadian rhythms in the mental performance of astronauts engaged in longer duration missions. Recent NASA-sponsored conferences have focused on the relevance of the circadian structure on medications, nutrients and work/performance in space.

Since the 1960's, many laboratory investigations have demonstrated that the tolerance and susceptibility of living systems to potentially noxious agents and dangerous stresses, whether they be physical, chemical or biological in nature, vary according to the time (circadian stage) during the 24 hours of the exposure (Smolensky & Reinberg, 1990). Circadian rhythms in the tolerance of human beings have been less studied, although day-night differences have been demonstrated in the symptom intensity and risk

of a large number of medical conditions (e.g., arthritis, asthma, cardiovascular and peptic ulcer disease). Other studies have evaluated circadian rhythms in the beneficial and adverse response to medications (e.g., aspirin, cancer agents, corticosteroids) and various diagnostic challenge tests (e.g., skin testing for allergen sensitivity, blood glucose tolerance testing, treadmill stress test) used in clinical medicine (Smolensky, 1996; Redfern & Lemmer, 1997).

Of great interest to us was the role the circadian time structure plays in the response to decompression exposure. Decompression-induced gas bubbles can result from pressure reductions that occur during the extravehicular activity (EVA) performed by astronauts (Balldin, 1976). Military missions of Navy and Air Force personnel and extravehicular activities of astronauts were now conducted at diverse times of the day and night; under emergency situations EVAs might even be conducted during the hours usually devoted to sleep. The risk of decompression sickness at any time is real, however, changes in susceptibility and outcome may be variable depending on circadian staging.

Venous gas embolism (VGE) resulting from decompression leads to mechanical obstruction of blood vessels by the circulating bubbles as well as pulmonary vasoconstriction. The vasoconstriction may be due in part to an initial neurogenic reflex as well as release of certain vasoactive mediators (Butler 1985; Wang et al., 1992). Secondary to these immediate responses were the increases in the total number of circulating leukocytes (neutrophils) reported with VGE (Albertine et al., 1984; Flick et al., 1981; Butler 1991) or decompression (Philp et al., 1974; Barnard 1981). In their activated state, these cells were involved with the release of vasoactive agents that lead to further vasoconstriction and endothelial cell membrane permeability changes (Flick et al., 1981; Wang et al., 1992). Our recent studies under this contract demonstrated both elevated white cells and neutrophil percentages with hypo and hyperbaric decompression as well.

The formation of decompression-induced VGE can lead to diffuse injury as a result of an interstitial inflammatory response and recurring injury to the vascular endothelium. Local tissue responses include pain, swelling and edema formation caused by release of microvascular permeability altering agents.

The granulocyte cell activation and chemotaxin release results in the production of circulating vasoactive metabolites, such as free arachidonic acid (AA) and the byproducts of its metabolism via either of two pathways. One pathway is catalyzed by cyclooxygenase, which results in formation of prostacyclin (PGI_2), which is a vasodilator and inhibits platelet aggregation. Other byproducts include the prostaglandins and thromboxane A_2 (TxA_2) which cause vasoconstriction, bronchoconstriction and platelet aggregation. The second pathway is catalyzed by lipoxygenases which give rise to the leukotrienes. Leukotriene B_4 (LTB_4) is chemotactic for neutrophils while leukotrienes C_4, D_4 and E_4 cause pulmonary and bronchial smooth muscle constriction and edema (Reeves et al, 1985). It is apparent that neutrophils likely play a significant role in the development of these bioactive mediators and possibly of decompression-induced injury (Philp et al. 72, Levin et al. 81). Circulating neutrophil counts were reported to vary

according to time of day (Labrecque et al., 1995). Furthermore, the inducement of inflammatory response, platelet activation, fluid shifts, etc. to antigen challenges is also circadian rhythm dependent (Reinberg and Smolensky, 1983). Application of this time of day rhythmicity has not been evaluated in conditions of decompression stress.

Blood-bubble interactions associated with DCS include 1) clumping of red blood cells; 2) microthrombus formation; 3) platelet aggregation; 4) neutrophil aggregation and activation; 5) alignment and denaturation of plasma proteins; and, 6) attraction of oriented phospholipids (Lee et al., 1971; Ohkuda et al., 1981; Neurath et al., 1944; Butler 1985). Platelet aggregation (Philp et al., 1972; Fratali et al., 1975; Philp et al. 1971; Tanoue et al., 1987) can result in release of ADP, serotonin and catecholamines (Philp et al., 1971) as well as further potentiate microvascular obstruction.

Although platelet activation may play a role in DCS through more direct effects, experimental evidence suggests that arachidonic acid products of platelet origin were less important in the lung pathophysiology caused by microembolization or complement activation (Snapper et al., 1985; Binder et al., 1980; McDonald et al., 1983). Neutrophils on the other hand were likely to play a more prominent role in DCS mediated tissue injury. Once activated, neutrophils become larger and less deformable (Rothe et al., 1990), Worthen, et al., 1989) which reduce their flow through the lungs in acute injury (Doerr et al., 1992). Emigration of neutrophils to the gas bubbles with subsequent sequestration in the pulmonary capillaries is triggered by a number of chemotactic factors (Kindt et al., 1991) of which complement activation, reportedly increased with decompression (Ward, et al., 1987) has been suggested. Wang et al. (1992) reported that VGE in isolated rat lungs did not increase the neutrophil count in the perfusate but did elevate the number of cells that were activated.

Activated neutrophils were also reported to stimulate production of oxygen radicals (Albertine et al., 1984, Flick et al., 1981) that can lead to damage to endothelial cells thereby altering the normal fluid balance of the lungs, thus resulting in pulmonary edema. These substances, including superoxide anions, hydrogen peroxide and hydroxyl radicals have been implicated in the acute injury associated with air embolism (Flick et al., 1983). Production of reactive oxygen species can lead to further neutrophil activation (Shasby et al. 1982) as well as stimulate lipid peroxidation (Chan et al. 1982) and phospholipase activation (Del Mastro et al. 1981).

The pulmonary hypertension and hypoperfusion reported with both VGE and DCS have an active vasoconstrictor component (Perkett et al., 1988, 1990) that is likely triggered by granulocytes and due to release of specific vasoactive agents. Wang et al. (1992) and Butler (1994), reported that VGE caused a significant elevation in thromboxane B_2, which is the stable and measurable metabolite of thromboxane A_2 (TxA_2), and which is a product of the cyclooxygenase pathway. TxA_2 is an unstable metabolite of arachidonic acid that is produced by thromboxane synthetase (Hamberg, 1975). The enzyme is found in platelets, neutrophils, monocytes, lymphocytes, and in various organs including the kidneys, heart, brain, GI and lungs (Granstrom, 1987). TxA_2 is also an active stimulant for platelet aggregation and can cause pulmonary

vasoconstriction. Bonsignore et al. (1986) and Fukushima et al. (1986) both reported that VGE increased thromboxane B_2; one in anesthetized and the other in unanesthetized sheep. Measurement of TxA_2 levels in any body fluid is not usually accurate because it is rapidly hydrolyzed to its primary stable metabolite thromboxane B_2. Measurement of the TxB_2 metabolite, 11-dehydrothromboxane B_2, especially in the urine however, reportedly gives a good approximation of TxA_2 synthesis (Reinke, 1992). Measurement of the 11dh thromboxane B_2 concentrations in urine potentially has additional advantages over blood because of the potential for platelet activation during the handling of the blood (Reinke, 1992). This can be particularly important in view of the degree of in vivo platelet activation that can already have occurred as a result of DCS-induced venous bubbles (Philp et al., 1972). In recent studies, we have reported elevated levels of TxB_2 and 11-dehyro TxB_2 levels in rats and dogs undergoing decompression (Butler & Little, 1994). These increases corresponded to pulmonary edema formation and increased levels of protein in the lungs, suggestive of microvascular membrane permeability changes. The periodicity of these mediator responses has not been studied however.

The other category of vasoactive mediators of interest with DCS includes the sulfidopeptide leukotrienes. Leukotrienes were produced in the lungs and if protein-bound have less direct effect but may function as a local hormone causing neutrophil chemotaxis and smooth muscle constriction and edema formation (Reeves et al., 1985). It is difficult to delineate the particular role leukotrienes play in DCS-induced injury as singular agents or in combination with other active substances. As a product of arachidonic acid metabolism via lipoxygenase, leukotrienes do have pulmonary vascular effects not dissimilar to acute DCS (i.e. edema, pulmonary hypertension, broncho-constriction and inflammation). Stimuli other than direct effects of circulating bubbles or neutrophil activation such as platelet activating factor (PAF, were also known to occur with DCS and may suggest a role of leukotrienes.

Evaluation of systemic levels of leukotrienes often involves blood samples but may include urinary sources as well (Kumlin et al., 1992). Keppler et al. (1990) reported that urinary excretion of LTE_4 is a reliable indicator of systemic production, especially by the lung, provided that hepatic and renal function are normal. Hepatic enzyme levels were not elevated with moderate altitude decompression (Jauchem et al., 1986). Further, several investigators have correlated urinary LTE_4 levels with adult respiratory distress syndrome, which often presents with a number of symptoms that were also not dissimilar to respiratory DCS [e.g. bronchoconstriction, pulmonary hypertension, lung compliance changes, hypoxemia and lung edema (Bernard et al., 1991)]. Similar comparisons have been reported with leukotriene levels and animal models of adult respiratory distress syndrome (Snapper et al., 1987). Additionally, Tagari et al. (1993) reported that the endogenously generated peptidoleukotriene metabolite LTE_4 in anesthetized dogs has a mean basal excretion rate that was very similar to the normal human levels and was an appropriate index for in vivo generation of 5-lipoxygenase products for that species. He further stated that the "actual urinary excretion of the endogenously generated metabolite LTE_4 is more comparable to that seen in humans, as seen in other animal models."

A significant source of circulatory leukotrienes includes eosinophils and neutrophil/endothelium interactions (Lagarde et al., 1989). The latter source is likely to play a key role in DCS-induced levels of systemic leukotrienes while the other two large sources were the liver and in particular the lungs (Kumlin et al., 1992) which were significantly impacted by venous bubbles. The lungs possess a considerable potential for LTC_4 production, especially in allergic conditions, which after sequential cleavage of its peptide residues results in the formation of LTD_4 and LTE_4. High concentrations of leukotrienes were also reported in bronchoalveolar lavage fluid (see Methods section) as well as lung edema fluid in subjects with adult respiratory distress syndrome (Matthay et al., 1984; Stephenson et al., 1988). Tagari et al. (1993) concluded that the lung source of LTE_4 was predominant. We have recently reported elevated levels of LTE_4 in rats after decompression have also showed signs of pulmonary edema (Butler & Little, 1994; Butler et al., 1995). Furthermore, we have also demonstrated that with attenuation of leukotriene and thromboxane production by pretreatment using an analogue of cAMP, the edema is blocked and symptoms were reduced in decompressed rats.

Presently, once-a-day decompression exposure animal model and human experiments are the norm. Typically, with rodent experiments, experiments were performed during the day, during the lights-on phase, of the animal's light-dark regimen of the housing chamber. Laboratory rodents were active during the night and rest (sleep) during the daytime. In terms of animal studies, this means that most, if not all, our understanding of the physiology of decompression stress is based on single time-point investigations carried out during the rest, rather than the activity span of the laboratory rodent to model the effects and response of human beings who were most likely to experience exposures during their diurnal activity span. Astronauts undergoing an EVA will remain at some risk for decompression sickness unless extraordinary procedures for denitrogenation and staged depressurization continue to be implemented. An acceptable level of risk is based upon exhaustive ground-based studies that provide incidence figures and data relating to the likelihood of venous bubble formation. Because some of the symptoms of decompression sickness, especially those involving the central nervous system were so insidious, a better understanding of the pathophysiology and underlying mechanism is warranted if "acceptable risk" is to be meaningful.

These series of animal experiments were implemented to assess the importance of the circadian time of decompression exposure in terms of tolerance to exposure (intensity of the symptoms of decompression sickness) and biochemical responses. Information regarding circadian rhythms in the tolerance and mechanisms of decompression sickness (DCS) is indispensable. Current oxygen prebreathe protocols to minimize DCS were under scrutiny in terms of duration and in combination with exercise. Regardless of the newer studies, the formation of VGE with decompression is likely to remain and therefore a clearer understanding of other underlying factors such as circadian rhythm dependent ones were important. The findings of these studies have identified the magnitude of the circadian rhythm response to decompression as well as the circadian times of greatest and least tolerance to decompression.

This proposal was intended to address the specific program emphasis of the NRA announcement (NRA 96-HEDS-04) that pertains to Environmental Health aspects of extravehicular activity and decompression sickness.

MATERIALS AND METHODS

This proposal involved the exposure of Sprague-Dawley rats to decompression and venous air embolism. The decompression exposures were selected to cause moderate to significant venous bubble formation. Previous studies have demonstrated the utility of this model for decompression evaluation.

Animal Care and Use: The following protocols were approved by the Animal Care and Use Committee of the University of Texas Health Science Center at Houston (Protocol #HSC-AWC-95-124 approval letter attached) and followed NIH-PHS guidelines regarding humane use of animals.

Decompression Procedures **(AIMS 1-3)**: The animals were placed in the compression chamber (see Facilities and Equipment section) and allowed 5 min. to acclimate. If in their dark cycle, the study was conducted in darkness with the chamber covered to maintain the lighting conditions. The chamber was compressed to 683 kPa over 11 min. The animals remained at 683 kPa for 60 min. and then were decompressed over 9.5 min. They were then removed for a 60 min. observational/recovery period. Any gross symptoms of DCS were recorded. (Please refer to Table for a listing of DCS symptoms)

This particular decompression exposure was selected to simulate saturation conditions and venous bubble formation in > 90% of the animals. The symptomology however, was far less than usually reported with rodent experimentation where death or recovery endpoints were selected. The purpose of the proposed studies required less severe symptomology for outcome evaluation.

Twelve different, but comparable (by mean body weight) groups of 12 adult male Sprague-Dawley rats each were studied in this series of experiments. Animals of six groups (experimentals) were exposed to 683 kPa for 60 minutes and the remaining 6 groups (controls) remained at atmospheric pressure for 60 minutes. The control and experimental groups were randomized for 60-minute timed exposures centered at 2, 6, 10, 14, 18 and 22 HALO. (Post decompression analyses were described in the sections below.)

Eicosanoid Assays: The concentrations of vasoactive mediators taken from the biological samples of animals undergoing decompression were measured with enzyme immunoassays (EIA), (Cayman).

Biological and biochemical data analyses were performed using analysis of variance with post hoc comparisons made using Fishers tests; $p < 0.05$ was considered to be statistically significant. Following collection of the optical density data for each enzyme assay test, the percent bound ligand for each standard and experimental sample was

determined. Standard curves were then generated by plotting % B/Bo as a function of the log concentration of the specific mediator. Percent B/Bo was plotted against pg/ml (mediator) standard per well (x-axis) on semi-log graph using the Biocalc software. Pg/well values were derived from the standard curve and corrected to sample volume.

Venous Air Infusions (VAE) **(AIM 4)**: Results of the decompression studies above identified the degree of risk of DCS that is influenced by circadian rhythm. To further clarify the influence of venous bubbles alone, versus other aspects of DCS, we selected two biological times (one corresponding to the circadian stage of least and the other corresponding to the stage of greatest tolerance to DCS as defined by the magnitude of pulmonary edema, symptoms and inflammatory mediator levels) to receive VAE infusions.

Surgical: For the VAE infusions, the rats had a silastic/tygon catheter placed into the right external jugular vein aseptically, with the tygon end tunneled under the skin and externalized at the nape of the neck. This procedure was conducted under pentobarbital sodium anesthesia (15-20 mg/kg IP). The catheter was flushed with saline and fused at the external end for prevention of blood loss. The surgical wound was closed with sterile suture and the rats were allowed to recover at least 72 hrs. prior to continuation of the protocol.

Air Infusion: For the venous air infusions, the catheters were connected to a syringe pump (Razel) and infused with room air at a rate of 0.015 ml/kg/min. The total amount of gas infused was approximately 1.35 mls over 3 hrs. This volume of gas represents not more than 5.4% of the total estimated blood volume of a 400-500 gm rat. This dose had been previously shown to produce a similar degree of hypomotility, tachypnea, labored breathing and EVLW as seen with DCS (Robinson, 1996). Post VAE analyses are described in the sections below.

Chronopharmacological Intervention **(AIM 5)**: Chronopharmacology is the study of biological rhythm-induced effects on the kinetics and dynamics of administration of medications according to administration time.

Results of the decompression studies (Aim 1) identified the extent of risk of DCS that is dependent on circadian rhythms or specific inflammatory mediators. From such data, the same as above in Aim 2, the two biological times (with reference to circadian rhythms in risk) - one corresponding to the greatest and the second to the least susceptibility - were selected to conduct dose-response investigations of drug effect both on overt variables and on the release of inflammatory mediators (eicosanoids). Three agents were studied. The first was aspirin for determining the role of cyclo-oxygenase products of arachidonic acid metabolism, namely thromboxane A_2 and its stable byproduct TxB_2. The second was zileuton [N-[1S-(2-benzothiophenyl)ethyl]-N-hydroxyurea], produced by Abbott labs which is specific to the 5-lipoxygenase pathway of arachidonic acid metabolic products, namely the leukotrienes. Zileuton actually blocks the 5-lipoxygenase enzyme rather than act as a leukotriene receptor antagonist. By selecting the appropriate cyclooxygenase and 5-lipoxygenase inhibitors above, and

evaluating their dose response effects at different time points, it is anticipated that circadian rhythm dependent patterns will emerge and thereby enable a better assessment of risk and intervention possibilities with DCS. The third was isoproterenol.

Surgical: The rats selected for the pharmacological studies had venous catheters inserted as described in the above (Venous Air Infusion, Surgical) section.

Procedure: Each of the drugs was evaluated in both control and experimental (decompressed) groups of rats. The Control Group for both drug groups received a vehicle infusion (normal saline) in an equal volume and rate as for the experimental rats.

The zileuton rats received oral doses at the same two time points. The infusions were controlled using the syringe pump previously described in the above section. Oral zileuton was given by gavage 60 mins. prior to the decompression exposure. These doses have been effective in blocking in vivo leukotriene production by 80% in other animal models when allergen stimulation was studied (Abraham et al., 1992; Smith et al., 1995). Drug doses were prepared such that the total volume of fluid infused will not exceed 500 υl. Post DCS analyses are described in the sections below.

ANALYSES:

Surgical Procedures: At 60 min. post-decompression, the rats were anesthetized with halothane (5%) in a rodent anesthesia box and anesthesia is maintained with 2% halothane in air through a hood-like ventilatory circuit. The 60 min. post exposure interval was selected for optimal response of post decompression eicosanoid levels (Butler & Little, 1994). Once surgical levels of anesthesia were achieved, an abdominal incision was performed for urine collection via direct bladder aspiration. Samples were stored in quadruplicate (50 υl aliquots) at -70°C until analyzed. An additional sample was stored at 5°C for creatinine concentration measurement.

The abdominal aorta was then cannulated with polyvinyl tubing (PE-50) for collection of arterial blood for white blood cell count (WBC), differential cell and platelet count, extravascular lung water ratio blood correction, plasma protein analysis, and eicosanoid analysis (See next section for analyses). Euthanasia occurred via exsanguination while under anesthesia. The presence of intravascular bubbles was recorded in the symptoms chart as well as a gross organ survey for DCS pathology.

The thorax was opened at the level of the diaphragm for collection of pleural fluid by aspiration of the dependent region of the thoracic cavity (see below for pleural fluid analysis). The trachea was then cannulated and the heart and lungs are removed en bloc. The heart and excess tissue were dissected free of the lungs for a fresh lung weight recording. Gross lung pathology was recorded.

Bronchoalveolar Lavage (BAL): For the BAL the freshly dissected and weighed lungs were degassed by vacuum three times. The airways were then lavaged with normal saline (5°C) via the tracheal cannula as follows: 10 mls was slowly infused (10 mls/min)

and washed in and out 3 times. This procedure was then repeated for a total of 5 times (50 mls) and all washes pooled. Recovered BAL was separated for protein and eicosanoid analysis and WBC counts.

Pulmonary Edema Measurement: The amount of blood-free extravascular lung fluid (edema) formation was measured using the methods of Pearce et al. (1965). The amount of extravascular lung water (EVLW), expressed as the extravascular fluid to blood-free dry weight ratio was then determined after correction for residual blood volume in the homogenate using a hemoglobin measurement correction.

Cell Counts: Total WBC and differential cell counts were performed on arterial blood, BAL and pleural fluid using a Neubauer hemocytometer. Differential cell counts were performed after Wright-Geimsa staining.

Protein Analysis: Total protein from arterial plasma, BAL and pleural fluid was measured using a modified Lowry (1951) method and adapted to a microplate reader at 750 nm. Samples were centrifuged to obtain a cell-free supernatant and stored at $5^{o}C$ until analysis.

Creatinine Analysis: Urine samples were stored air-tight at $-20^{o}C$ until analysis. Creatinine was measured using an alkaline picrate method with colorimetric analysis. Measurement of creatinine allows for normalization of the excretion of urinary metabolites (Christman et al. 92) when expressing eicosanoid levels in the urine.

RESULTS:

CIRCADIAN RHYTHM STUDIES

TABLE I: Circadian characteristics for each variable and site were listed below:

Variable	Site:Units	Time-effect:	ANOVA	Cosinor: 24h* or 24h+12h model			
		Mesor ±SE	p	p	Amp	Orthophase	Bathyphase
EVLW	wet/dry ratio	3.56±0.04	0.051	0.029	0.24	04:48h	11:48h
Protein	B:mg/g wt	18.53±0.76	<0.001	0.001	7.16	18:28h	01:24h
	P:mg/ml	17.84±0.53	0.002	0.010	3.37	19:20h	12:16h
WBC	A:cells/cu mm	2959±74	0.063	0.033	381	09:28h	15:56h
	B:cells/cu mm	359±11	0.007	0.004	70	23:32h	15:24h
	P:cells/cu mm	68295±1830	0.096	0.481*	3074	09:52h	21:52h
Neutrophils	A:cells/cu mm	551±20	0.024	0.046	104	09:20h	02:16h
	B:cells/cu mm	1.32±0.21	0.050	0.045	0.95	11:24h	17:32h
	P:cells/cu mm	12064±449	0.086	0.022*	1819	12:37h	00:37h
TXB2	A:pg/ml	171±22	0.038	0.028	125	12:24h	19:08h
	B:pg/g wt	1534±53	<0.001	0.001	570	05:48h	12:52h
	U:pg/mg/creat	599±28	0.234	0.055*	99	20:52h	08:52h
11dhTXB2	A:pg/ml	18.1±1.14	<0.001	0.035	5.8	16:12h	09:16h
	B:pg/g wt	50.0±4.0	<0.001	0.001	33	12:28h	18:48h
	U:pg/mg/creat	1943±120	<0.001	0.001	1231	14:04h	21:00h
LKE4	A:pg/ml	1089±107	<0.001	0.001	950	01:40h	08:04h
	B:pg/g wt	1738±152	<0.001	0.013	871	01:28h	18:24h
	U:pg/mg/creat	82±4	<0.001	0.001	38	13:40h	20:20h
NO	A:nM	21.79±0.68	0.451	0.190*	1.89	13:55h	01:55h

Mesor=24hr mean; amp=amplitude=1/2 peak-trough difference; orthophase=peak time; and bathyphase= trough time. Orthophase and bathyphase referenced to local midnight.

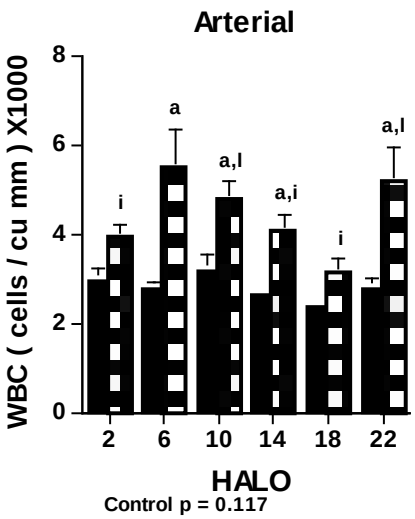
Conclusions: Nearly all baseline indices of DCS demonstrated circadian time Dependencies

TABLE II: Circadian characteristics for each variable were listed below (units=% of control):

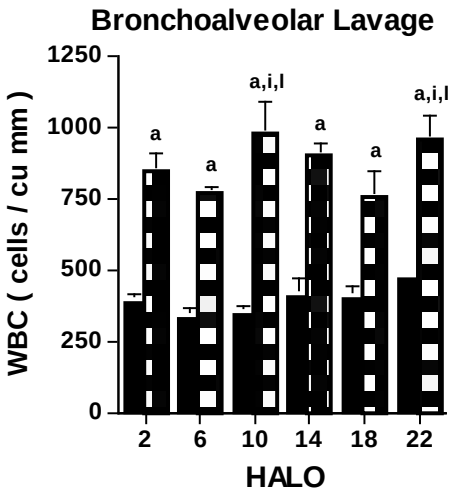
Variable	Site:orig Units	Time-effect:	ANOVA	Cosinor: 24h* or 24h+12h model			
		Mesor ±SE	p	p	Amp	Orthophase	Bathyphase
EVLW	wet/dry ratio	165±12	0.022	0.076*	40	00:18h	12:18h
Protein	B:mg/g wt	243±17	0.008	0.232*	41	22:15h	10:15h
	P:mg/ml	140±5	0.003	0.049*	20	10:51h	22:51h
WBC	A:cells/cu mm	147±5	0.100	0.664	13	22:36h	16:00h
	B:cells/cu mm	210±6	0.003	<0.001	36	14:44h	00:20h
	P:cells/cu mm	92±10	0.237	0.482*	17	18:12h	06:12h
Neutrophils	A:cells/cu mm	235±13	0.012	0.084	64	20:08h	13:00h
	B:cells/cu mm	1289±195	<0.001	<0.001	1527	06:08h	23:40h
	P:cells/cu mm	116±5	0.001	0.041	25	23:32h	07:12h
TXB2	A:pg/ml	143±11	0.052	0.118	49	04:24h	10:52h
	B:pg/g wt	106±6	0.024	0.179*	15	15:15h	03:15h
	U:pg/mg/creat	325±23	<0.001	<0.001	176	13:28h	19:52h
11dhTXB2	A:pg/ml	108±4	0.026	0.022	20	19:40h	12:20h
	B:pg/g wt	173±14	<0.001	<0.001	92	05:40h	12:00h
	U:pg/mg/creat	200±14	<0.001	<0.001	130	21:32h	14:44h
LKE4	A:pg/ml	305±27	<0.001	<0.001	308	09:52h	02:36h
	B:pg/g wt	386±27	0.117	0.098	136	12:56h	20:20h
	U:pg/mg/creat	172±10	0.019	0.017	52	00:08h	16:28h
NO	A:nM	55±2	0.007	<0.001*	10	07:39h	19:39h

Mesor=24hr mean; amp=amplitude=1/2 peak-trough difference; orthophase=peak time; and bathyphase= trough time. Orthophase and bathyphase referenced to local midnight.

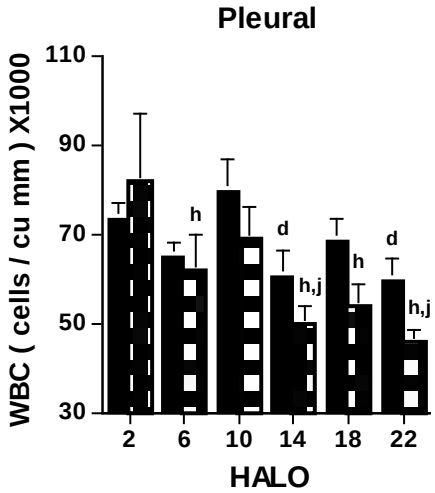
Conclusions: Nearly all indices of DCS symptomology were influenced by circadian time dependencies



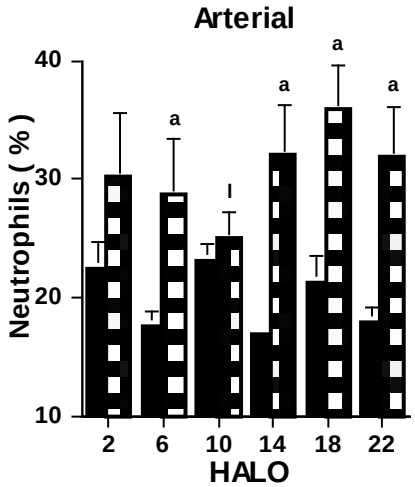
Control p = 0.117
DCS p = 0.215
DCS as % Control p = 0.598



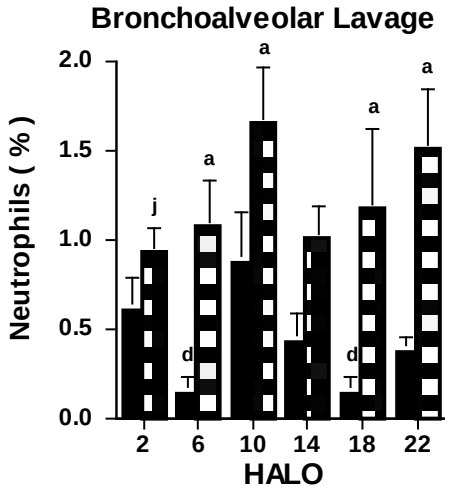
Control p = 0.159
DCS p = 0.153
DCS as % Control p = 0.023



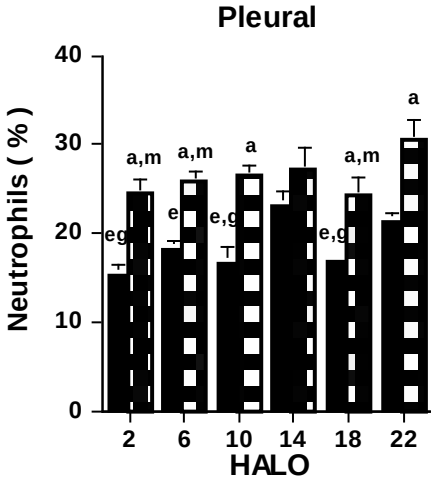
Control p = 0.617
DCS p = 0.237
DCS as % Control p = 0.299



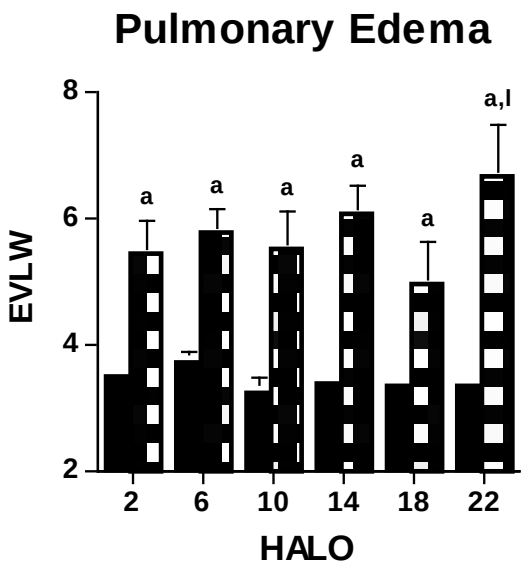
Control p = 0.923
DCS p = 0.441
DCS as % Control p = 0.385

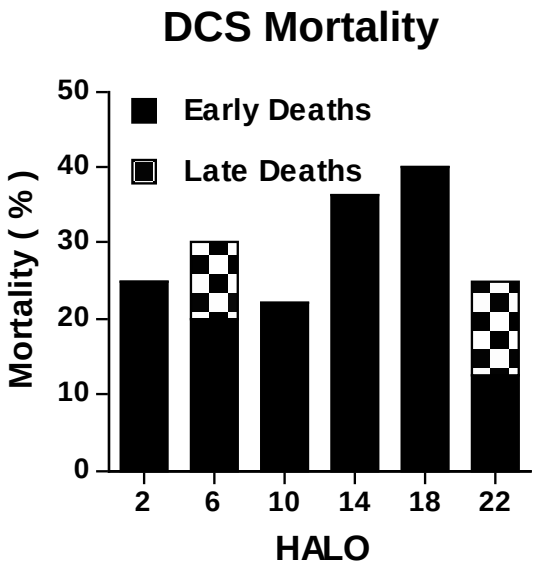


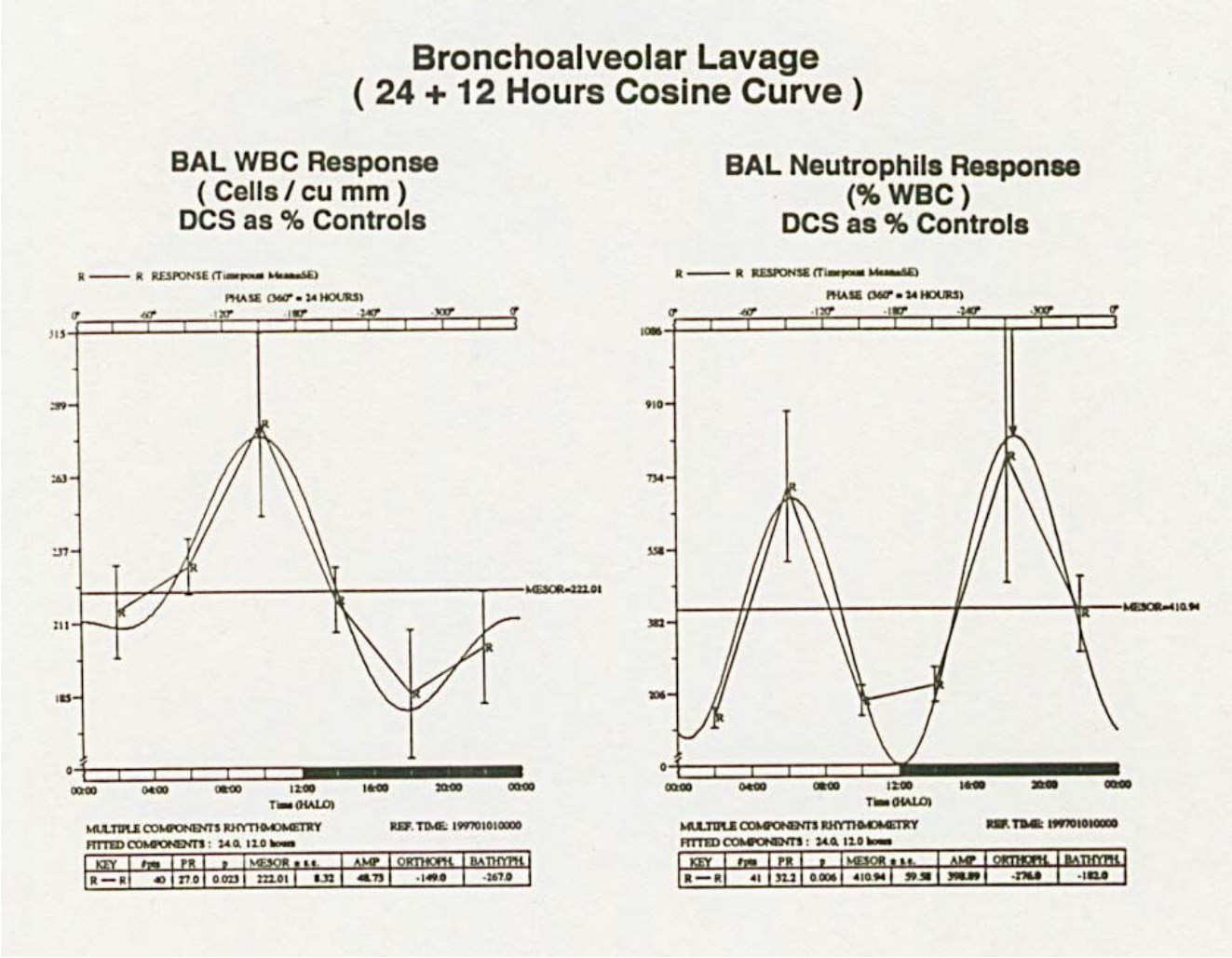
Control p = 0.045
DCS p = 0.338
DCS as % Control p = 0.006

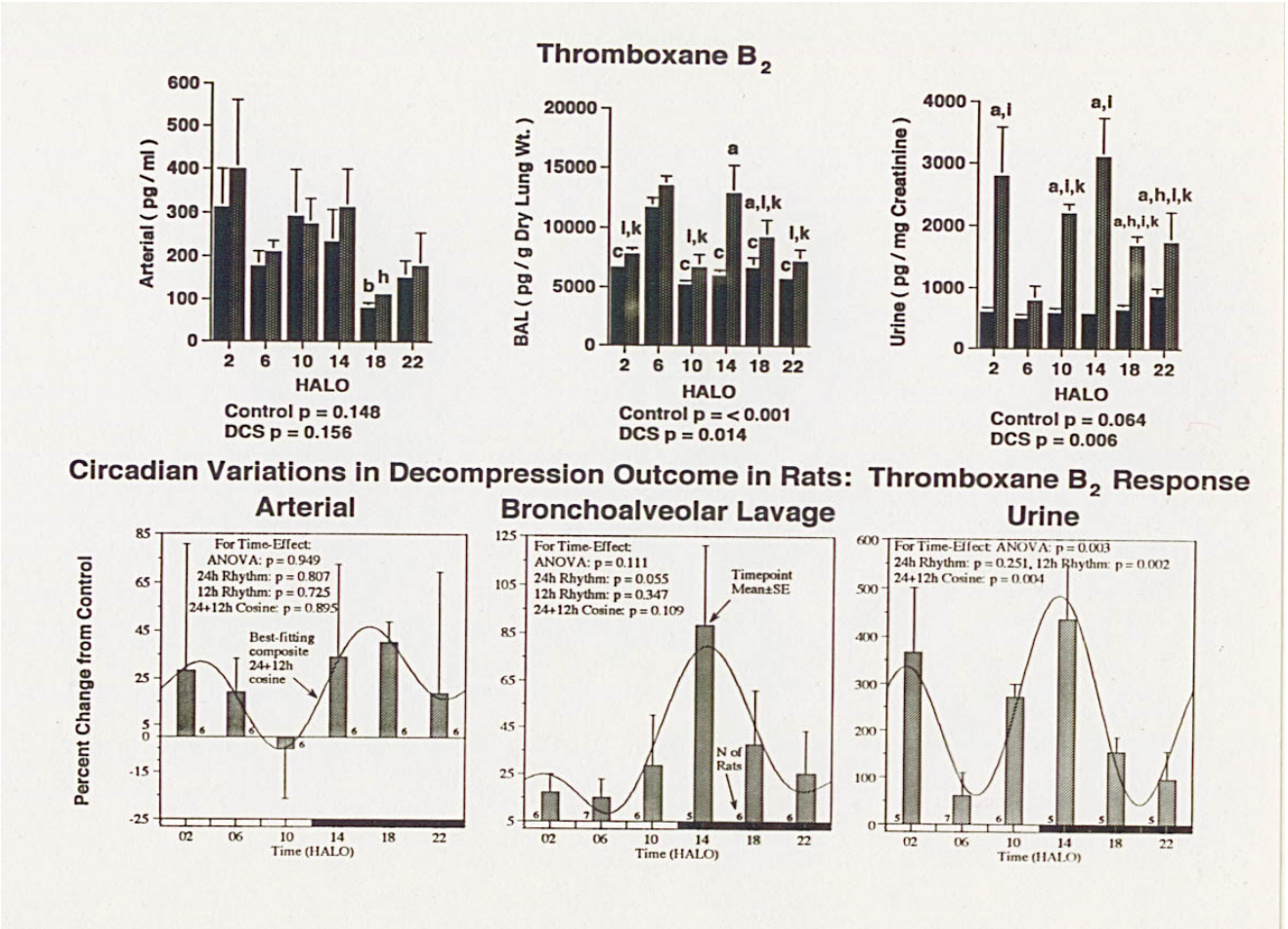


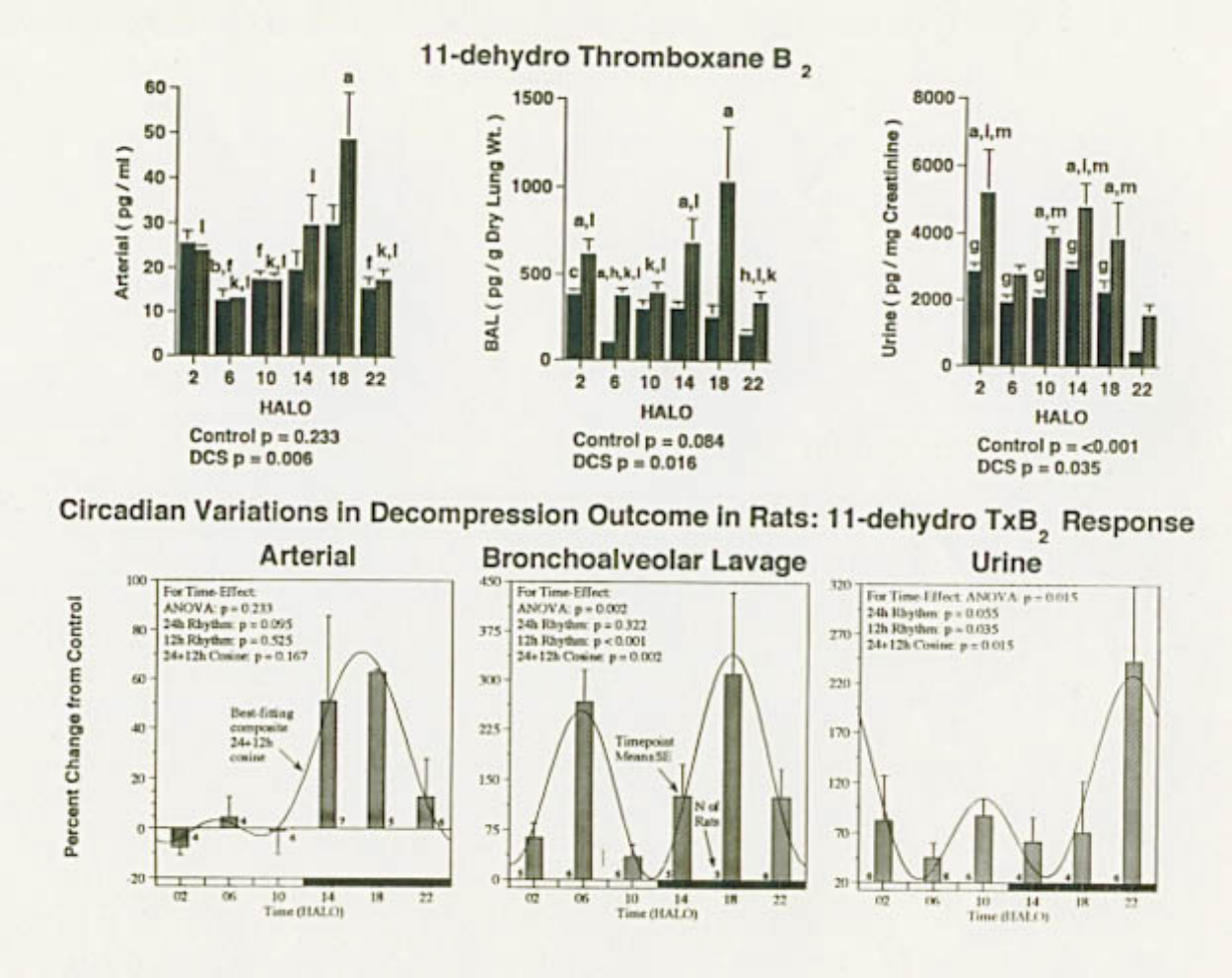
Control p = 0.161
DCS p = 0.462
DCS as % Control p = 0.449

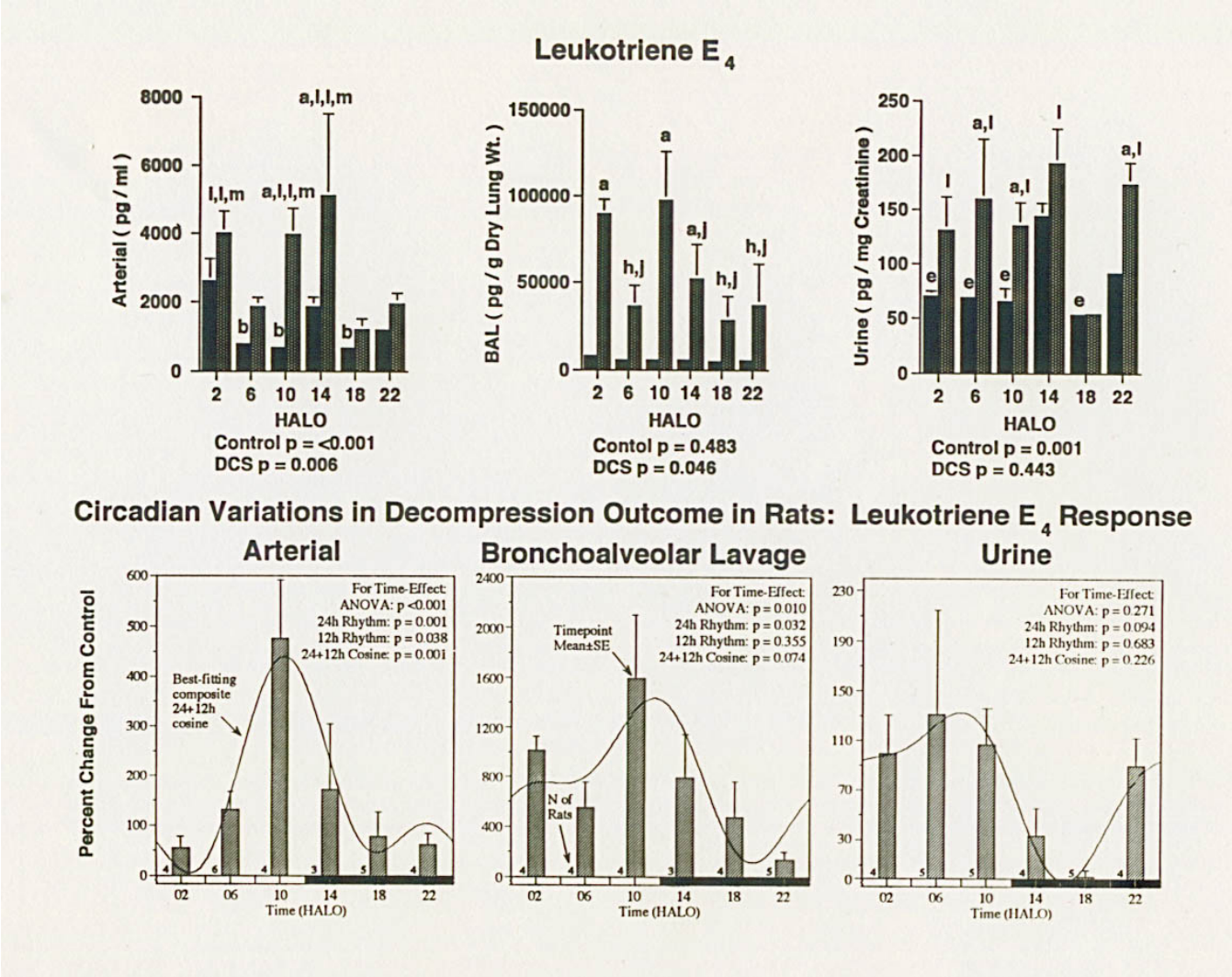

Pulmonary Edema
EVLW
8
6
4
2
a
a
a
a
a
a,l
2
6
10
14
18
22
HALO


DCS Mortality
Mortality (%)
50
40
30
20
10
0
Early Deaths
Late Deaths
2
6
10
14
18
22
HALO

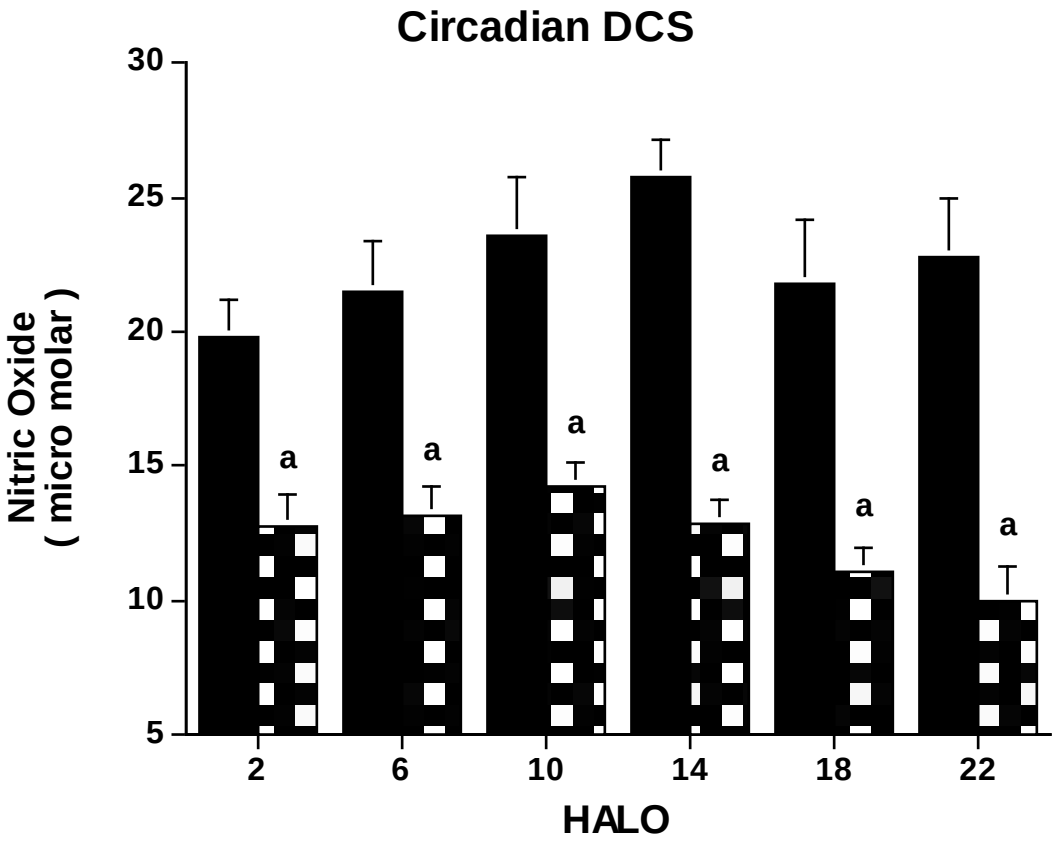

Bronchoalveolar Lavage
(24 + 12 Hours Cosine Curve)
BAL WBC Response
(Cells / cu mm)
DCS as % Controls
BAL Neutrophils Response
(% WBC)
DCS as % Controls
MESOR=222.01
MESOR=410.94
Time (HALO)
MULTIPLE COMPONENTS RHYTHMOMETRY
FITTED COMPONENTS : 24.0, 12.0 hours
KEY | PR | p | MESOR | AMP | ORTHOPH. | BATHYPH.
R — R | 40 | 27.0 | 0.023 | 222.01 | 8.32 | 48.75 | -149.0 | -267.0
R — R | 41 | 32.2 | 0.006 | 410.94 | 59.58 | 398.89 | -276.0 | -182.0


Thromboxane B2
Arterial (pg / ml)
HALO
Control p = 0.148
DCS p = 0.156
BAL (pg / g Dry Lung Wt.)
HALO
Control p = < 0.001
DCS p = 0.014
Urine (pg / mg Creatinine)
HALO
Control p = 0.064
DCS p = 0.006
Circadian Variations in Decompression Outcome in Rats: Thromboxane B2 Response
Arterial
Bronchoalveolar Lavage
Urine
Percent Change from Control
For Time-Effect:
ANOVA: p = 0.949
24h Rhythm: p = 0.807
12h Rhythm: p = 0.725
24+12h Cosine: p = 0.895
Best-fitting composite 24+12h cosine
For Time-Effect:
ANOVA: p = 0.111
24h Rhythm: p = 0.055
12h Rhythm: p = 0.347
24+12h Cosine: p = 0.109
Timepoint Mean±SE
N of Rats
For Time-Effect: ANOVA: p = 0.003
24h Rhythm: p = 0.251, 12h Rhythm: p = 0.002
24+12h Cosine: p = 0.004
Time (HALO)


11-dehydro Thromboxane B 2
Arterial (pg / ml)
BAL (pg / g Dry Lung Wt.)
Urine (pg / mg Creatinine)
HALO
Control p = 0.233
DCS p = 0.006
Control p = 0.084
DCS p = 0.016
Control p = <0.001
DCS p = 0.035
Circadian Variations in Decompression Outcome in Rats: 11-dehydro TxB2 Response
Arterial
Bronchoalveolar Lavage
Urine
Percent Change from Control
Best-fitting composite 24+12h cosine
Timepoint Means±SE
N of Rats
Time (HALO)


Leukotriene E 4
Arterial (pg / ml)
BAL (pg / g Dry Lung Wt.)
Urine (pg / mg Creatinine)
HALO
Control p = <0.001
DCS p = 0.006
Contol p = 0.483
DCS p = 0.046
Control p = 0.001
DCS p = 0.443
Circadian Variations in Decompression Outcome in Rats: Leukotriene E 4 Response
Arterial
Bronchoalveolar Lavage
Urine
Percent Change From Control
For Time-Effect:
ANOVA: p <0.001
24h Rhythm: p = 0.001
12h Rhythm: p = 0.038
24+12h Cosine: p = 0.001
Best-fitting composite 24+12h cosine
For Time-Effect:
ANOVA: p = 0.010
24h Rhythm: p = 0.032
12h Rhythm: p = 0.355
24+12h Cosine: p = 0.074
Timepoint Mean±SE
N of Rats
For Time-Effect:
ANOVA: p = 0.271
24h Rhythm: p = 0.094
12h Rhythm: p = 0.683
24+12h Cosine: p = 0.226
Time (HALO)

Nitric Oxide



DRUG STUDIES

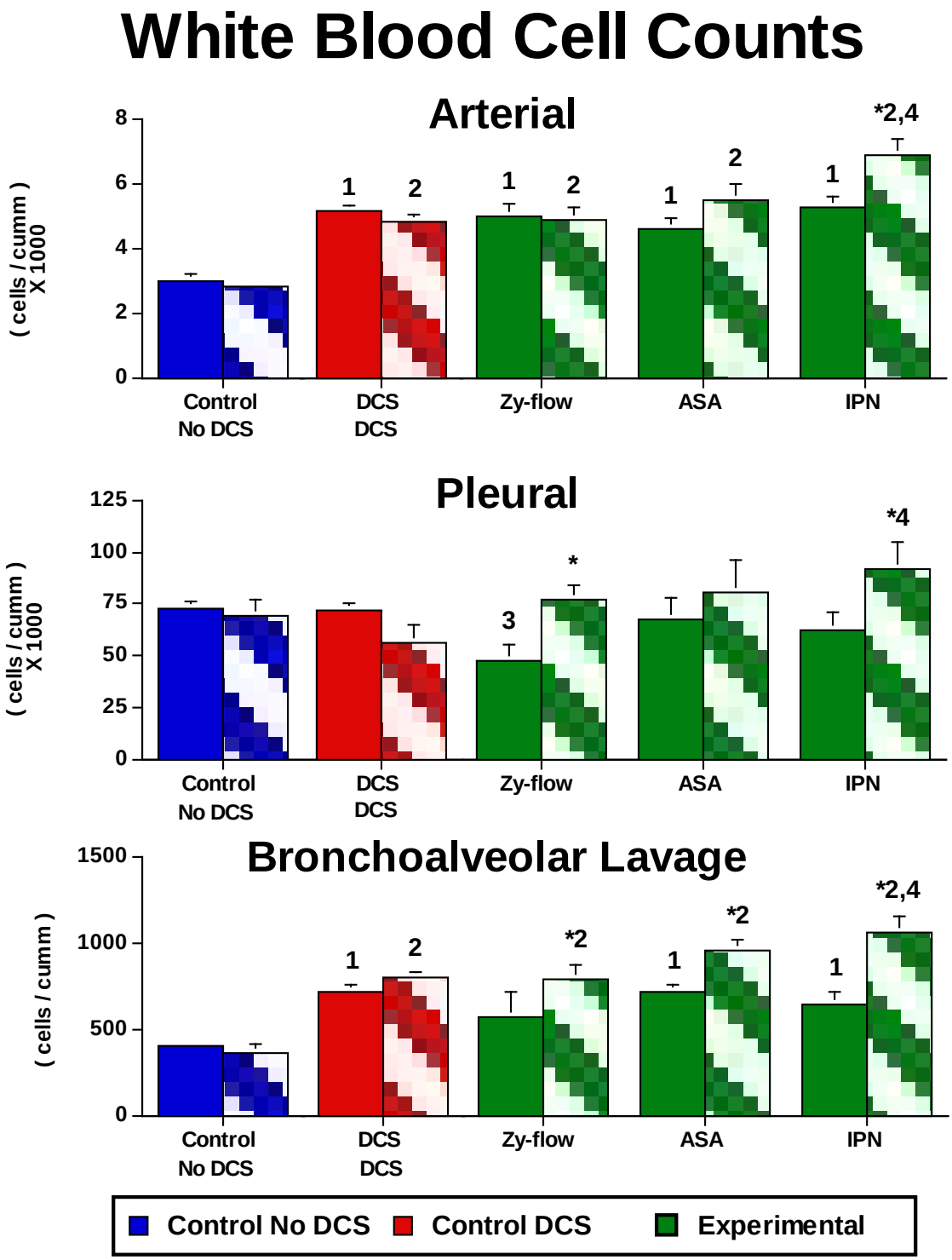
TABLE III: Drug Studies The time of day influence on drug effects on DCS was apparent as shown in the table.

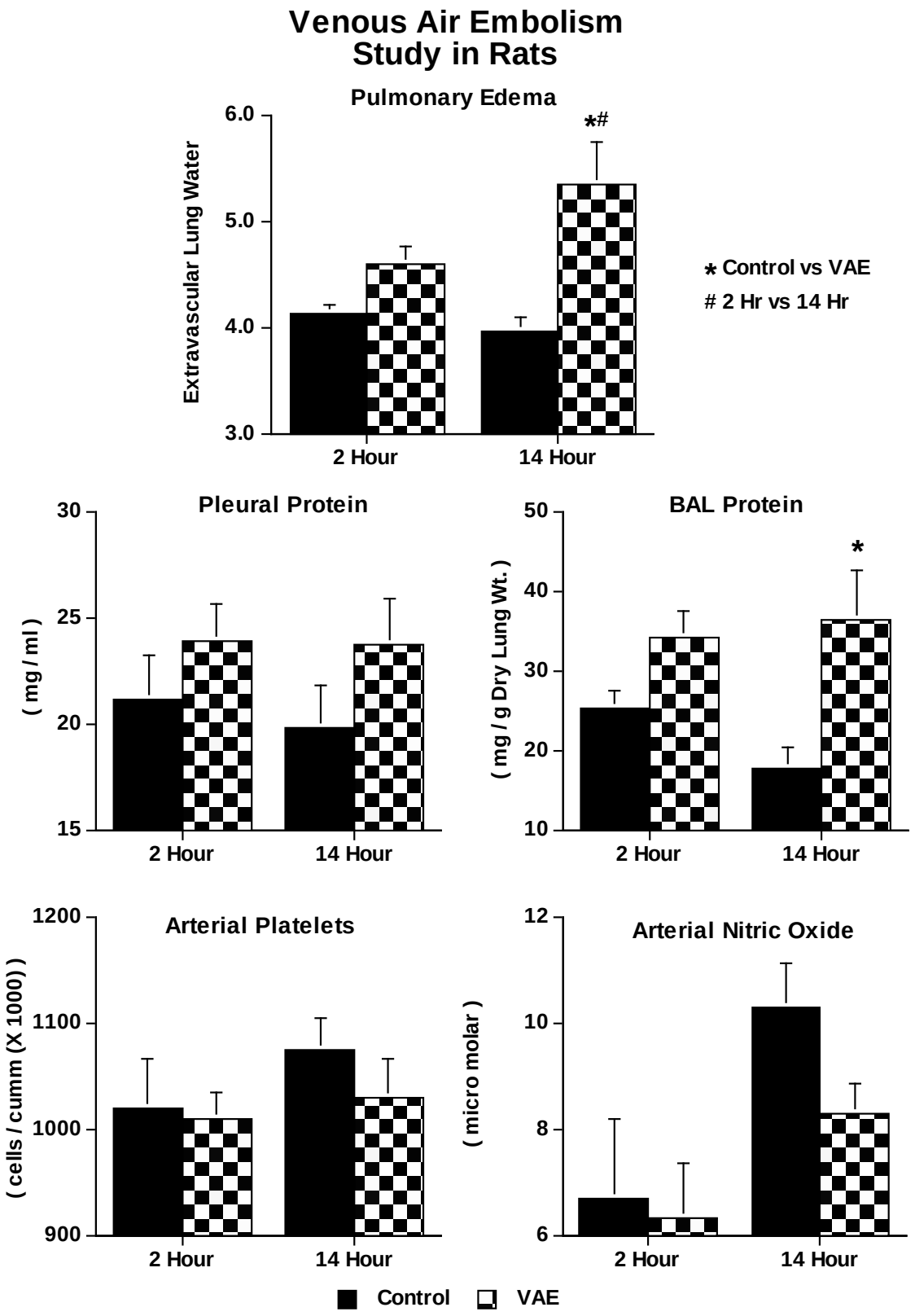
	Control DCS		Zy-flow		Aspirin		Isoproterenol	
HALO	**2**	**14**	**2**	**14**	**2**	**14**	**2**	**14**
Wet/Dry	**4.926**	**5.161**	**3.856**	**4.929**	**5.698**	**5.515**	**5.344**	**7.250**
Arterial								
WBC	**5144**	**4831**	**4991**	**4884**	**4590**	**5495**	**5275**	**6842**
Neutrophils	**30.4**	**40.2**	**42.5**	**35.6**	**32.4**	**36.3**	**70.5**	**65.3**
Pleural								
Protein	**23.03**	**14.79**	**18.05**	**14.57**	**24.39**	**18.88**	**19.00**	**23.76**
WBC	**71277**	**56125**	**47747**	**77000**	**67750**	**80548**	**62250**	**91575**
Neutrophils	**25.2**	**24.7**	**21.3**	**21.0**	**25.7**	**22.9**	**23.2**	**24.6**
BAL								
Protein	**35.57**	**29.01**	**23.28**	**24.80**	**43.50**	**43.47**	**31.38**	**67.60**
WBC	**727**	**802**	**578**	**798**	**720**	**963**	**649**	**1067**
Neutrophils	**2.1**	**1.7**	**1.7**	**1.0**	**1.3**	**2.1**	**2.6**	**2.1**
TxB_2								
Arterial	**497**	**403**	**120**	**342**	**35**	**43**	**247**	**703**
Urine	**2890**	**2518**	**1653**	**2193**	**1810**	**2153**	**1974**	**2991**
11dh-TxB_2								
Arterial	**15.6**	**21.9**	**11.2**	**12.6**	**14.4**	**11.1**	**13.5**	**9.9**
BAL	**948**	**1854**	**3255**	**20561**	**273**	**333**	**997**	**2242**
Urine	**3778**	**2648**	**492.3**	**2054**	**1172**	**1349**	**1582**	**1921**
LKE_4								
Arterial	**3087**	**3948**	**479**	**195**	**1114**	**1071**	**826**	**3161**
BAL	**35740**	**40970**	**4893**	**4278**	**61438**	**119081**	**29753**	**92360**
Urine	**137.1**	**74.1**	**87.3**	**85.6**	**164.3**	**189.1**	**72.4**	**131.7**
Nitric Oxide	**12.0**	**11.8**	**9.0**	**12.9**	**13.7**	**12.1**	**22.3**	**17.1**

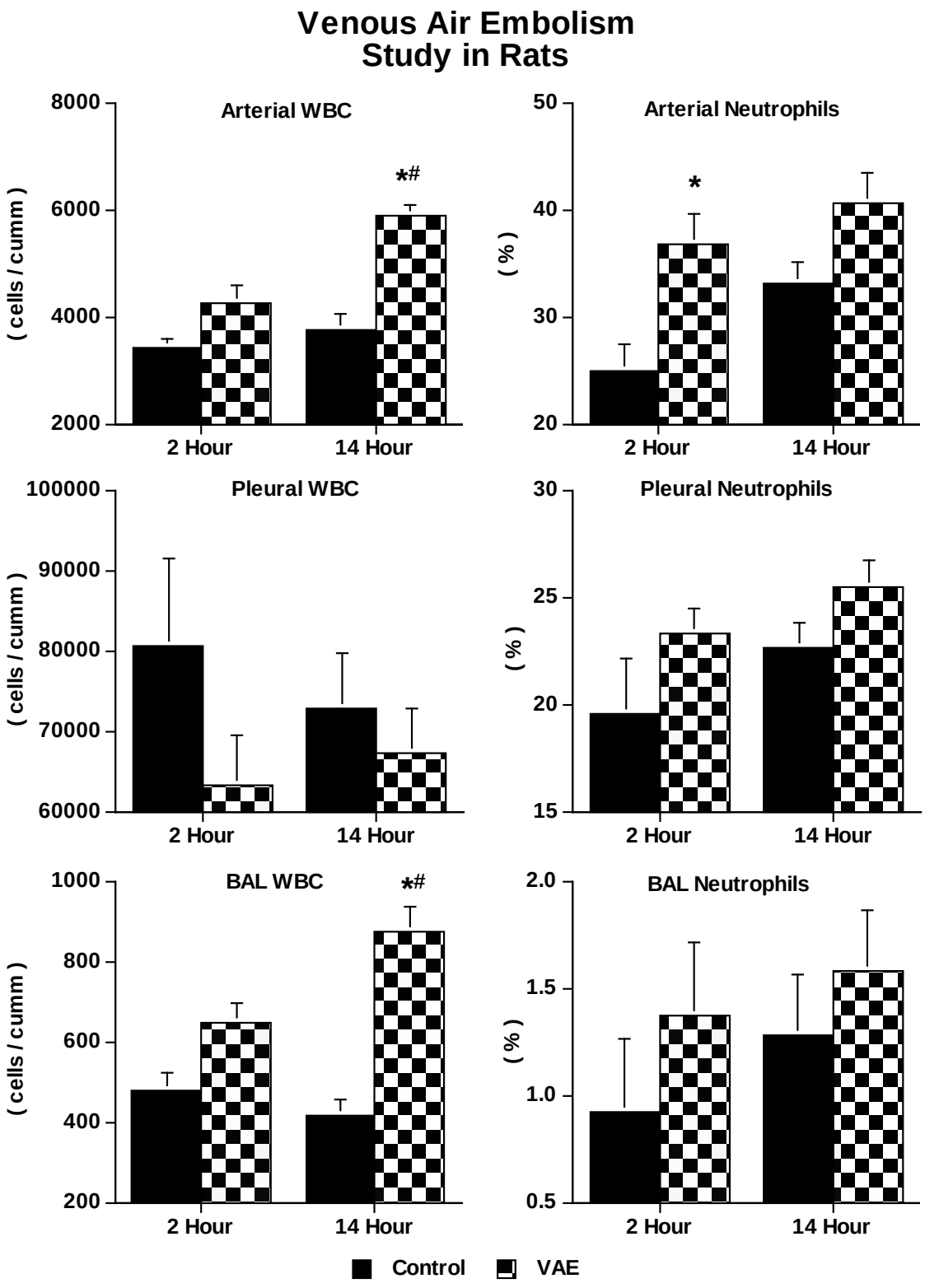
TABLE IV: PHARMACOLOGICAL INTERVENTION. SIGNIFICANT CHANGES (+), TREATED VERSUS CONTROLS:

	Acc	Zil	Indo	ASA	ISP
EVLW	+	+			
Art WBC / neutrophils	+/+		+/	+/	
Pleural WBC / neutrophils	+/+	+/+	/+		/+
Pleural Protein	+	+			
BAL WBC / neutrophils	+/+	+/		/+	
BAL Protein	+	+			
Art TxB2 /11dhTxB2 / LKE4	/+/	/ /+	+/ /	+/ /	/ /+
BAL TxB2 /11dhTxB2 / LKE4	/ /+	/ /+	+/+/	+/+/+	+/ /
Urine TxB2 /11dhTxB2 / LKE4	/ /	/ /	+/+/+	+/+/	+/+/

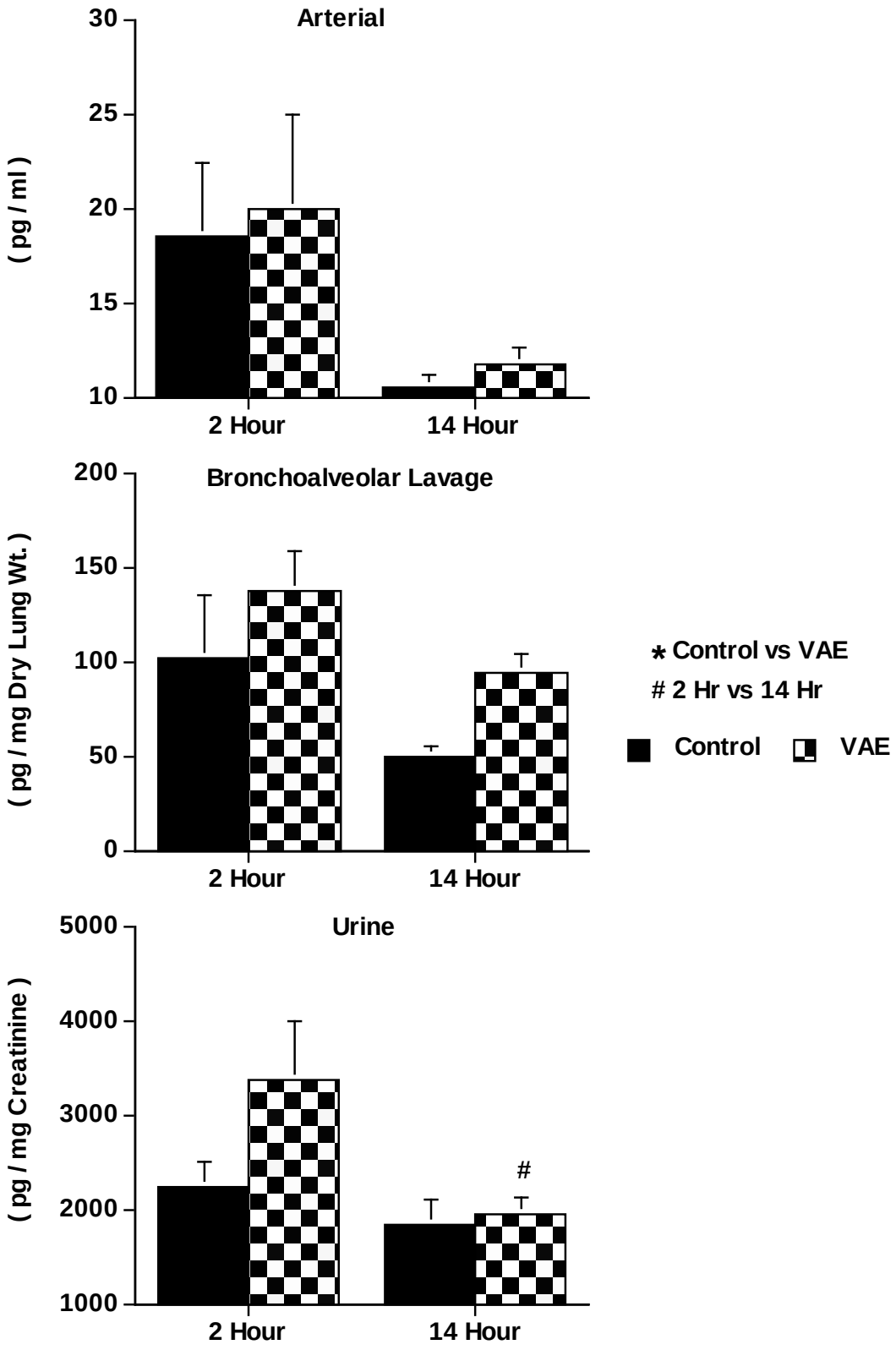
VENOUS AIR EMBOLISM STUDIES

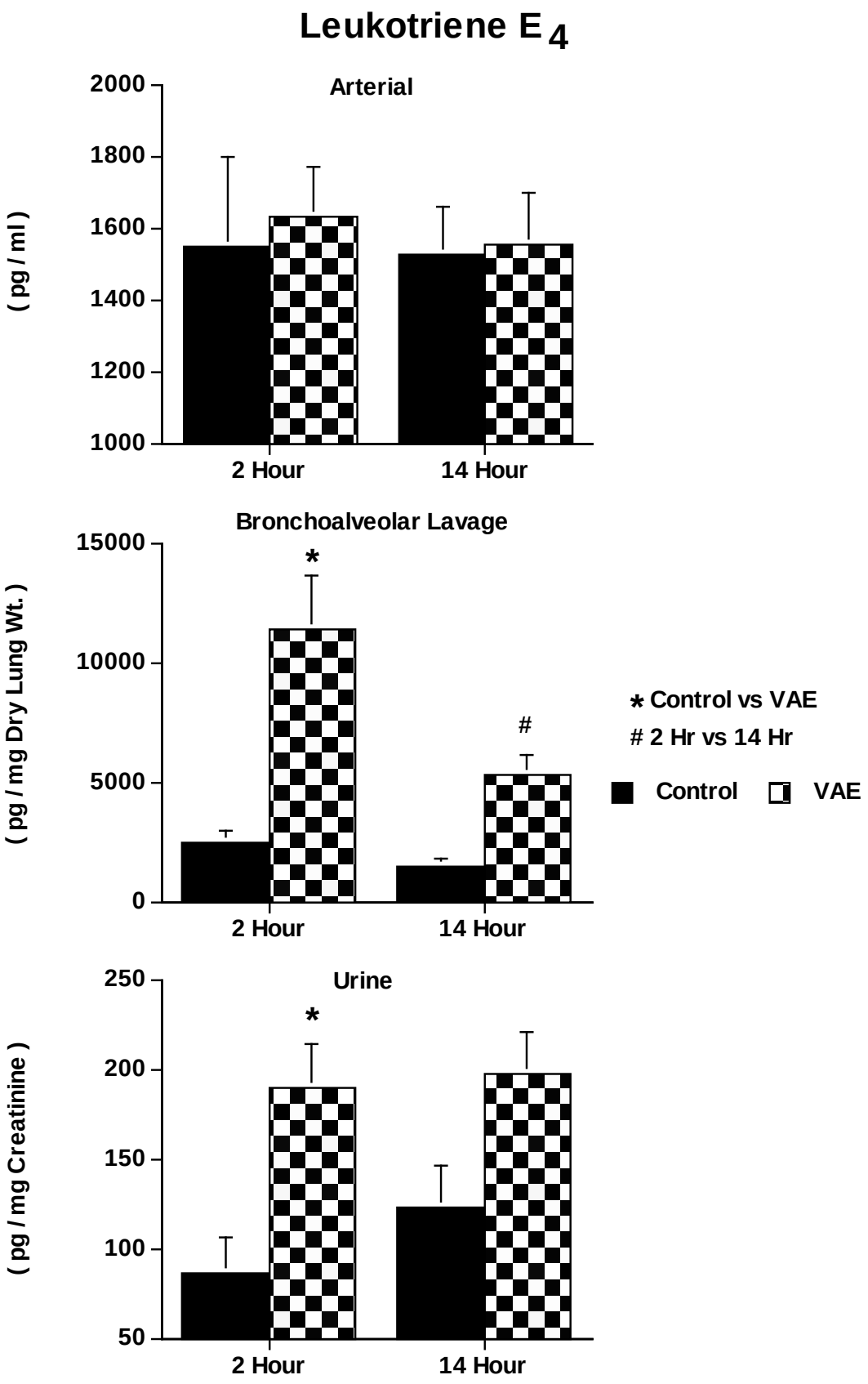



Venous Air Embolism
Study in Rats
Pulmonary Edema
Extravascular Lung Water
6.0
5.0
4.0
3.0
*#
2 Hour
14 Hour
* Control vs VAE
# 2 Hr vs 14 Hr
Pleural Protein
(mg / ml)
30
25
20
15
2 Hour
14 Hour
BAL Protein
(mg / g Dry Lung Wt.)
50
40
30
20
10
*
2 Hour
14 Hour
Arterial Platelets
(cells / cumm (X 1000))
1200
1100
1000
900
2 Hour
14 Hour
Arterial Nitric Oxide
(micro molar)
12
10
8
6
2 Hour
14 Hour
Control
VAE


Venous Air Embolism
Study in Rats
Arterial WBC
(cells / cumm)
8000
6000
4000
2000
*#
2 Hour
14 Hour
Arterial Neutrophils
(%)
50
40
30
20
*
2 Hour
14 Hour
Pleural WBC
(cells / cumm)
100000
90000
80000
70000
60000
2 Hour
14 Hour
Pleural Neutrophils
(%)
30
25
20
15
2 Hour
14 Hour
BAL WBC
(cells / cumm)
1000
800
600
400
200
*#
2 Hour
14 Hour
BAL Neutrophils
(%)
2.0
1.5
1.0
0.5
2 Hour
14 Hour
Control
VAE

11-dehydro Thromboxane B_2



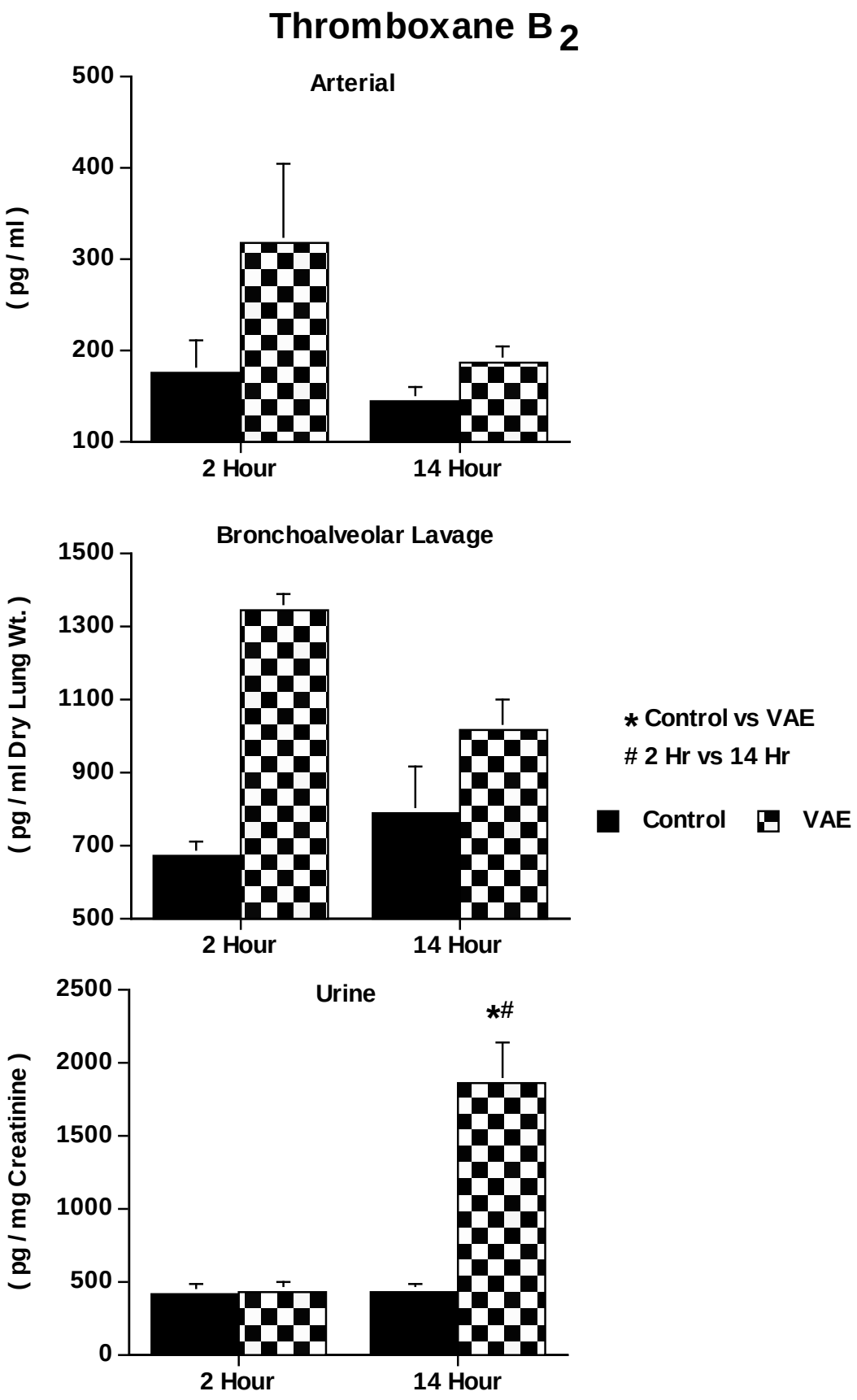

Leukotriene E4
Arterial
(pg / ml)
2000
1800
1600
1400
1200
1000
2 Hour
14 Hour
Bronchoalveolar Lavage
(pg / mg Dry Lung Wt.)
15000
10000
5000
0
*
#
2 Hour
14 Hour
* Control vs VAE
# 2 Hr vs 14 Hr
Control
VAE
Urine
(pg / mg Creatinine)
250
200
150
100
50
*
2 Hour
14 Hour

Occurrences of DCS Symptoms

Venous Air Embolism

Symptoms	HALO (Hrs)	2	14
n		12	12
Tachypnea [1]		9	10
Cyanosis [1]	9	12	
Dyspnea [1]		1	5
Limb paralysis [3]		0	0
Chokes [3]		0	0
Cheyne-Stokes [1]	0	0	
Pulmonary Arterial Gas Emboli [2]		0	0
IVC Gas Emboli [2]	0	0	
Thoracic Aortic Bubbles [3]		0	0
Blebs [1]		2	8
Petechiae [2]		7	4
Hematomas [2]		11	10
Perivascular Cuffing [2]		0	8
Atelectasis [2]		0	0
Alveolar Edema [3]		0	0
Seizure [3]		0	0
Death [4]		0	0
Total Symptom Score		57	79
Weighted Score Based on Severity		4.8	6.6

[ ] Symptom Scale 1-4


Thromboxane B2
Arterial
(pg / ml)
500
400
300
200
100
2 Hour
14 Hour
Bronchoalveolar Lavage
(pg / ml Dry Lung Wt.)
1500
1300
1100
900
700
500
2 Hour
14 Hour
* Control vs VAE
# 2 Hr vs 14 Hr
Control
VAE
Urine
(pg / mg Creatinine)
2500
2000
1500
1000
500
0
*#
2 Hour
14 Hour

DISCUSSION

Decompression-induced venous bubbles can lead to obstruction of blood vessels, alteration in vessel wall permeability and vasoconstriction. Apart from the mechanical obstruction induced by the bubbles, these effects are partially caused by release of arachidonic acid metabolites, such as thromboxanes and leukotrienes, resulting from bubble-activated neutrophils and endothelial cell injury. Circadian rhythm-dependencies in the tolerance to DCS were evident in most of the parameters studied. These observations concur with other studies examining the circadian rhythm-dependencies of inflammatory-like reactions to various stresses. Pharmacological intervention on the effects of inflammatory-like responses to DCS showed reductions, however, the hemodynamic and mechanical effects of the intravascular bubbles may also require specific intervention. It is concluded that DCS risk is affected by the circadian time structure and should be taken into consideration for properly assessing the underlying mechanisms of DCS as well as the efficiency of preventive and interventions strategies.

CRITICAL PATH ROADMAP DISCUSSION

This study addresses the Critical Question contained in the July 30, 2003 Bioastronautics Critical Path Baseline Roadmap Document. Under section 7.11 of Clinical Capabilities, and Risk No. 47, the Critical Question 11.33 states, “What are the strategies to mitigate the risk of Decompression Sickness during all NASA operations (i.e., extravehicular activities from the International Space Station and future habitats beyond LEO, during training activities in underwater facilities, or altitude chambers, during flight using NASA aircraft, and in research designed to prevent decompression sickness?’ The Critical Question priority is ‘1’, meaning it has the highest priority from the Countermeasures/Medical Diagnosis and Treatment Critical Question & Risk Mitigation/CM Category. Since the Time of Day effects on DCS outcome were clearly indicated, such measures might be well suited for further study to determine if operational Extravehicular Activities (EVAs) might be scheduled at those time points where least susceptibility to DCS occurs.

OTHER INFORMATION AND MATERIALS

ABSTRACTS:

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2002 NASA Bioastronautics Investigator's Workshop, Circadian rhythm influences on drug actions in experimental decompression sickness (DCS), Galveston, TX