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14. ABSTRACT In this project, we have developed and verified experimental rat models capable of reproducing physiological responses to extreme environmental conditions, such as simulated high-altitude hypoxia, acute heat stress, and chronic cold stress, comparable to conditions in military relevant scenarios. The major goal of the project was to experimentally test a drug treatment using a heat-shock protein co-inducer (Arimocloamol®; CytRx, Los Angeles, CA) in these rat models of extreme environmental exposures. We measured parameters of metabolic and tissue integrity of vital organs, and tested motor function and cognitive performance in animals exposed to simulated high-altitude hypoxia, acute heat stress or chronic cold					
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Report Title

Improving Warfighters' Sustainment and Performance in Extreme Environmental Conditions

ABSTRACT

In this project, we have developed and verified experimental rat models capable of reproducing physiological responses to extreme environmental conditions, such as simulated high-altitude hypoxia, acute heat stress, and chronic cold stress, comparable to conditions in military relevant scenarios. The major goal of the project was to experimentally test a drug treatment using a heat-shock protein co-inducer (Arimoclomol®; CytRx, Los Angeles, CA) in these rat models of extreme environmental exposures. We measured parameters of metabolic and tissue integrity of vital organs, and tested motor function and cognitive performance in animals exposed to simulated high-altitude hypoxia, acute heat stress or chronic cold stress, and treated either with the drug or vehicle immediately before the stress exposure. Our results demonstrated that the drug, given immediately to the animals before being exposed to the extreme condition: 1) significantly reduced the stress-induced reduction in both motor function and cognitive performance; 2) reduced stress-induced damage (such as oxidative stress and inflammation, among others) in vital organs (heart, liver, kidneys, and brain); and 3) increased the body's tolerance to these extreme environmental conditions. Based on these preliminary results, we suggest continuation of the research in a form of a larger-scope 3-year project, which would include both experimental and clinical studies, to fine-tune the experimental models and validate the beneficial role of the drug in human population.

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- 2) Heat-shock protein co-inducer significantly modifies the simulated high altitude hypoxia-induced pathological changes (J. LaManna & I. Cernak);
- 3) Heat-shock protein co-inducer significantly modifies the chronic cold-induced pathological changes (Z. Sun & I. Cernak).

These manuscripts are prepared but not submitted. Immediately after receiving the Public Release, these manuscripts will be submitted. The ARO will be informed about the journals.

Number of Manuscripts: 3.00

Number of Inventions:

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Jodie Haak	0.05
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Ibolja Cernak	0.13	No
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Names of Under Graduate students supported

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FTE Equivalent:	0.05
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This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

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The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

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The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

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1 a. University of Iowa

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Sub Contractor Numbers (c): 929366

Patent Clause Number (d-1): 52.227-10

Patent Date (d-2): 4/1/1984 12:00:00AM

Work Description (e): Develop a rat model of heat stress, and test the effects of a heat-shock protein co-inducer on physiolo

Sub Contract Award Date (f-1): 7/5/2007 12:00:00AM

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Patent Clause Number (d-1): 52.227-10

Patent Date (d-2): 4/1/1984 12:00:00AM

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Patent Clause Number (d-1): 52.227-10

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Work Description (e): Develop a rat model of simulated high-altitude hypoxia, and test the effects of a heat-shock protein c

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Inventions (DD882)

FINAL REPORT

Improving Warfighters' Sustainment and Performance In Extreme Environmental Conditions (Contract No. W911NF-07-C-0053)

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The Johns Hopkins University Applied Physics Laboratory

Collaborators:

- Case Western University (Cleveland, OH)
- University of Iowa (Iowa City, IO)
- University of Oklahoma Health Sciences Center (Oklahoma City, OK)

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I PROGRAM OVERVIEW

I.1. STATEMENT OF WORK

Notes and Assumptions: This statement of work covers the proposed activities performed as part of the DARPA BAA 07-21. The period of performance is from June 08, 2007 to February 07, 2008.

Objectives: The goal of this effort is to define and experimentally test a treatment that will boost the physiological capabilities of the organism during a very short period of time by inducing heat-shock proteins (HSPs).

Tasks:

1. The JHU/APL designed experiments to test potential protective effects of a heat-shock protein co-inducer, arimoclomol, in extreme heat, cold, and high-altitude hypoxia conditions;
2. The JHU/APL supervised and coordinated the animal testing in the collaborating laboratories (Integrative Physiology Laboratory, Department of Exercise Science, University of Iowa, Iowa City, Iowa; Department of Physiology, University of Oklahoma Health Sciences, Oklahoma City, Oklahoma; and CASE Western School of Medicine Cleveland, Ohio);
3. The JHU/APL integrated the results and prepared the final report with recommendations for further actions (research, clinical utilization, etc.) depending on the experimental findings.

In conducting these tasks, JHU/APL acted under the guidance and at the behest of the government, and made technical recommendations to the government who makes all final acceptance, selection and subsequent procurement decisions. JHU/APL shall have a continuing obligation to inform the government of known problems and issues in development and deployment of systems, and make disclosure of known risks associated with the system's performance and with any of the other activities to be conducted under this task. Deliverables and recommendations shall be provided for government review and approval and provided only for the environments and specified conditions for which they were intended and/or tested. All efforts under this task shall be conducted in accordance with established Laboratory quality assurance policy and procedures.

Deliverables: Monthly follow up reports and a final report on treatment feasibility.

I.2. PROGRAM PROGRESS

Figure 1 demonstrates the milestones, progress, and deliverables of our program.

ID	Task Name	Start	Finish	Duration	Q3 07				Q4 07			Q1 08			
					Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	
1	Award Announced	6/8/2007	6/8/2007	.2w											
2	Finalized Contract	6/21/2007	6/21/2007	.2w											
3	Kick-off Meeting	7/6/2007	7/6/2007	.2w											
4	Animal Protocol Approval Process	7/24/2007	10/12/2007	11.8w											
5	High-altitude Hypoxia Experimental Model Verification	10/15/2007	11/1/2007	2.8w											
6	High-altitude Hypoxia Experiments	11/2/2007	1/21/2008	11.4w											
7	Heat Stress Experimental Model Verification	10/12/2007	11/16/2007	5.2w											
8	Heat Stress Experiments	11/19/2007	1/21/2008	9.2w											
9	Cold Exposure Experimental Model Verification	10/12/2007	11/28/2007	6.8w											
10	Cold Exposure Experiments	11/29/2007	1/21/2008	7.6w											
11	Final Data Analysis	1/22/2008	2/8/2008	2.8w											
12	First (Quarterly) Report	10/23/2007	10/23/2007	.2w											
13	Second (Quarterly) Report	12/21/2007	12/21/2007	.2w											
14	Final Technical Report (DARPA) *	2/19/2008	2/19/2008	.2w											
15	Final Report (Technical & Financial) (ARO & DARPA) *	4/16/2008	4/16/2008	.2w											

Figure 1. Milestones, progress, and deliverables of the program

I.2.1. Kick-off Meeting (July 06, 2007)

The team including the Johns Hopkins University Applied Physics Laboratory (JHU/APL), represented by Dr. Ibolja Cernak: systems integration, project design and supervision; Case Western Reserve University, represented by Dr. Joseph LaManna: high-altitude hypoxia research; University of Iowa, represented by Dr. Kevin Kregel: heat stress/shock study; and the University of Oklahoma, represented by Dr. Zhongjie Sun: cold stress study; had a successful kick-off meeting on July 06, 2007. The experimental design and protocols have been discussed, defined and synchronized in details.

I.2.2. Arimoclomol® (CytRx, Los Angeles, CA) Release

According to the NIH recommendations concerning Investigational Agents, two administrative forms have been developed: 1) a **Transfer Form** acknowledging the amount of the drug transferred from the APL to collaborating research laboratories, signed by the receiving Principal Investigator; and 2) an **Investigational Agent Accountability Form**, which ensures the proper recording of every dispensed dose. The required amount of the drug is released by the CytRx and shipped to the JHU/APL.

I.2.3. Animal Protocol Approval Process

The animal protocols for the program "Improving Warfighters' Sustainment and Performance in Extreme Environmental Conditions" are granted full approval by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rats and will remain so until its expiration or cancellation.

- The protocol concerning the high-altitude hypoxia experiments, approved by the Case Western Reserve University School of Medicine's IUCUC, was accepted by the ACURO on September 20, 2007;
- The protocol concerning the heat stress/shock experiments, approved by the University of Iowa's IACUC, was accepted by the ACURO on October 11; and
- The protocol concerning the cold stress experiments, approved by the University of Oklahoma's IACUC, was accepted by the ACURO in October 12.

1.2.4. Experimental Studies

As suggested by the DARPA Program Manager, Dr. Geoffrey Ling, the experiments are designed as pilot studies; thus six rats per group will be used to analyze different environmental conditions.

1.2.4.1. High-Altitude Hypoxia Study

On October 15, the Program's Systems Integrator, Dr. Ibolja Cernak, traveled to the Case Western University, delivered the projected amount of Arimoclomol[®] to Dr. LaManna, the Principal Investigator of the high-altitude hypoxia study, and ensured that the experimental conditions in the laboratory are in compliance with the JHU/APL's contract and relevant regulations and guidelines. Also, Dr. Cernak supervised the drug solution preparation and first injections. The model verification was completed on November 1, 2007, the high-altitude hypoxia studies commenced on November 2, 2007 and completed on January 21, 2008.

1.2.4.2. Acute Heat Stress Study

On November 19, the Program's Systems Integrator, Dr. Ibolja Cernak, traveled to the University of Iowa, delivered the projected amount of Arimoclomol[®] to Dr. Kregel, the Principal Investigator of the acute heat stress study, and ensured that the experimental conditions in the laboratory are in compliance with the JHU/APL's contract and relevant regulations and guidelines. Also, Dr. Cernak supervised the drug solution preparation and first injections. The model verification was completed on November 16, 2007, the acute heat stress studies started on November 19, 2007 and completed on January 21, 2008.

1.2.4.3. Chronic Cold Stress Study

On November 29, the Program's Systems Integrator, Dr. Ibolja Cernak, traveled to the Oklahoma University Health Science Center, delivered the projected amount of Arimoclomol[®] to Dr. Sun, the Principal Investigator of the chronic cold stress study, and ensured that the experimental conditions in the laboratory are in compliance with the JHU/APL's contract and relevant regulations and guidelines. Also, Dr. Cernak supervised the drug solution preparation and first injections. The model verification was completed on November 28, 2007, the cold stress studies started on November 29, 2007 and completed on January 21, 2008.

1.2.5. Program Finalization

All experiments were concluded on January 21, 2008, and the final data analysis completed on February 8, 2008. The projected date for Technical Final Report delivery is February 19, 2008, whereas the projected date for both Technical and Financial Final Report is April 16, 2008.

I.2.6. Planned-versus-Actual Expenditure

The budget planned for individual laboratories were committed and transferred to the corresponding Universities. The project is finalized and the actual expenditure reached the planned level. Due to incoming invoices, the final financial report will be delivered on April 16, 2008. Please see the Spending Control Report (Appendix I), Incurred Cost Report on the Commitment Basis (Appendix II), and the Incurred Cost Report on the Expenditure Basis (Appendix III), for the period from March 2007 to February 2008.

I.3. FINAL RESULTS

1. We developed and verified experimental rat models capable of reproducing physiological responses to extreme environmental conditions, such as simulated high-altitude hypoxia, acute heat stress, and chronic cold stress, comparable to conditions in military relevant scenarios;
2. We defined and experimentally tested a drug treatment using a heat-shock protein co-inducer (Arimoclomol[®]; CytRx, Los Angeles, CA) in animal models of simulated high-altitude hypoxia, acute heat stress, and chronic cold stress;
3. We measured parameters of metabolic and tissue integrity of vital organs, and tested motor function and cognitive performance in animals exposed to simulated high-altitude hypoxia, acute heat stress, or chronic cold stress, and treated either with the drug or vehicle immediately before the stress exposure;
4. We have shown that the drug, given immediately to the animals before being exposed to simulated high-altitude hypoxia, acute heat stress, or chronic cold stress: 1) significantly reduced the stress-induced reduction in both motor function and cognitive performance; and 2) reduced stress-induced damage (such as oxidative stress and inflammation, among others) in vital organs (heart, liver, kidneys, and brain); and 3) increased the body's tolerance to these extreme environmental conditions;
5. Based on these preliminary results, we suggest continuation of the research in a form of larger-scope 3-year project, which would include **both experimental and clinical studies**. The *experimental studies* would: 1) refine the existing models to address the most vital problems experienced by our Warfighters; 2) provide in-depth analysis of the key mechanisms involved in extreme environment-induced pathobiology; 3) and identify the optimal therapeutic regime dependent on the type of the stress. The aim of the *pilot clinical studies* would be to validate Arimoclomol[®] as a potential therapeutic and/or preventive measure for high altitude hypoxia-, acute heat stress-, or chronic cold stress-induced functional deficits in volunteers exposed to these extreme environmental conditions. The continuous feedback between the basic experimental studies and clinical research will ensure the fastest possible way to final deliverables that could be transferred immediately or in a short period of time into military arena.

II HIGH-ALTITUDE HYPOXIA

(PI Joseph LaManna; Case Western Reserve University)

II.1. INTRODUCTION

The normal function of the brain is entirely dependent on an adequate supply of oxygen for provision of ATP via energy metabolism[1]. The brain response to mild and moderate hypoxia requires acute and chronic adaptive mechanisms, which involve systemic and central metabolic and vascular processes that are mediated by various signaling pathways[1-3]. These responses include increased brain blood flow, increased hematocrit [4], increased cerebral metabolic rate for glucose [5] and the activation of molecular cell survival pathways *via* activation of erythropoietin (EPO), vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1 (HIF-1)[6], glycolytic enzymes and glucose transporters. The increased O₂ delivery through enhanced erythropoiesis, angiogenesis and metabolic adaptations facilitate glycolytic ATP production [6-8]. These mechanisms are continuously active during normal physiological adaptation, and also play a significant role in the protective/restorative, as well as, the pathological responses to oxidative challenges. In this study, we hypothesize that the heat-shock protein (HSP) co-inducer, Arimoclomol[®] (CytRx, Los Angeles, CA) treatment will: 1) improve the tolerance to mild hypoxia (0.5 ATM); 2) shorten the hypoxic-adaptive response time; and 3) improve brain functions, thus functional outcomes, measured as overall physiological response and motor / cognitive functions using behavioral tests and immunocytochemical analyses for heat shock proteins HSP 70, intercellular adhesion molecule-1 ICAM-1, and neuronal cell death in rats subjected to simulated altitude exposure.

II.2. METHODS

II.2.1. *Animal preparation*

Male Sprague-Dawley rats (300-350g) were randomly assigned into 4 groups: 1) rats exposed to hypoxia for 10 days, receiving vehicle (saline, applied intraperitoneally); 2) rats exposed to hypoxia for 10 days, receiving drug (Arimoclomol[®], 200 mg/kg, dissolved in saline, intraperitoneally); 3) rats exposed to normoxic environment, receiving vehicle (saline, applied intraperitoneally); and 4) rats exposed to normoxic environment, receiving drug (Arimoclomol[®], 200 mg/kg, dissolved in saline, intraperitoneally). The corresponding treatments were applied immediately before and over the course of hypoxia exposure.

II.2.2. *Simulated altitude exposure (Hypobaric hypoxic exposure)*

Hypoxic rats were kept in hypobaric chambers for 10 days at a constant pressure of 0.5 ATM (380 mmHg, ~10% O₂), except for a maximum of 3 hours daily when the pressure was returned to atmosphere for cage cleaning, water, food and weighing and behavioral tests. The normoxic control rats were housed in the same room next to the hypobaric chamber to ensure identical ambient conditions[4].

II.2.3. Behavioral tests

All the behavioral tests were performed by the Rodent Behavior Core of Case Western Reserve University. Tests included:

- 1) T- maze test**, performed on 2, 4, and 8 days of exposure). This test is used to establish general cognitive function, and it is based on the innate preference of animals to explore an arm that has not been previously explored (spontaneous alternations). The number of arms entered and the sequence of entries were recorded, and a test score was calculated to determine the alternation rate (degree of arm entries without repetitions) for all groups.
- 2) Object recognition test**, performed on day 9 of exposure. This test is based on the natural tendency of rodents to investigate a novel object instead of a familiar one as well as their innate tendency to re-start exploring when they were presented with a novel environment. The time spent exploring the open field (movement/inactivity), number of exploration, and length of time inspecting the objects over the different trials were calculated in this test.
- 3) Inclined screen test**, performed on 1, 3, 7, and 10 days of exposure. This test was used to test balance, muscle strength and coordination. The test was carried out at a 60 and 90 degree incline levels. Latency to climb to the top of the screen and/or latency to fall were recorded, and an overall score was calculated.
- 4) Adhesive (Sticky-tape) removal test**, performed on 1, 3, 7, and 10 days of exposure. This test was used to measure somatosensory deficits. Two adhesive-backed paper tapes, about 18 mm in diameter were placed bilaterally on the wrist of each forelimb of rat, and the time to remove sticky tapes from both limbs was recorded.

II.2.4. Immunohistochemistry of HSP 70, ICAM-1 and TUNEL staining

At the day 11 post-exposure, rats were deeply anesthetized with 5% pentobarbital, perfused intracardially and perfusion fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and embedded with paraffin and sectioned on a microtome. Coronal sections (6 μ m) at levels of cortex, hippocampus and cerebellum were processed for detection of HSP 70 and ICAM-1, as described previously [9].

Corresponding primary antibodies (e.g. anti-heat-shock-protein-70, Chemicon) were used. TUNEL staining is used to assess apoptotic cell death. The paraffin sections were stained via an in situ technique (terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end-labeling [TUNEL] reaction). The Promega kit was used for the detection of DNA fragmentation and apoptotic cell bodies in tissue. Positive cells were visualized with a fluorescence microscope (Nikon).

II.3. RESULTS

II.3.1. Physiological Variables

Body weights were measured daily for all experimental groups. As seen in Figure 1, the body weights of normoxic rats remained unchanged, while the body weights of normoxic rats decreased for 12-17% at the end of the exposure. When the whole period of exposure is analyzed together, there was no significant difference between the body weight of drug-treated

and the vehicle groups exposed either to normoxic or hypoxic conditions. Nevertheless, if analyzed separately, on day 1 of exposure, the hypoxic rats had significantly lower (t-test, $p < 0.05$) body weights compared to their matched normoxic controls. Consistent with previously reported [4] hypoxia-induced increase in hematocrit, on day 10 of exposure, significantly increased (t-test, $p < 0.05$) hematocrit was found in both drug-treated (35% increase) and vehicle (30% increase) groups, compared to their normoxic controls. There was no significant difference between the drug-treated and the vehicle groups in any of these conditions (Fig. 2).

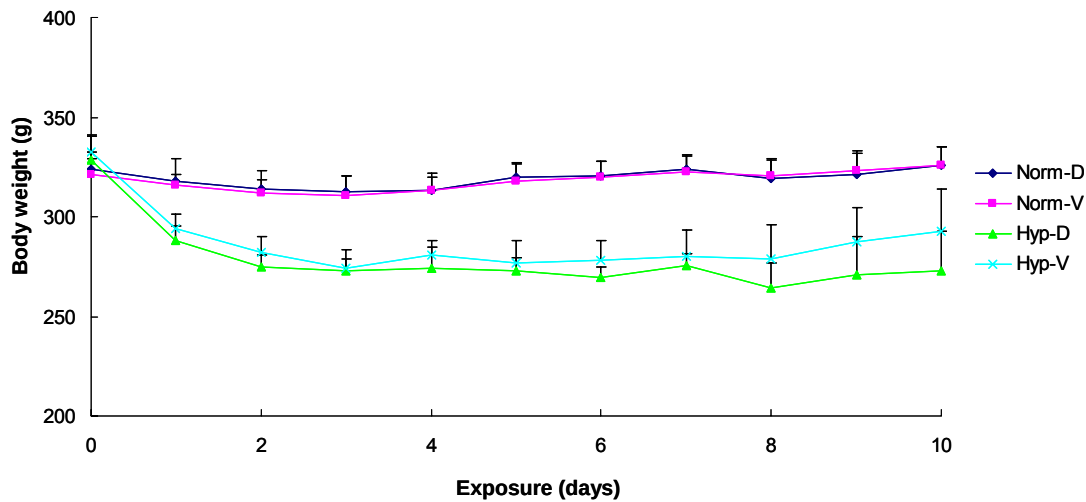


Figure 1: Body weights during 10 days of normoxic or hypoxic exposure. Hyp-D: hypoxic drug-treated group; Hyp-V: hypoxic vehicle group; Norm-D: normoxic drug-treated group; Norm-V: normoxic vehicle group; values are mean $\pm$ SD, $n = 6$ in each group. Hypoxic rats (drug-treated and vehicle groups) had significantly lower body weights compared to their normoxic controls starting from the first of exposure.

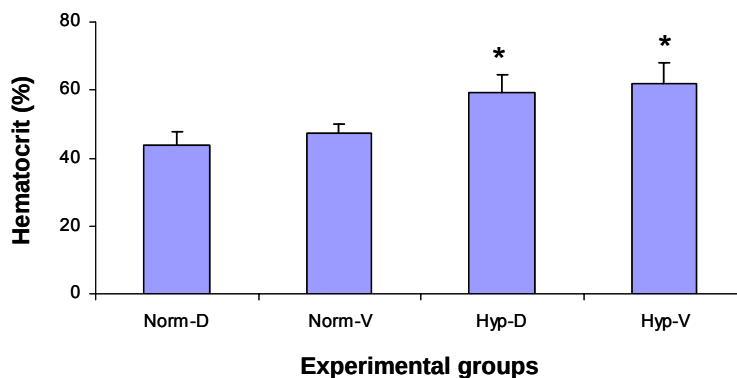


Figure 2: Hematocrit after 10 days of exposure. Hyp-D: hypoxic drug-treated group; Hyp-V: hypoxic vehicle group; Norm-D: normoxic drug-treated group; Norm-V: normoxic vehicle group; values are mean $\pm$ SD, $n = 6$ in each group. * = significantly difference compared to the normoxic control with same treatment condition (t-test, $p < 0.05$).

II.3.2. Behavioral performance

II.3.2.1. Cognitive function

T-maze test:

The T-maze function has been shown a valuable tool to detect hippocampal damage, memory deficits induced by drugs, and effects of gene manipulations involved in cognition as a treatment for neurodegenerative diseases such as AD, among others. A high alternation rate is indicative of sustained cognition as the animals must remember which arm was entered last to not re-enter it. We tested all rats at 2, 4, and 8 days of exposure, and we found that two normoxic groups had similar alteration rates, while the drug-treated hypoxic group had a significantly higher alteration rate compared to the vehicle-treated hypoxic group (see Figure 3).

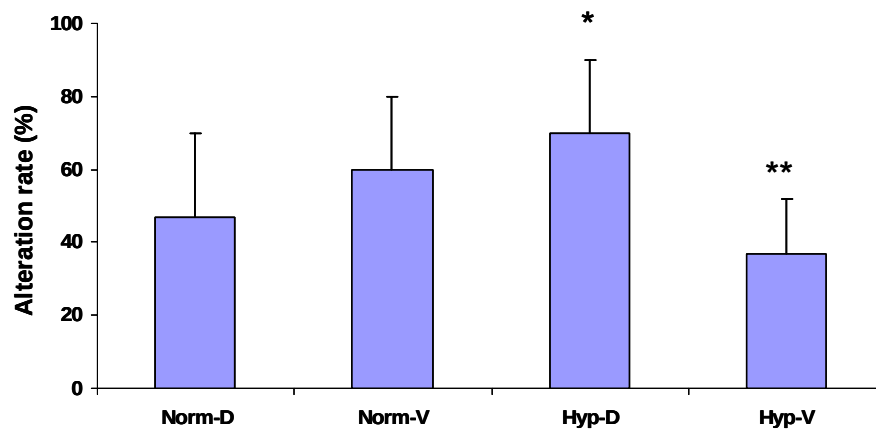


Figure 3: T-maze function (alteration rates) on day 4 of exposure. Hyp-D: hypoxic drug-treated group; Hyp-V: hypoxic vehicle group; Norm-D: normoxic drug-treated group; Norm-V: normoxic vehicle group; values are mean $\pm$ SD, $n = 3, 5, 4, 6$, respectively. *, ** =significantly difference compared to vehicle group under same exposure condition, and normoxic controls with the same treatment, respectively (t-test, $p < 0.05$).

Object Recognition Test

In this test, the choice of an animal to explore a novel object as well as the reactivation of exploration after object displacement reflects the capability to learn and recall previous information (memory). The number of times and length of time for inspecting the objects (old and new) was calculated. The percent of exploration of a new object is higher with good cognitive function. Our data showed that the percent of exploration of a new object is significantly decreased (40%) in the vehicle-treated hypoxic group, compared to its normoxic control group, whereas the drug treatment significantly improved the cognitive function in hypoxic rats (Figure 4).

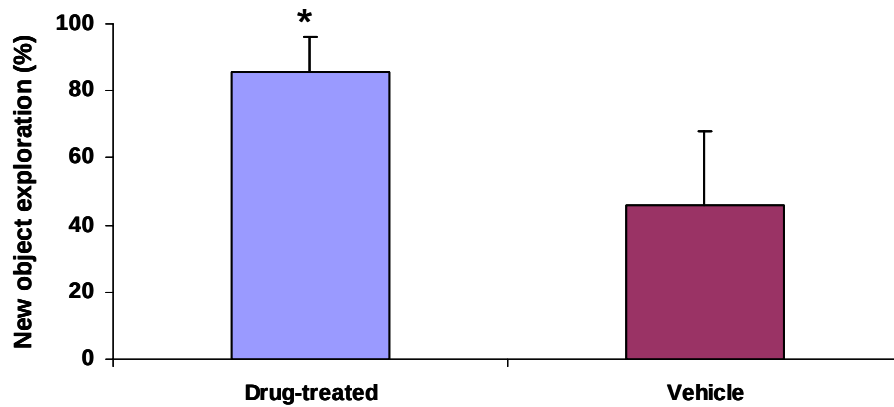


Figure 4: Object recognition function on day 9 of hypoxic exposure. New object exploration (%) = [new object exploration time /total exploration time (both old and new)]*100. Values are expressed as mean $\pm$ SD, n = 6 in each group. * indicates significant difference compared to the vehicle group (t-test, $p < 0.05$).

II.3.2.2. Motor Function

Inclined Screen Test

This test was used to measure balance, muscle strength and coordination. The test was carried out at 60- and 90-degree incline levels for all groups at 1, 3, 7, and 10 days of exposure. Latency to climb to the top of the screen and/or latency to fall was recorded, and an overall score was calculated. For the hypoxic rats, there was a trend of increase in overall score in the drug-treated group (Figure 5).

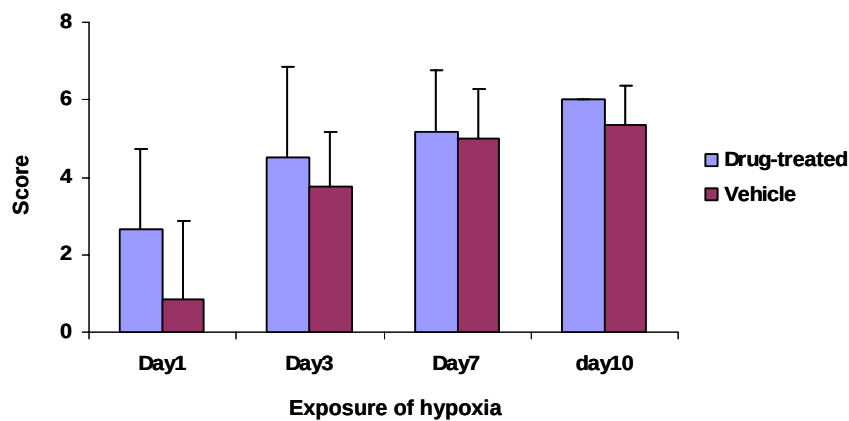


Figure 5: The score of the 90-degree inclined screen test in hypoxic rats. Values are expressed as mean $\pm$ SD, n = 6 in each group. Scoring system: 2 = climb to the top successfully; 1 = fail to climb to the top but stay on the screen; and 0 = fall. The results are averaged for 3 trials per rat; 1 minute per trial.

The Adhesive (sticky) Tape Removal Test

This test was used to measure somatosensory function: the faster to remove the tapes, the better the function. There were no significant differences among the groups at any condition during exposure. However, for the hypoxic rats, the drug-treated rats seemed to have better performance on adhesive removal test, i.e., they used less time to remove the sticky tapes (Figure 6)

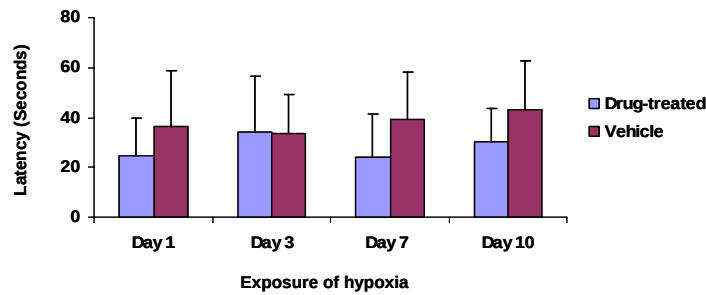


Figure 6: Results of adhesive removal test in the hypoxic rats. Values are expressed as mean $\pm$ SD, n = 6 in each group, three trials per day. On 1, 7, and 10 days of hypoxic exposure, the drug-treated rats used less time to remove the adhesive tapes than the vehicle-treated rats.

II.3.2.3. Immunohistochemistry

HSP 70

HSP 70 was detected in cortex (Figure 7-A), cerebellum (Figure 7-B) and hippocampus (Figure 7-C-1, 7-C-2, and 7-C-3) during hypoxic exposure. In the hypoxic vehicle-treated rats, the positive staining of HSP70 appeared in cortex and cerebellum on day 3 of exposure, and was enhanced on day 10 of exposure. In hippocampus, CA2-3 and CA3 regions had an earlier and stronger response, starting from days 1-3 of exposure) compared to the CA1 region (shown on day 10). Drug treatment resulted in increased HSP70 at 1 day exposure, but attenuated the hypoxic induced increases at 3 and 10 days. The drug treatment seemed to suppress the expressions of HSP70 in all these regions at 10 days.

Figure 7-A: HSP 70 in cortex

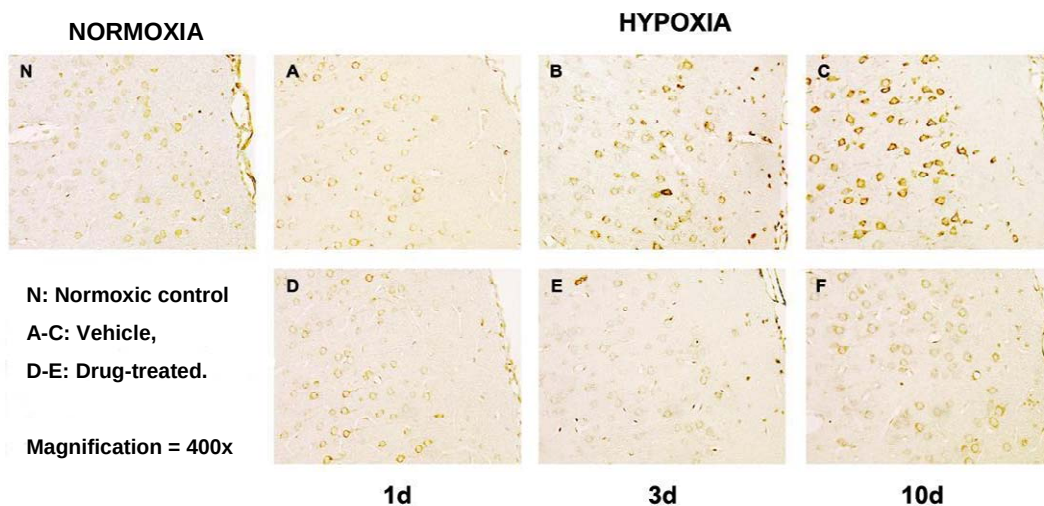


Figure 7-B: HSP 70 in cerebellum

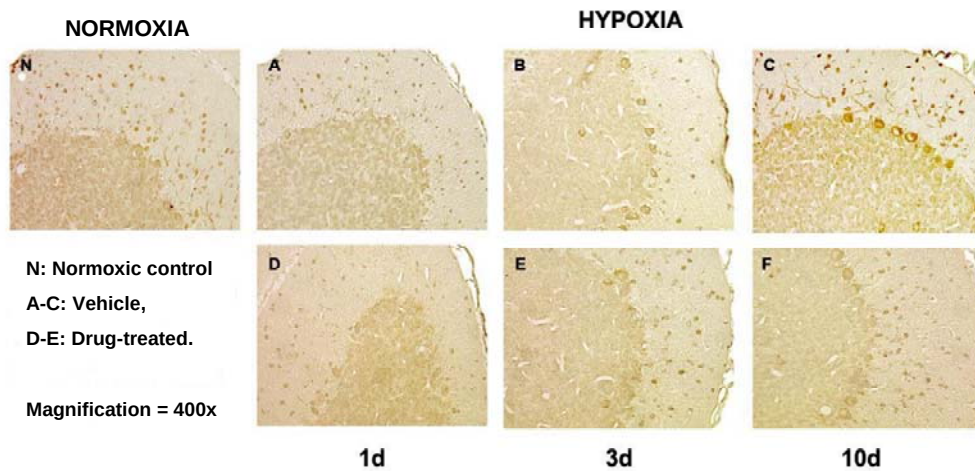


Figure 7-C-1: HSP 70 in CA1 region of hippocampus

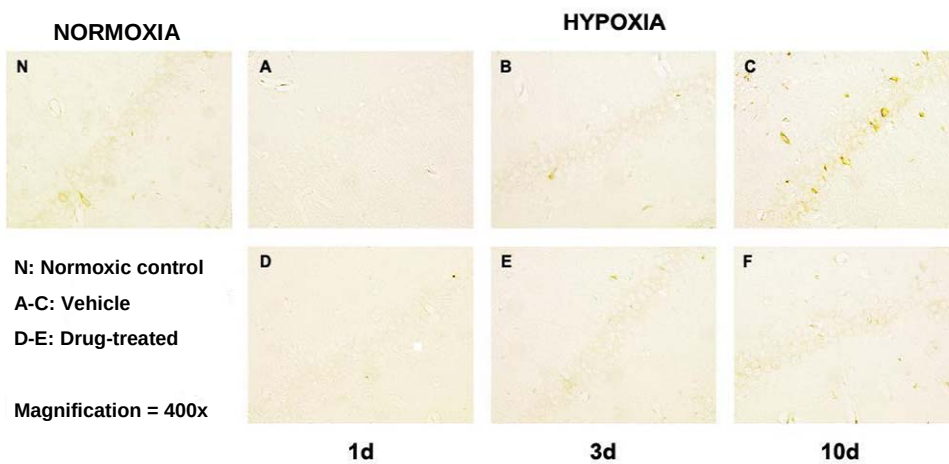


Figure 7-C-2: HSP 70 in CA2-CA3 regions of hippocampus

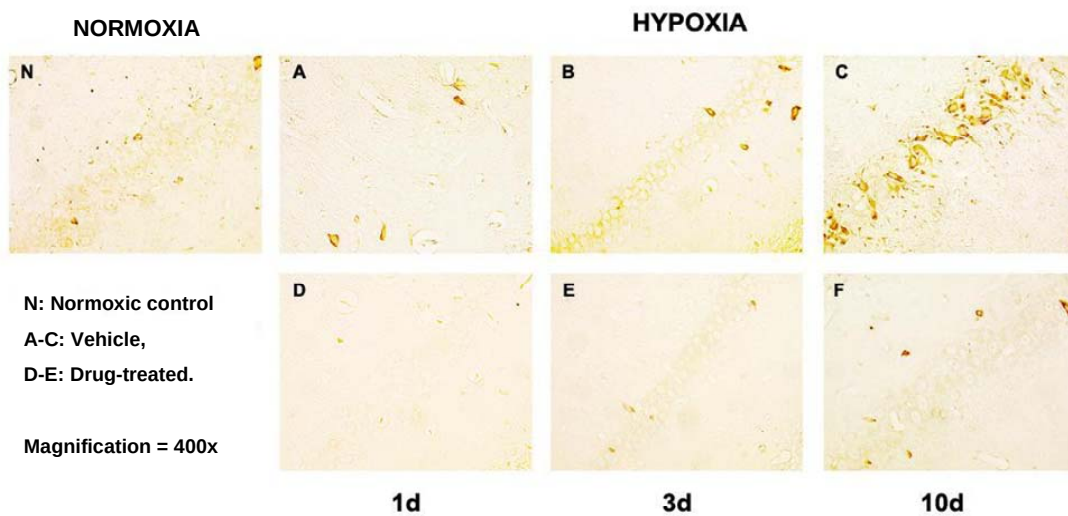


Figure 7-C-3: HSP 70 in CA3 region of hippocampus

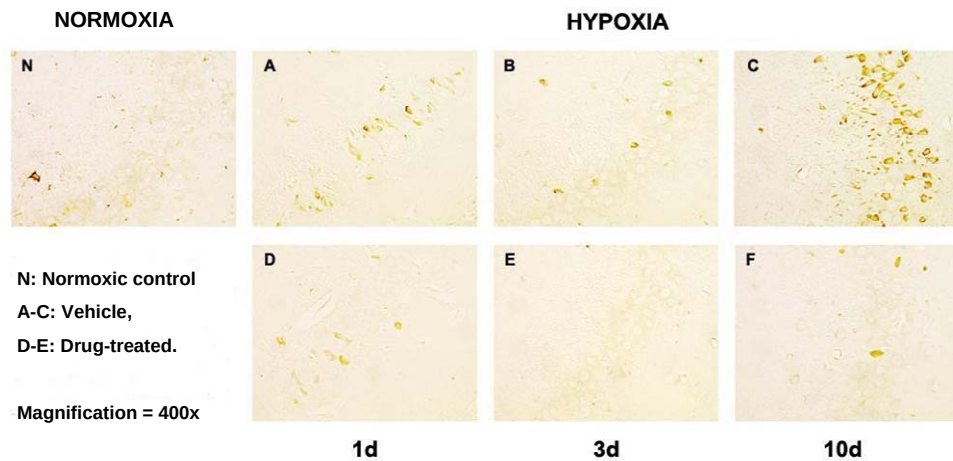


Figure 7: Immunohistochemistry of HSP 70 in cortex (7-A), cerebellum (7-B) and hippocampus (7-C-1, 7-C-2, and 7-C-3) during hypoxic exposure. N: normoxic control; A-C: vehicle; D-F: drug-treated. HSP 70 positive staining started to show at 1 day of hypoxia (e.g. hippocampal CA3 region), and peaked at 10 days in all regions in the vehicle rats. Drug treatment suppressed the HSP 70 expression in all regions.

ICAM-1

Intercellular adhesion molecule-1 (ICAM-1) plays an important role in inflammatory responses. ICAM-1 was detected in cortex on days 1, 3, and 10 of hypoxic exposure (Figure 8). ICAM-1 positive staining, most of it located at the endothelium, was found in the vehicle-treated group exposed to hypoxia, whereas the drug treatment suppressed ICAM-1 expression.

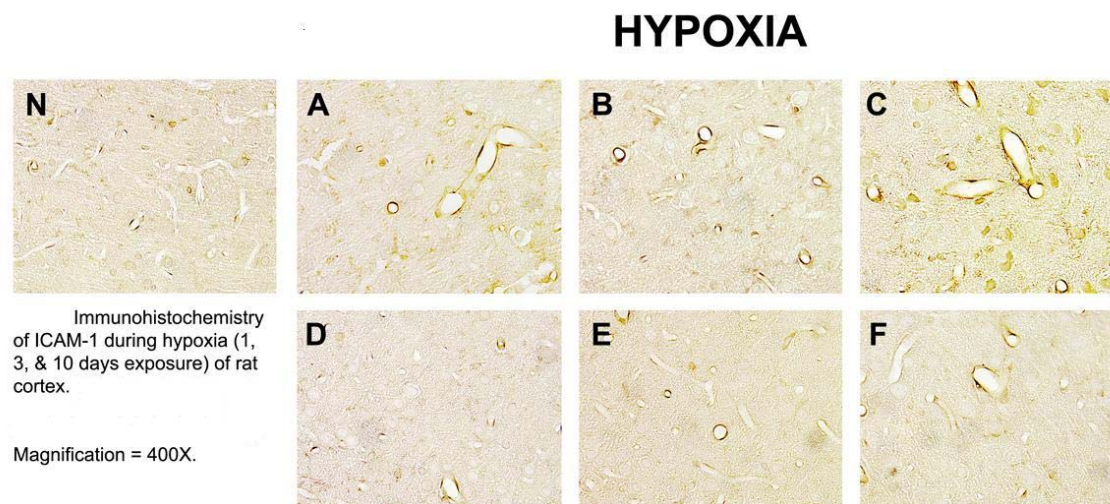


Figure 8: Immunohistochemistry of ICAM-1 in cortex during hypoxic exposure (1, 3, and 10 days). N: normoxic control; A-C: vehicle; D-F: drug-treated; Magnification = 400x. ICAM-1 positive staining mainly located on endothelial cells of vehicle brains (A-C), less positive staining was found in the drug-treated brains (D-F).

TUNEL staining

TUNEL staining was used to evaluate the hypoxia-induced apoptotic cell death. Figure 9 shows TUNEL-positive cells in hippocampal CA3 region in vehicle-treated rat on day 3 of hypoxic exposure (B), whereas drug treatment significantly reduced the number of TUNEL-positive cells (E).

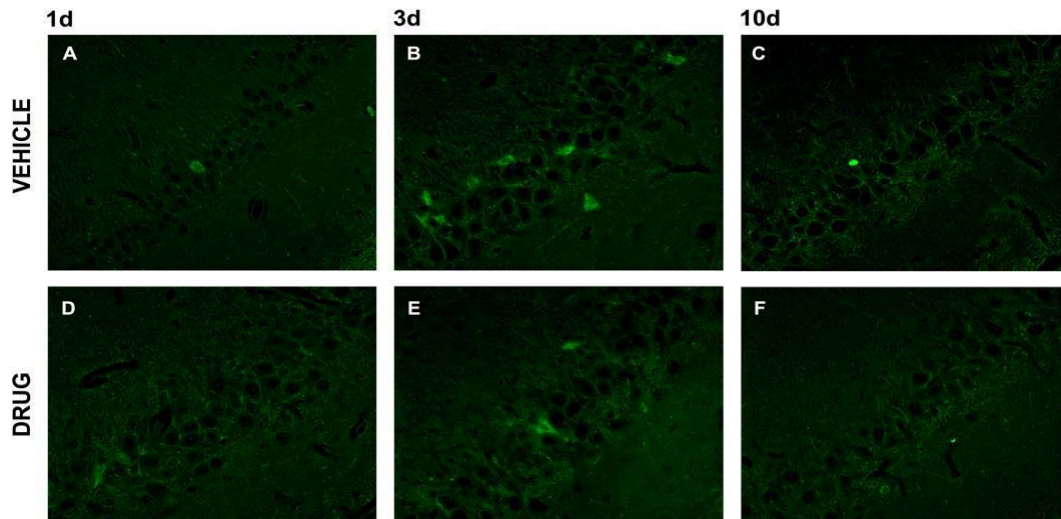


Figure 9: Representative fluorescent TUNEL staining in hippocampal CA3 region during hypoxic exposure (on days 1, 3 and 10 of exposure). A-C; vehicle-treated animal exposed to hypoxia; D-F: drug-treated animal exposed to hypoxia. Magnification = 400x. On day 3 of hypoxic exposure evident TUNEL-positive cells were found in the brain of vehicle-treated animal exposed to hypoxia (B) compared to the drug-treated brain (E).

II.4. CONCLUSIONS

In this study, we investigated the effect of Arimoclomol[®], a HSP co-inducer, on the adaptive response toward hypoxia, using a rat model of simulated high altitude. We found that the treatment: 1) improved overall behavioral performance (both cognitive and motor functions) during hypoxic adaptation; 2) reduced the hypoxia-induced cell death; and 3) suppressed the hypoxia-induced inflammation. Our preliminary data suggests that Arimoclomol[®] reduced the hypoxia-induced stress in brain, and enhanced cerebral tolerance to hypoxia, thus, improving functional outcome.

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II. 6. FUTURE RESEARCH DIRECTIONS

The military routinely conducts missions in extreme conditions such as those at high altitude (HA), which requires the human body to acclimatize to hypobaric hypoxia. The process of acclimatization is often accompanied by headache, nausea and other symptoms, more commonly known as high-altitude illness. This includes acute mountain sickness (AMS), high-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE). The best current treatment option for high-altitude illness is acetazolamide (Diamox). Although it relieves the headache symptoms of AMS, it is an overall unsatisfactory approach due to its associated side effects (e.g. tingling of extremities). The central nervous system, especially the brain, is physiologically vulnerable to hypoxia due to its high demand for oxygen. The physiological and pathological changes induced by HA lead to physical and mental dysfunction and reduced operational readiness. Some abnormalities may still persist even after descent to a normoxic environment. There are adaptive mechanisms during hypoxic exposure that control oxygen delivery to maintain coupling with tissue oxygen consumption for normal brain function. However, the mechanisms of deadaptation responses are less understood and remain unclear. **Thus, we propose a study using a model of simulated altitude exposure in rat to investigate both adaptation and deadaptation responses to hypoxia.** Moreover, parallel with our experimental studies, we propose a clinical study including volunteers exposed to high-altitude hypoxia, and analyze the effects of Arimoclomol[®] on their physiological and behavioral performance.

II.6.1. Rationale

Brain adaptive responses to hypoxia include an acute transient increase in blood flow, increased packed red cell volume (hematocrit), and activation of hypoxia-inducible factor-1 (HIF-1). Chronic responses involve systemic, central metabolic and vascular processes such as brain angiogenesis. Reoxygenation during deadaptation may cause oxidative stress to the brain, which is a consequence of returning to a normal atmosphere from HA environments. Heat shock proteins (HSPs) protect cells from a wide variety of physiological and pathological stressors through cytoprotective properties related to anti-apoptotic and anti-oxidant defenses. Thus, activation of HSPs is critical for adaptation to hypoxia and for enduring oxidative stress associated with reoxygenation. Inducing HSPs may be a therapeutic approach to improve tolerance to HA.

II.6.2. Hypothesis

We hypothesize that an HSP co-inducer (Arimoclomol[®]; CytRx, Los Angeles, CA) treatment will prevent or attenuate cellular injury during hypoxic adaptation and deadaptation. Therefore, treatment will result in an improved tolerance to hypoxia and shorten hypoxic-adaptive response time and improve the Warfighter's performance. Behavioral function (motor/cognitive) analysis will be used to assess the functional outcome, while immunocytochemical analysis will be used to assess neuronal cell death, neurological

inflammation, and heat shock proteins. Physiological response will also be evaluated.

II.6.3. Specific Aims

II.6.3.1. Specific Aim #1

To investigate the effect of an HSP co-inducer treatment strategy on the adaptive response to mild (0.5 ATP, 10% O₂) and moderate hypoxia (0.4 ATM, 8% O₂). 8% hypoxic exposure is used to increase the severity of the insult and counted for species difference.

Hypothesis: Inducing heat shock proteins by HSP co-inducers reduces hypoxia-induced stress and neurodegeneration. This will result in a shortened hypoxic adaptation time during mild and moderate hypoxic exposure and reduced tissue stress through activation of HIF alpha and HSP pathways.

II.6.3.2. Specific Aim #2.

To investigate the effect of HSP co-inducer treatment on the recovery following 3-week period of mild or moderate hypoxia.

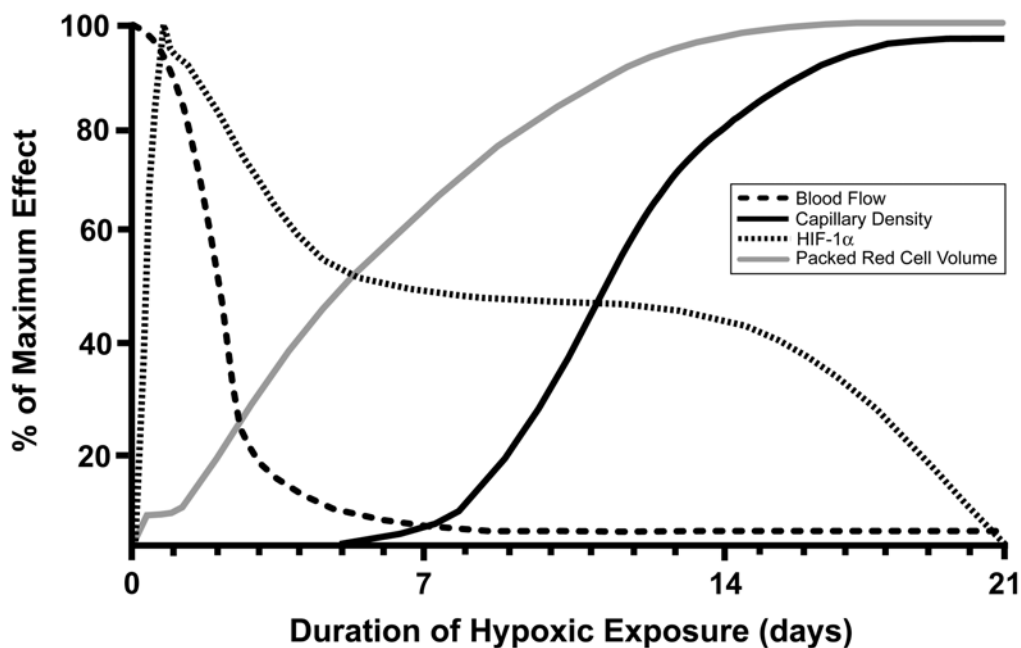
Hypothesis: Inducing heat shock proteins using a HSP co-inducer improves recovery following mild or moderate hypoxic exposure through reducing the oxidative stress as a result of reoxygenation during deadaptation.

II.6.4. BACKGROUND AND SIGNIFICANCE

Military personnel routinely conduct missions at high altitude (HA), such as the U.S. military in Afghanistan, and sometimes need be deployed at an altitude of approximately 11,000 feet (3,355m) and above[1]. Frequently these missions can last days to weeks. Travel to a high altitude requires human body acclimatization to hypobaric hypoxia. Failure to acclimatize results in three common, but preventable, maladies known collectively as high-altitude illness: acute mountain sickness (AMS), high-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE)[2]. About 20% of those ascending about 9,000 feet have experienced high altitude illness and the percent continues to rise with increased elevations[1]. The physiological responses to HA include decreased physical and mental performance. Many measurable behavioral functions are impaired at various altitudes. For example, the memory test results showed a decline at 8,000 feet (2,440m), with a more pronounced decrease at above 12,000 feet (3,660m). It has been recognized that sensory impairment starts at an altitude of about 5,000 feet (1,525 m), mental impairment at 12,000 feet, and impairment of motor function above 16,000 feet (4,880 m). Yet, some abnormalities may still persist after descent to normoxic environment[3].

The brain is a very high energy consumer and is completely reliant on oxygen for oxidative energy metabolism[4]. Yet, oxygen excess can become toxic[5]. There are mechanisms which allow the brain to maintain its function under low oxygen conditions (hypoxia)[6]. The brain response to hypoxia requires acute and chronic adaptive mechanisms, which involve systemic, central metabolic, and vascular processes mediated by various signaling pathways[7-9]. The acute responses include increased ventilation, blood flow, packed red cell

volume (hematocrit)[10], cerebral glucose metabolic rate [11], and the activation of various molecular cell survival pathways (e.g. HIF-1 activation)[12-14]. If the hypoxia persists, the angiogenesis will develop[7;15]. One of the main effects of HIF-1 activation is to mediate hypoxia-induced angiogenesis resulting in increased capillary branching and density. This decreases intercapillary and diffusion distances, restoring tissue oxygen tensions to baseline[8;16]. Acclimatization to mild hypoxia (0.5 ATM, equivalent to 10% normobaric oxygen, or an altitude of ~5,000 m) through natural physiological responses can take up to three weeks before complete adaptation (normalization of local tissue PO_2). The relative time course of blood flow, capillary density and HIF-1 in brain are shown in the scheme (SCHEME 1). Blood flow initially responds rapidly (minutes), then returns to baseline by the fourth or fifth day[10]. Hematocrit has begun to increase by day 3, reaching about 80% of maximum by 7 days[10]. Angiogenesis begins at about 1 week and completed within 3 weeks of exposure onset. HIF-1 α , indicative of tissue hypoxia, is initially elevated, falling to about half after 4 days and then back to baseline by 3 weeks[12]. The mechanisms of deadaptation responses during recovery are less understood. It's not clear how brain blood flow changes during reoxygenation after hypoxic exposure. The hematocrit and capillary density remain elevated for about the first 2 weeks of recovery and then return to normoxic baseline in about 3 weeks[16]. Apoptotic response has been shown in rat brain during recovery [16], suggesting that reoxygenation during deadaptation may result in oxidative stress in the brain.



SCHEME 1: The relative time courses of responses of brain blood flow, capillary density and HIF-1 alpha during hypoxic exposure. Blood flow responds rapidly and reaches its maximum in minutes; then returns to baseline by fourth or fifth day. Packed red blood cell volume has begun to increase by day 3, reaching about 80% of maximum by 7 days. Angiogenesis starts at about 1 week and completed within 3 weeks of exposure. HIF-1 α , is initially elevated, falling to about half after 4 days and then back to baseline by 3 weeks.

High-altitude illnesses, including AMA, HACE and HAPE, are often accompanying the process of acclimatization. Capillary leakage in the brain (AMS/HACE) or lungs (HAPE) accounts for these syndromes[2]. High altitude exposure increases oxidative stress as indicated by increased parameters of lipid peroxidation and DNA damage in young men[17]. One of the major pathophysiological mechanisms underlying HA-induced oxidative damage is the elevated metabolic demand that heightens mitochondrial free radical production, which in turn induces cycles of relative ischemia and reperfusion leading to bursts of oxidative activity[18]. Increased free radical production in HA has been suggested to cause decreased physical performance, implying that scavenging free radicals with supplemental antioxidants (AO) may improve exercise performance at altitude[19].

Activation of heat shock proteins (HSPs) is critical to adaptation to low oxygen levels (hypoxia) and for enduring the oxidative stress of reoxygenation[20]. HSPs prevent protein aggregation and restore denatured proteins to their native state. Moreover, the cytoprotective role of HSP includes a direct stabilization of macromolecular structure of proteins and lipids and structure of mRNA, as well as direct support of anti-apoptotic signaling molecules and anti-oxidant defense. Chaperone induction by various types of harmful insults is mediated at the transcriptional level by an autoregulatory feedback loop that involves release of HSF-1 from the repressing HSP90/HSP70/HSP40 complex and a subsequent activation of heat shock gene transcription[21]. It has been found that there is an evolutionally conserved altitudinal differentiation in altitude-related stress, suggesting that the expression level of the heat-shock protein 70 (HSP 70) can be directly related to altitudinal clines of heat-stress resistance[22]. Interestingly, experimental studies demonstrated the increased resistance of rat heart to ischemia/reperfusion injury after intermittent hypoxia exposure is directly related to the HSP 70 induction[23]. Because HSPs protect the cells from a wide variety of physiological and pathological stressors, inducing HSPs may be a therapeutic approach to improve tolerance to HA[24].

On a short term basis (less than three weeks of hypoxia), such as during conditions of warfare, we hypothesize that induction of HSPs using HSP co-inducers would provide improved performance at high altitude by increasing tissue resistance toward hypobaric hypoxia. This study will focus on brain function using behavioral, physiological, and immunocytochemical analyses (neuronal cell death, neurological inflammation and heat shock proteins) in a rodent model of simulated altitude exposure. The analyses of this study will provide an understanding of the role of heat shock proteins in the activation of hypoxic-adaptation, as well as for regulating cell survival pathways. The goal of this effort is to define and experimentally test a treatment that will boost the physiological capabilities of the organism during a very short period of time by inducing heat-shock proteins.

II.6.5. RESEARCH DESIGN

II.6.5.1. *Specific Aim #1.*

To investigate the effect of an HSP co-inducer treatment strategy on the adaptive response to mild (0.5 ATP, 10% O₂) and moderate hypoxia (0.4 ATM, 8% O₂). 8% hypoxic exposure is used to increase the severity of the insult and counted for species difference.

Hypothesis: Inducing heat shock proteins by HSP co-inducers reduces hypoxia-induced stress and neurodegeneration. This will result in a shortened hypoxic adaptation time during mild and moderate hypoxic exposure and reduced tissue stress through activation of HIF alpha and HSP pathways

Rationale: Acute brain adaptive responses to hypoxia include a transient increase in blood flow, an increase in hematocrit, whereas chronic responses involve the activation of hypoxia-inducible factor-1, which mediates hypoxia-induced angiogenesis. HSPs are protective against hypoxic-induced stress, therefore, induction of HSP 70 using HSP co-inducers shortens adaptive responses to hypoxia, reduces pathological responses such as expression of stress proteins and apoptotic cell death, and thus, improves overall behavioral performance.

Questions to be addressed: 1) Does HSP co-inducer treatment affect renormalization of brain blood flow and hematocrit during hypoxia? 2) Does the treatment result in reduced tissue stress and apoptosis? 3) Does HSP co-inducer treatment result in higher capillary density? What are its effects on HIF-1 α and HSP 70? 4) Does the treatment improve the overall physiological responses and behavioral outcome?

Experiment #1-1: To determine acute adaptive responses related to brain blood flow and hematocrit changes during acute hypoxia. Brain blood flows (on 3 h, and 1, 3, 5 days of exposure, n = 6 for each group) will be measured; and changes of brain blood flow will be correlated to hematocrit change.

Experiment #1-2: To determine the expressions of hypoxia-induced stress proteins and apoptosis during hypoxic exposure. Expressions of HSP70, ICAM-1 will be detected using immunohistochemistry at 1, 3, 10 and 21 days of exposure (n = 3 for each group). Apoptotic cell death is determined by TUNEL staining and caspase-3 immunohistochemistry.

Experiment #1-3: To measure capillary density and determine the changes of HIF alpha and HSP 70 during hypoxic exposure. Capillary density were assessed using by GLUT-1 immunohistochemistry at 14 and 21 days of exposure; tissue content of HIF 1 alpha and HSP 70 proteins will be analyzed by western blot analyses at 1, 3, 7 days of exposure (n = 4 for each group).

Experiment #1-4: To test behavioral performance during hypoxic exposure. Motor function will be tested on before and 1, 3, 5, and 20 days of exposure; cognitive function will be tested

on before and 2, 4, 6, and 21 days of exposure (n= 12 for each group).

Minimum number of rats will be used in Experiment #1 (n = 360):

	Blood flow	Immunohistochemistry	Western blot	Behavioral tests
Normoxic control	48	24	24	24
10% O₂	48	24	24	24
8%	48	24	24	24

II.6.5.2. Specific Aim #2.

To investigate the effect of HSP co-inducer treatment on the recovery following 3-week period of mild or moderate hypoxia.

Hypothesis: Inducing heat shock proteins using a HSP co-inducer improves recovery following mild or moderate hypoxic exposure through reducing the oxidative stress as a result of reoxygenation during deadaptation.

Rationale: In circumstances of returning to normal atmosphere from hypoxic environment after successful adaptation, the brain tissue is most likely under hyperoxic exposure since the hematocrit and capillary density remain elevated during early phase of deadaptation. Reoxygenation during deadaptation may result in oxidative stress to the brain. Induction of HSP 70 using HSP co-inducer reduces oxidative stress during deadaptation and improves behavioral performance.

Questions to be addressed: 1) Does HSP co-inducer treatment affect changes in brain blood flow and hematocrit during recovery? 2) Does the treatment result in less stress in brain tissue and reduced neurodegeneration, e.g. apoptotic cell death? 3) What is the effect of HSP co-inducer treatment on HIF-1 α , HSP 70, and capillary density during recovery? 4) Does the treatment improve the overall physiological responses and behavioral outcome?

Experiment #2-1: To determine acute deadaptive responses related to brain blood flow and hematocrit changes during recovery. Brain blood flows will be measured on 0, and 1, 7, 14 days of recovery following 3-week exposure (n = 6 for each group); and changes of blood flows will be correlated to hematocrit change.

Experiment #2-2: To determine the expressions of hypoxia-induced stress proteins and apoptosis during recovery. Expressions of HSP70, ICAM-1 will be detected using immunohistochemistry at 1, 3, 10 and 21 days of recovery (n = 3 for each group). Apoptotic cell death is determined by TUNEL staining and caspase-3 immunohistochemistry.

Experiment #2-3: To measure capillary density and determine the changes of HIF alpha and HSP 70 during recovery. Capillary density were assessed using by GLUT-1

immunohistochemistry at 14 and 21 days of recovery; tissue content of HIF 1 alpha and HSP 70 proteins will be analyzed by western blot analyses at 1, 3, 7 days of recovery (n = 4 for each group).

Experiment #2-4: To test behavioral performance during recovery. Motor function will be tested on before and 1, 3, 5, and 20 days of recovery; cognitive function will be tested on before and 2, 4, 6, and 21 days of recovery (n= 12 for each group).

Minimum number of rats will be used in Experiment #2 (n = 360): same as the number of rats will be used in experiment #1.

II.6.5.3. Specific Aim #3.

Perform a clinical study including volunteers exposed to high-altitude hypoxia, and analyze the effects of Arimocloamol[®] on their physiological and behavioral performance.

II.6.6. METHODS

II.6.6.1. Animal preparation

Adult male Sprague-Dawley rats (250-350g) are purchased and allowed to acclimate in the CWRU animal facility for one week before used in experiments. All rats are housed two-three in a cage, maintained on a 12:12 light-dark cycle with standard rat chow and water available *ad libitum*. Rats will be randomly assigned to 6 groups, four groups of rats exposed to hypoxic exposure (10% O₂ and 8% O₂, respectively,) drug-treated and vehicle groups; and two groups of rats exposed to normoxic environment, drug-treated and vehicle-treated groups. All drug-treated rats are administered Arimocloamol[®] (200 mg/kg, dissolved in normal saline) intraperitoneally daily for the expected experimental period; all vehicle-treated rats are given same dose of saline instead.

II.6.6.2. Simulated altitude exposure (Hypobaric hypoxic exposure) [4]

Hypoxic rats are kept in hypobaric chambers for up to 21 days at a constant pressure of 0.5 ATM (380 mmHg, ~10% O₂) for mild hypoxia and 0.4 ATM (300 mmHg, ~8% O₂) for moderate hypoxia. The pressure of the chamber is allowed to return to atmosphere for maximum of 3 hours daily for the purpose of cage cleaning, water, food and weighing and behavioral tests. The normoxic control rats will be housed in the same room next to the hypobaric chamber to ensure identical ambient conditions.

II.6.6.3. Brain blood flow measurement [10]

Anesthesia is induced by 3% isoflurane in air, rat is intubated orotracheally with a 14-gauge catheter, which is attached to a rodent ventilator (20-25% O₂ for the normoxic and 8 or 10% O₂ for the hypoxic rats; tidal volume: 10cc/kg; respiratory rate: 70 breaths/min). Cannulae are placed in: (i) femoral artery using polyethylene tubing (PE-50, 0.023" i.d., 0.038" o.d.) for the purpose of monitoring of systemic arterial blood pressure and obtaining blood samples (ii) an external jugular vein into the right atrium using a Silastic catheter (0.025" i.d., 0.047" o.d.) for administration of drug and indicator substances. Regional brain blood flows are measured by

using a single pass n-[¹⁴C]-butanol uptake method[25]. A bolus (0.15ml) containing n-[¹⁴C]butanol (10 µCi; 1.0 mCi/mL, American Radiolabeled Chemicals, St. Louis, MO) plus normal saline is injected in the jugular vein catheter three seconds after a femoral artery withdrawal pump (1.6 ml /min) is started. The animal is decapitated and the pump is stopped simultaneously ten seconds after bolus injection. The brain is removed rapidly and bilateral samples (frontal and parietal cortex, hippocampus, brainstem and cerebellum) and withdrawn arterial blood are weighed and solubilized in pre-weighed scintillation vials. Aliquots of arterial blood and brain samples are measured for their radioactive contents on a β-scintillation counter (1066 TR Liquid Scintillation Analyzer, Packard). Blood flow (BF, ml/g/min) is calculated from: $BF = (F_s \times [^{14}C]_{tissue}) / ([^{14}C]_{arterial} \times \text{tissue weight})$, where F_s is the calibrated withdrawal rate of the syringe (ml/min); [¹⁴C]_{tissue} and [¹⁴C]_{arterial} are the radioactivity (dpm) of the brain sample and the withdrawn arterial blood.

II.6.6.4. Motor and Cognitive Tests

All the behavioral tests will be performed by the Rodent Behavior Core of Case Western Reserve University, see website (<http://neurosciences.case.edu/crbc/index>). For the exposure phase, the animals will be tested during hypoxic or normoxic exposure (motor function on 1, 3, 7, and 20days of exposure; and cognitive function on day 2, 4, 8 and 21 days of exposure), same time tests will be performed at the same time points for the recovery phase. All hypoxic rats will be tested in a hypoxic chamber (10% O₂ or 8% O₂, oxygen concentration is controlled by the proportion of nitrogen and oxygen). Tests include:

1) Rotarod: This is the most widely used test to assess sensorimotor coordination and motor overall motor function in rodents. This test is affected by experimental damage to the basal ganglia and cerebellum as well as genetic manipulations and drugs that effect motor function. Rats are placed on a rotating rod in which the speed at which the rod rotates is gradually increased. Latency of the animal to fall of the rotating rod is recorded. This test can be carried out as a single measure or multiple times to determine cerebellar learning.

2) Inclined screen test: This test is used to test balance, muscle strength and coordination. The test is carried out at a 60 and 90 degree incline. Latency to climb to the top of the screen and/or latency to fall is recorded, and overall score is calculated.

3) Adhesive (Sticky-tape) removal test: This test is used to measure somatosensory deficits. Two adhesive-backed paper tapes, about 18mm in diameter are placed bilaterally on the wrist of each forelimb of rat, and the time to remove sticky tapes from both limbs is recorded.

4) T- maze test: This test is used for general cognitive function. This test is based on the innate preference of animals to explore an arm that has not been previously explored (spontaneous alternations); the number of arms entered as well as the sequence of entries is recorded and a score is calculated to determine alternation rate (degree of arm entries without repetitions).

5) Object recognition test: This test is based on the natural tendency of rodents to investigate

a novel object instead of a familiar one as well as their innate tendency to re-start exploring when they are presented with a novel environment. The time spent exploring the open field (movement/inactivity) as well as number of times and length of time inspecting the objects over the different trials is calculated.

II.6.6.5. Immunohistochemistry and TUNEL staining [26;27]

Rats are deeply anesthetized with 5% pentobarbital, perfused intracardially and perfusion fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain is removed and embedded with paraffin and sectioned on a microtome at levels of cortex, hippocampus and cerebellum. Coronal serial sections (6µm) are deparaffinized, hydrated and subjected to antigen retrieval at 90°C for 15 min using a Target Retrieval Solution (Dako). Sections are incubated with 3% H₂O₂ in PBS and ~5% blocking serum for 2 h and are exposed with corresponding primary antibodies (e.g. anti-heat-shock-protein-70, anti-ICAM-1, Chemicon), for overnight at 4 °C. Sections are then exposed to appropriate biotinylated IgG secondary antibody (1:200, Vector) for 1 h and incubated with avidin-biotin horseradish peroxidase solution (ABC kit, Vector Laboratories) for 30min. Staining is visualized by using 0.025% 3,3'-diaminobenzidine (DAB, Sigma) and 0.075% H₂O₂ in PBS. For active caspase-3 immunohistochemistry, sections are incubated with a 1:200 dilution of the primary antibody (Cell Signaling Tech) and then incubated with the Oregon green-conjugated secondary antibody. This antibody properly recognized the large fragment of activated caspase-3 (~17 kDa) by Western blot analysis of 5-day-old rat cerebellum. TUNEL staining is used to assess apoptotic cell death. The paraffin sections are stained via an in situ technique (terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end-labeling [TUNEL] reaction). This method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA and ensuing synthesis of a polydeoxynucleotide polymer using a fluorescent nucleotide. The Promega kit will be used for the detection of DNA fragmentation and apoptotic cell bodies in tissue. The slides are pre-equilibrated with the reaction buffer and incubated with the nucleotide mixture and TdT Enzyme at 37°C for 1 hour. The reaction is stopped by washing the sections with 2X SSC. Negative controls will be performed using water instead of TdT Enzyme. Positive cells are visualized with a fluorescence microscope (Nikon).

II.6.6.6. Capillary density [26;27]

Cerebral capillaries are identified by GLUT-1 immunostaining and their density quantified by counting the number of positive capillaries per unit area. Immunohistochemical staining for GLUT-1 is performed (see method above) for assessing brain microvascular density. Three sections (6 µm thick) cut 150 µm apart corresponding approximately to *plates 10-14* in a rat brain atlas (Paxinos and Watson) are stained using a goat polyclonal anti-GLUT-1 antibody (1:200, Santa Cruz) and a biotinylated secondary antibody (Vector Lab). Color detection is carried out with the use of avidin-biotin horseradish peroxidase solution and the diaminobenzidine peroxidase substrate kit (Vector Lab). Images spanning the full depth of the parietal cortex are digitized with a SPOT digital camera connected to a Nikon E600 Eclipse microscope with a ×20 objective. A computer-aided image analysis system (ImageProPlus) is used to determine the number of GLUT-1-positive capillary profiles per unit area that are < 20

µm in diameter.

II.6.6.7. Western Blot Analyses

Western blot analysis is used to detect HSP70 and HIF-1α proteins. Rats are deeply anesthetized and decapitated; brains are removed and dissected. Samples of cortex, hippocampus and cerebellum are prepared by buffer containing 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl. Samples (100µg of protein) are electrophoresed on 10% SDS-polyacrilamide gels. The proteins on the gels are transferred to nitrocellulose membranes then incubated with 5% skim milk blocking buffer for 1 hour (room temperature). Specific proteins are detected by incubating the membranes with a 1:500 dilution of polyclonal corresponding primary antibodies (e.g. anti-heat-shock-protein-70, Chemicon) overnight (4 °C) followed by incubation with horseradish peroxidase- conjugated anti-rabbit IgG (1:5000) for 1 hour (Jackson ImmunoResearch). The primary antibody immunoreactive protein bands are visualized using enhanced chemiluminescence detection system (ECL kit, Amersham).

II.6.6.8. Statistical Methods

All values are presented as mean ± SD. Statistical analyses are performed using SPSS v13.0 for Windows. Group comparisons are made by one-way analysis of variance (ANOVA) using Tukey's statistic or t-test. Significance is considered at the level of $p < 0.05$.

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III ACUTE HEAT STRESS

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III.1. INTRODUCTION

Acute exposure to environmental extremes (e.g., high ambient temperatures) or conditions that significantly raise core body temperature (e.g., exertion, specialized protective clothing, transport of heavy equipment, placement in confined spaces) can have serious health consequences and negatively impact a Warfighter's performance. The goal of these preliminary studies was to assess whether pharmacological induction of heat shock proteins (HSPs) could be beneficial to an organism in coping with extreme heat exposure.

It is well known that heat-shock protein (HSP) induction is an important cellular mechanism of adaptation in organisms that are undergoing stress or extreme environmental conditions. Importantly, this induction can occur over a very rapid time frame (i.e., within a few hours) and expression of these proteins can prevent cellular damage from environmental extremes in a variety of ways. For instance, HSPs act as molecular chaperones by preventing aggregation of misfolded proteins and by restoring denatured proteins to their native state. They also act to directly stabilize macromolecular structures of various proteins and lipids, as well as the structures of mRNA. Preconditioning of subjects by sub-lethal stress has been shown to cause intracellular induction and accumulation of HSPs, which subsequently protects a subject from injury or death during future exposure.

We used a rat model of acute heat stress exposure that closely mimics the conditions encountered by military personnel who conduct operations in a field setting (e.g., hot ambient conditions, soldiers who are not heat acclimatized). The aim of these pilot studies was to determine the potential protective effects of utilizing a rapid-acting pharmaceutical HSP co-inducer (Arimoclomol[®]) shortly before and over the course of several days after exposure to acute heat stress. Specifically, we hypothesized that enhanced induction of Hsp70 would protect rodents against cellular damage associated with acute hyperthermia in critical organs and tissues such as the brain, liver and skeletal muscle, and reduce heat stress-induced neurological impairments. Bearing in mind the nature of heat-stress induced pathology, we expected significant systemic effects and less neurological impairments due to hyperthermia.

III.2 METHODS

III.2.1. Military Relevance of the Heat Stress Protocol

Acclimatization to extreme temperatures occurs after approximately two weeks of increasing chronic exposure to this specific stressor. In a military setting, there is often not enough time for soldiers to become acclimatized to harsh conditions before being deployed, resulting in decreased performance and increased risk of injury. Utilizing pharmaceutical interventions when situational conditions preclude traditional acclimatization has the potential to greatly

reduce heat-related injuries (e.g., heat stroke, neurological impairments) and increase the performance of military personnel under rapid deployment circumstances.

The heating protocol utilized in these studies was designed to correlate with 'real life' deployment scenarios involving exposure of soldiers to hot ambient (e.g., desert) conditions. They are also relevant to situations that involve work/exertion in warm ambient conditions. Young healthy rats were utilized, and research from our laboratory and other investigators has established this to be an excellent model of heat stress and heat stroke in humans. Physiological adjustments (e.g., cardiovascular, thermoregulatory, fluid balance, stress protein induction) in rats undergoing this heating protocol are quite similar to those observed in humans. Moreover, pathophysiological alterations are comparable between human heat-induced injury/stroke cases and our rodent model (e.g., cellular alterations such as protein denaturation and necrosis, CNS dysfunction, dehydration, circulatory failure).

III.2.2. Animal Model

Previous studies have determined that both heat- and work-induced mortality curves are similar between humans and rats. In the current studies, a maximal T_{co} of 41.5°C was the target endpoint, because it approximates the minimal lethal temperature for death due to non-exertional heating in the rat. Similar to humans, rats achieving a T_{co} of 41.5°C will typically have mild to moderate systemic and CNS injury that is associated with moderate declines in performance. It is also well-documented from epidemiological data and anecdotal accounts that a T_{co} of 41-42°C is well within the range observed in young healthy humans who have suffered heat stroke injuries due to exposure to hot ambient conditions (exertional and non-exertional). Therefore, this rodent model of heat stroke is an excellent choice for simulating conditions that can be faced by military personnel in extreme environments, as well as the cellular and systemic responses that are manifested from this type of challenge.

III.2.3. Experimental Groups

Male Sprague-Dawley rats (~300 g) were randomly assigned to one of the following treatment groups:

- 1) heat stressed + drug (arimoclomol)
- 2) heat stressed + vehicle (saline)
- 3) sham-heated + drug
- 4) sham-heated + vehicle

III.2.4. Drug Treatment

Rats in the drug-treatment groups received an intraperitoneal (i.p.) injection (200 mg/kg) of the molecular chaperone co-inducer Arimoclomol® (CytRx, Los Angeles, CA) one hour before the initiation of the heating protocol. These rats continued to receive a single i.p. injection of arimoclomol once per day for the duration of their designated recovery period (1, 3, 5 or 9 days) following an acute heat stress protocol. Arimoclomol was dissolved in sterile saline and prepared fresh daily. Vehicle injections, which were administered on the same schedule, were of the same volume and consisted of sterile saline only.

Animals in the four treatment groups underwent behavioral testing on days 1, 2, 3, 4, and 5 after heat stress. Half of these animals were euthanized on day 5 (n=3 per group), while the other half continued to receive daily injections (drug or saline) and were euthanized on day 9. Additional rats in each of the four groups were euthanized on days 1 and 3 (n=3 per group) in order to assess physiological changes over a shorter recovery period. We have determined from previous studies in our laboratory that there is substantial heat-related injury (both CNS and peripheral) within the first few hours to days after an acute hyperthermic challenge, and considerable insight can be gained by assessing both shorter (i.e., 1 to 3 days) and longer (i.e., 5 to 9 days) durations of recovery and drug treatment. A general timeline for the experiments is depicted below.

Day	0	1	2	3	4	5	6	7	8	9
Heat Stress	+									
Behavioral Testing		+	+	+	+	+				
Euthanization		+		+		+				+
N per treatment group		3		3		3				3

III.2.5. Heating Protocol

All rats were handled daily and familiarized with a colonic temperature probe during the week before the heat-stress protocol. On the day of the heating protocol, each rat was fitted with a thermistor temperature probe for the measurement of core body temperature (T_{co}) and then placed, conscious and restrained, in a plastic cage. A baseline T_{co} (37.0-38.0°C) was established for each rat, followed by an established and well-characterized heating protocol. An infrared lamp was positioned ~40 cm above each rat and either raised or lowered to obtain an ambient temperature of 38-40°C. Movement of the lamp permitted a constant heating rate (~0.06°C/min) to be attained in each animal, and this rate was consistent within and between all treatment groups. When T_{co} reached 41.5°C (~60 min), rats were maintained at this temperature for 40 min. At the end of this period, rats were allowed to passively cool in a cage at room temperature. Sham-heated rats were handled identically to the experimental rats (e.g., colonic probe, placement in cage for similar duration), with the exception that ambient temperature was maintained at 22-24°C.

III.2.6. Behavioral Tests

Four different tests were utilized over the 5-day recovery period following heat stress to assess potential behavioral changes associated with the challenge and/or drug intervention. On days 1, 3 and 5 following heat stress, rats in all four groups performed inclined screen and adhesive tape tests to evaluate motor function. The **inclined screen test** measured the time it took a rat to climb to the top (or fall to the bottom) of a wire screen that was placed at 60° and 90° inclines (several trials per rat). For the **adhesive tape test**, small circular stickers were placed on the forepaws of each rat, and the time it took for the animal to remove the sticker was measured (several trials per rat). Cognitive assessments were made on days 2 and 4 utilizing a **T-maze** test (several trials per rat) and an **object recognition test**.

For detailed description of these methods, please refer to section I.2.3.

III.2.7. Tissue Preparation and Analysis

On the designated day, rats were euthanized with sodium pentobarbital (i.v.) and tissues were harvested. Brains were removed and processed for immunohistochemical analysis. Brain sections (cortex, hippocampus, cerebellum) will initially be assessed immunohistochemically for ICAM-1 (an adhesion molecule that is expressed in the brain in high concentrations with inflammation) and Hsp70 (stress protein with chaperone and thermotolerance properties). These immunohistochemical assessments will provide insight into potential neurological changes associated with heat stress and the effects of arimoclomol treatment on these responses.

We also harvested liver, skeletal muscle, heart, kidney and blood samples from each animal for evaluation of protein expression and cellular damage. Because of the short time frame allocated for these pilot studies, we have chosen to focus our initial efforts on Hsp70 and ICAM-1 expression in the liver, skeletal muscle and heart of the four treatment groups at different time points after heat stress. This approach allows us to gain some important insight into the duration of action for arimoclomol in a rodent model of acute heat stress in terms of its ability to co-induce Hsp70, as well as evaluate the time course of expression of a protective stress protein (Hsp70) following hyperthermic challenge and its relationship to a marker of inflammatory damage (ICAM-1) in selected organs that are critical to overall function and homeostasis of an organism.

III.3. RESULTS

III.3.1. Protein Analyses

III.3.1.1. Hsp70 Expression in the Liver, Skeletal Muscle and Heart

One of the primary focuses of this pilot project was to evaluate the physiological responses (e.g., protein expression, cell damage, organ dysfunction) known to occur in peripheral tissues and organs with hyperthermic challenge, and test whether arimoclomol treatment would alter these pathological alterations due to acute heat stress.

Immunoblots were performed on liver samples from day 1, 3, 5 and 9 rats (i.e., 1, 3, 5 and 9 days after an acute heat exposure), and some very consistent (within groups) and provocative results were produced. There was robust Hsp70 expression on day 1 in heated rats that received the Hsp70 co-inducer arimoclomol (n=3), while heated rats that received vehicle (n=3) had a very modest level of Hsp70 expression at the same time point (see figure below). Sham rats (i.e., not heat stressed) receiving the Hsp70 co-inducer had no Hsp70 expression in the liver (note: we are measuring the inducible form of Hsp70, so this protein should not be present in cells that are unstressed). The results for day 3 rats followed a very similar trend, with Hsp70 still elevated in heat stressed rats that had received arimoclomol compared to their heat-stressed counterparts that received saline. Another very interesting observation was that Hsp70 expression was still present in the liver of heat stressed rats that had received

arimoclomol on day 5 of recovery (data not shown). At the 9-day post-heating time point, no Hsp70 expression was observed in this cohort.

These observation demonstrates that co-induction of Hsp70 with daily administration of arimoclomol, in conjunction with acute heat stress, can induce an extremely high level of expression of the **protective stress protein Hsp70** in critical organs such as the liver. Furthermore, intracellular Hsp70 levels can be maintained for at least six days following a single heat challenge, suggesting that long-duration protection can be manifested at a systemic level with the administration of an Hsp70 co-inducer (Fig. 10).

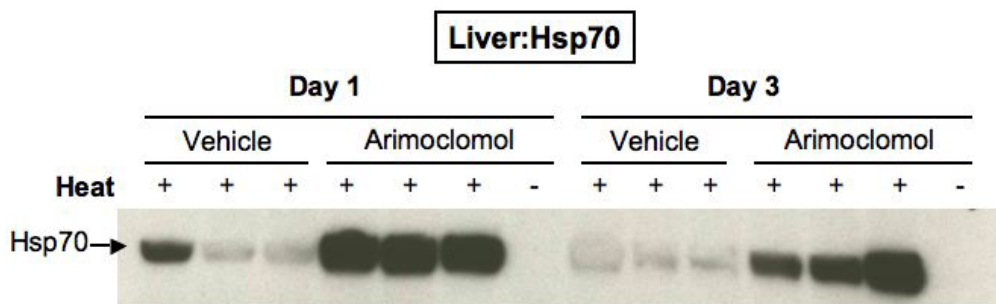


Figure 10. Liver Hsp70 immunoblot.

Hsp70 expression in liver samples from rats on days 1 and 3 after an acute heat stress. Vehicle + heat produced modest Hsp70 responses, but arimoclomol + heat produced strong Hsp70 expression. Arimoclomol alone (no heat stress) did not induce Hsp70. Each lane in the blot represents a sample from a different animal.

Very similar responses were observed for quadriceps muscle (Fig. 11). Hsp70 was expressed on days 1 and 3 in heated animals, and this response was clearly enhanced in rats treated with arimoclomol. Low levels of Hsp70 expression were still noted on day 5 in the heat plus arimoclomol group (data not shown). No Hsp70 expression was noted in muscle samples from sham-heated rats that received the drug.

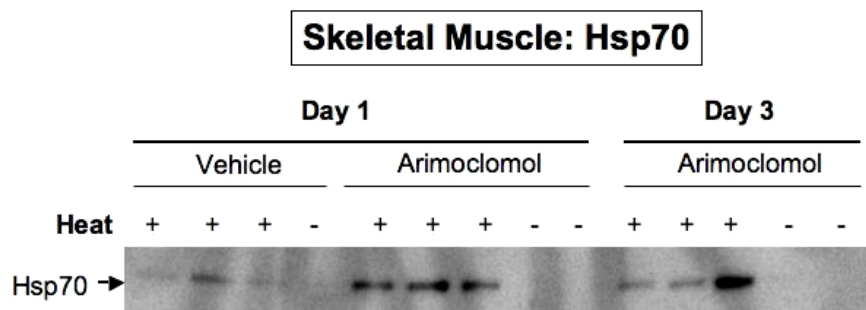


Figure 11. Skeletal muscle Hsp70 immunoblot.

Hsp70 expression in quadriceps samples from rats on days 1 and 3 after an acute heat stress. Vehicle + heat produced low levels of Hsp70 expression, but arimoclomol + heat produced greater Hsp70 expression in the muscle on day 1. By day 3 post-heating, Hsp70 was still present in muscle from arimoclomol + heat rats, but there was no expression in vehicle + heat rats (not shown on the blot). Arimoclomol alone (no heat stress) did not induce Hsp70. Each lane in the blot represents a sample from a different animal.

Results in the heart were less dramatic than as those observed in the liver and muscle, although the trends between treatment groups in the heart were similar to those observed in liver and muscle. Hsp70 expression was increased to a greater level in heat stressed rats receiving arimoclomol on days 1 and 3 than those receiving vehicle, but the response was less robust than that in the other two organs and the difference between treatment groups was not as striking (data not shown).

III.3.1.2. ICAM-1 Expression in the Liver

We also performed western blot analyses for ICAM-1 in the liver. ICAM-1 expression on day 1 was fairly similar between the different treatment groups (Fig. 12), and it appeared that neither heat stress nor arimoclomol had an effect on ICAM-1 expression. However, on day 3 post-heating, there was a trend for rats in the heat + vehicle group to have increased expression compared to rats in the heat + arimoclomol group. The two sham-heated groups had low levels of ICAM-1 expression that were similar to the heat + arimoclomol group.

These results suggest that an inflammatory response to heat-induced injury in the liver is primarily manifested on day 3 of recovery, and that the injury and inflammation responses are blunted in heat-stressed rats that received arimoclomol compared to those that were vehicle-treated. Further, the time course of the inflammatory response (i.e., elevations at 48 to 72 hours after heat stress) is consistent with our own previously published observations (e.g., cytokine levels, histopathological assessments) and reported data in humans. Finally, there is a clear association (especially on day 3) between increased levels of Hsp70 expression and reduced levels of inflammation in the liver following a severe heat challenge.

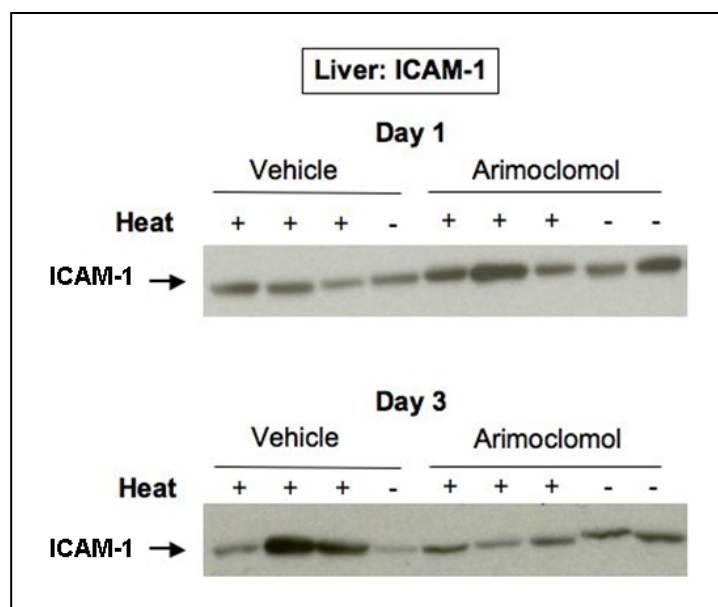


Figure 12. Liver ICAM-1 immunoblot.

ICAM-1 expression in liver samples from rats on days 1 and 3 after an acute heat stress. Low levels of ICAM-1 expression were noted in all treatment groups on day 1 after heat stress. On day 3 post-heating, there was a trend for increased ICAM-1 expression in liver samples from heat + vehicle rats compared to heat + arimoclomol rats.

III.3.1.3. Additional Assessments

Since Hsp70 expression within cells that are undergoing stress is associated with prevention and repair of damage to intracellular proteins, based on preliminary measurements of inflammatory and oxidative injury markers, we would postulate that the robust Hsp70 response observed in tissues of rats that received arimoclomol potentially prevents the widespread cellular damage, inflammation, and organ dysfunction that typically accompanies acute heat stress.

III.3.1.2. Protein Expression in the Brain

We are currently able to report on Hsp70 and ICAM-1 staining in the cortex of rats in the four treatment groups. There was consistent moderate-to-strong Hsp70 staining throughout the cortex of heat-stress rats that received the Hsp70 co-inducer arimoclomol. This staining was present on days 1 and 3 post-heating. In contrast, the brains of heat-stress plus vehicle-treated rats had much lower levels of Hsp70 expression. Brain sections from the two sham-heated groups had only trace levels of Hsp70 expression.

Immunohistochemical staining for the presence of ICAM-1 was low in the cortex of heat stressed rats that received arimoclomol. In contrast, moderate to strong levels of ICAM-1 were present in brains of heat stress plus vehicle rats. These differences were consistent on days 1 and 3 post-heating, with the greatest amount of ICAM-1 expression observed on day 3. Since ICAM-1 is thought to play a key role in the inflammatory response to heat-induced injury in the brain, these preliminary results suggest that co-induction of Hsp70 with arimoclomol treatment reduces the degree of injury and inflammation in the brain with an acute heat challenge.

III.3.2. Functional Tests

There were several limitations in the study design of these pilot experiments that likely impacted the behavioral results. First, the tests used were, by necessity in these preliminary studies, somewhat unsophisticated and lacking in sensitivity. Second, these types of behavioral tests are most effective and insightful when large numbers of subjects are assessed per group. While it is apparent from the preliminary results generated that the heat stress protocol did manifest some degree of neural impairment that would translate into motor and cognitive dysfunction, the simple tests used and the low animal numbers were likely responsible for the inconsistent results.

III.3.1.1. Cognitive Outcome

T-maze Test:

As mentioned previously, the T-maze function has been used to detect hippocampal damage, as well as to establish memory deficits and memory improvement induced by drugs. A high alternation rate suggests a sustained cognition as the animals must remember which arm was

entered last and not re-enter it. We tested all rats at 2 and 4 days of exposure, and didn't find significant differences between groups (Fig. 13).

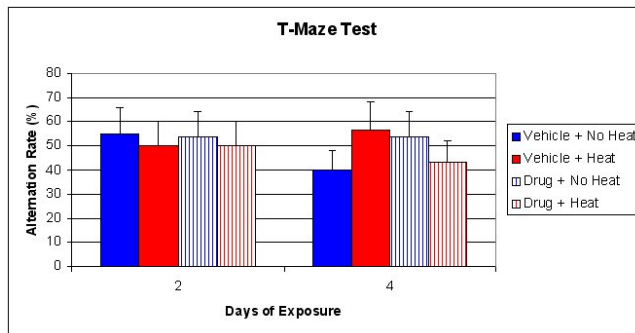


Figure 13. T-maze test results expressed as alternation rates (%) measured on days 2 and 4 of exposure. No statistical difference was found between the groups.

Object Recognition Test

In this test's task, the animal explores a novel object and reactivates the exploration after the object is displaced. Thus test reflects the learning capability and ability to recall previous information (memory). The number of times and length of time for inspecting the objects (old and new) was calculated. The percent of exploration of a new object is higher with good cognitive function. Our data showed that the percent of exploration of a new object is significantly decreased (40%) in the vehicle-treated hypoxic group, compared to its normoxic control group, whereas the drug treatment significantly improved the cognitive function in hypoxic rats (Figure 14).

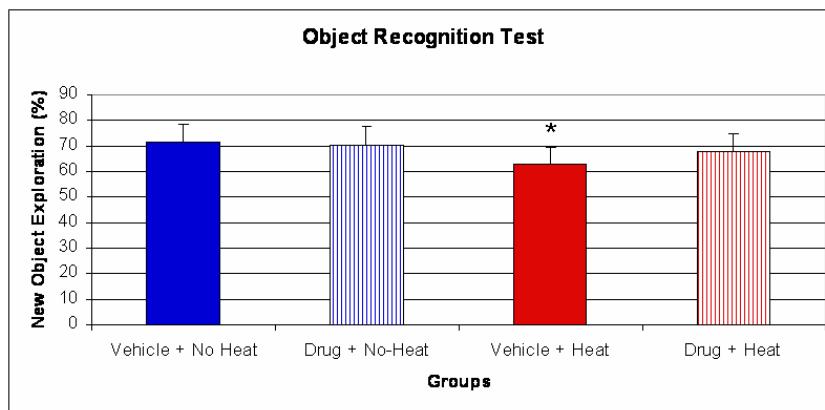


Figure 14. Object recognition test showing the rats' activity explore new objects.

III.3.1.2. Motor Function

The Inclined Screen Test

This test was used to measure balance, muscle strength and coordination. The test was carried out at 60- and 90-degree incline levels for all groups at 1, 3, 7, and 10 days of exposure. Latency to climb to the top of the screen and/or latency to fall was recorded, and an overall score was calculated. For the heat stressed rats, there was significant increase in overall score in the drug-treated group 1 day of exposure (Figure 15), and a trend toward improved motor function during the entire heat stress period.

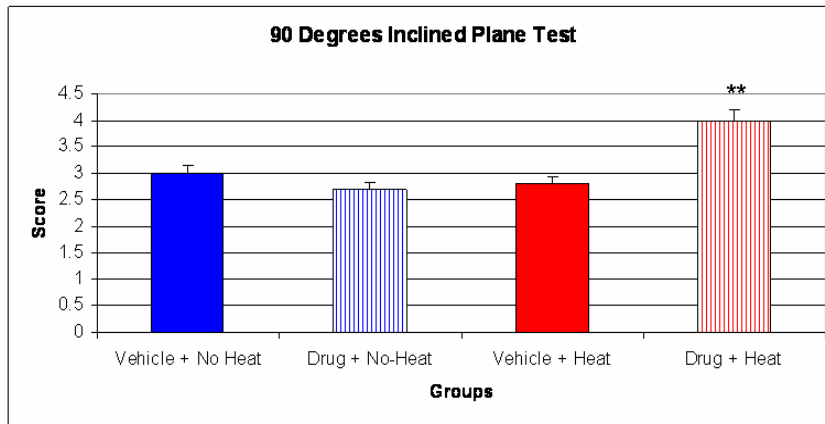


Figure 15. 90° inclined plane test measured after one day of heat stress exposure. The drug-treated heat stressed animals showed significantly improved balance, muscle strength and coordination compared to non-treated animals ($p < 0.01$)

The Adhesive (sticky) Tape Removal Test

This test was used to measure somatosensory function: the faster to remove the tapes, the better the function. There were no significant differences among the groups at any condition during exposure (Fig. 16).

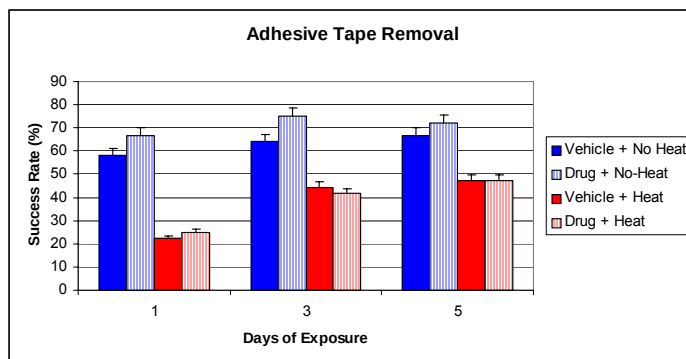


Figure 16. Success rate (%) in adhesive tape removal task measured on days 1, 3, and 5 of exposure. No statistical difference has been found between treated and non-treated animals subjected to heat stress.

III. 4. CONCLUSIONS

These preliminary studies demonstrate the feasibility of using a rodent model to reproduce the complex physiological adjustments and neurological impairments in soldiers who are exposed to a stressor such as warm ambient conditions. This model also allowed us to assess the potential benefit of arimoclomol-induced HSP accumulation in a mammalian system that closely mimics the heat-induced pathophysiological responses observed in humans.

The induction of HSP expression is one of the most important mechanisms available to an organism in adapting to extreme environmental conditions, and the observation that the Hsp70 co-inducer arimoclomol markedly enhances the Hsp70 response in both peripheral organs (liver, skeletal muscle, heart) and the brain, and maintains this response for several days after a severe heat stress episode suggests that this therapeutic approach may be beneficial as a booster of Warfighters' adaptive and functional fitness levels.

III.5. FUTURE DIRECTIONS – EXERTIONAL HEAT INJURY STUDIES

III.5.1. *Rationale*

The results we obtained in preliminary acute heat stress studies demonstrate that the Hsp70 co-inducer, Arimoclomol[®], markedly enhances intracellular Hsp70 expression in both the brain and key peripheral organs (liver, skeletal muscle) of rats; this increased HSP 70 expression is maintained for several days after a heat challenge. Importantly, animals that received the Hsp70 co-inducer also demonstrated improved motor and cognitive functions over several days of recovery from hyperthermic challenge. In addition, the increased Hsp70 expression in key organs such as the brain, liver, and skeletal muscle was associated with reduced tissue inflammation. These findings suggest that Hsp70 co-induction is viable as both a protective and therapeutic approach to heat-related injuries, and may be beneficial as a booster of Warfighters' adaptive and functional fitness levels.

III.5.2. *Approach*

We envision two specific interventional approaches (aims) that will be conducted and completed over a three-year time frame. The focus of these studies will be on the potential therapeutic effect of Hsp70 co-induction (via arimoclomol treatment) on exertional heat injury, and will involve **both an animal model and an expedited human clinical component**. This design is important because it will involve protocols that combine the effects of heat exposure and exertion (e.g., exercise, work, marching, etc.), which more accurately matches the challenging circumstances encountered by Warfighters and the pathophysiological conditions that can develop in these individuals (e.g., reduced performance, declines in motor skills and decision-making, heat-induced injury and heat stroke).

We will also be able to utilize existing protocols and data involving non-exertional heating to rapidly and efficiently develop exertional heating protocols in both rodents and human. An additional component of these studies will be the inclusion of a weight load to some subjects undergoing exertional heat stress. This combination of heat exposure, exercise, and the added burden of carrying a weight load will more closely mimic the conditions faced by Warfighters that contribute to a wide range of deleterious outcomes. Another benefit of this exertional heat injury design is the opportunity to investigate mechanisms contributing to the development of muscle injury and **rhabdomyolysis** in humans, as well as the possibility that Hsp70 co-induction may help mitigate this serious condition. Rhabdomyolysis is becoming a greater concern for the Warfighter as military deployments increase to hot environments. For all these studies, we will utilize several state-of-the-art experimental technologies to determine the mechanisms responsible for the high levels of morbidity and mortality and behavioral/performance deficits associated with exertional heat injury in the Warfighter.

III.5.3. Specific Aims

III.5.3.1. Aim 1

This task will involve an acute exertional heat injury protocol in rodents. These studies will be similar in design to our recently conducted pilot experiments (which involved only non-exertional heating), and include groups of animals that are treated with either vehicle or the Hsp70 co-inducer Arimoclomol[®]. Animals will be assessed at several different time points in the recovery phase after the induction of heat injury (i.e., several hours to several days post-stress) aiming to accurately characterize the temporal profile of both injury and potential protection with the Arimoclomol[®] treatment.

Different levels of exertion (i.e., walking/running on a treadmill in a warm environment) (Fig. 17) will be employed that mimic those conditions faced by soldiers in the field. The additional exertion component will involve different amounts of weight to animals undergoing these exercise protocols (Fig. 18). A range of specific assessments at molecular, cellular, organ, and systemic levels will be employed, and a battery of behavioral and cognitive tests will be performed to delineate the potential beneficial effect of arimoclomol treatment on exertional heat injury.

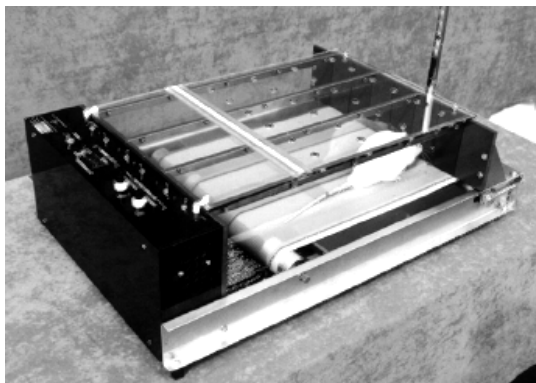


Figure 17. Rat exercising on a treadmill in extreme heat conditions



Figure 18. A custom design vest to add different amount of weight to animals undergoing heat + exertion protocols

III.5.3.2. Aim 2

In this aim, we will develop an exertional heat injury model in humans that will run in parallel to the animal studies and build on our preliminary results. These studies will be designed to simulate conditions faced by Warfighters in the field (i.e., heat, exertion, carrying additional weight loads). Similar to the studies in animals, young healthy subjects (both male and female) will be treated with the Hsp70 co-inducer, Arimoclomol[®] or vehicle prior to an exertional heating protocol. The study design will include heat stress alone, exhaustive exercise alone, or a combination of the two stressors. These experiments will be designed to evaluate the effects of a range of parameters, including: 1) different exertion levels (e.g., mild, moderate, severe); 2) the impact of exertion when combined with heat stress; 3) gender differences in heat stress response; 4) fitness level; and most importantly 5) the Arimoclomol[®]

treatment on physiological and neurological outcome of these stress conditions. Several clinically relevant parameters (e.g., thermoregulatory, cardiovascular, renal and skeletal muscle variables, serum markers of injury) will be assessed in subjects over several days of recovery from the acute exertional heating protocol. In addition, multiple behavioral and cognitive tests will be performed.

III.5.4. Summary of the Proposed Studies

In these proposed studies, we will develop and implement *in vivo* models of exertional heat injury that transition previous findings from *in vitro* and whole animal studies to more relevant human clinical experiments. This design will provide more **direct extrapolation to the Warfighters' condition**. The ability to rapidly induce Hsp70 will both enhance heat tolerance and reduce exertional heat injury in Warfighters, and will be of great benefit for rapid military deployment to hot climates.

IV COLD STRESS

(PI Zhongjie Sun, The Oklahoma University Health Science Center)

IV.1. INTRODUCTION

A change in environmental temperature is one of the most common stresses experienced throughout evolution. Cold shock adaptation at temperature above freezing has been extensively studied in *E. coli* and *B. subtilis*, among others ¹. Cold temperatures have adverse effects on human health. The mortality rate significantly increases in cold regions or in winter.

One of the universal responses to low temperature is a change in the lipid composition of membranes. When the temperature drops, membranes that are normally in a liquid crystalline form, undergo a transition to a gel phase state ². Numerous organisms have developed mechanisms to compensate for the transition from the liquid crystalline to gel phase. The first adaptive mechanism, homeoviscous adaptation, includes increasing the proportion of unsaturated fatty acids in the membrane lipids, since phospholipids with unsaturated fatty acids have lower melting points and greater degree of flexibility than phospholipids containing saturated fatty acids ². Similar homeoviscous response at the cellular level has been observed in hibernating animals, changing the degree of saturation of membrane phospholipids in heart membranes. Stabilization of secondary structures of RNA and DNA and subsequent difficulties in translating mRNA and synthesizing necessary proteins are among significant harmful effects of low temperature. It is noteworthy that near freezing temperatures have been found to induce heat shock protein response in plants, yeast and *E. coli* ^{3 4}. Thus, the heat shock proteins should be more appropriately called temperature-stress proteins. It seems that HSPs are not unique to high-temperature stress: they appear to be required to maintain protein folding at low temperature as well ⁵.

It has been shown that for every 10°C drop in temperature, the rate of most biochemical reactions reduces by a factor of approximately two. The fact that cold temperatures also decrease physical and cognitive performances is of great importance for soldiers who are often exposed to extreme-cold environment. The heat-shock protein has been shown to protect cells under stress conditions.

The purpose of this project was to evaluate if HSP co-inducer (arimoclomol) can trigger the heat-shock response and booster adaptive ability. Specifically, we will evaluate the effect of arimoclomol on body weight gain, cognitive behavior, and adverse biochemical changes in cold-exposed arts.

IV. 2. METHODS

IV.2.1. Animals

Four groups of male Sprague-Dawley rats (180-200 g) were used (12 rats/group). Following a 3-day control period, animals were exposed to moderate cold (5°C) continuously. Animals were kept in a cold climate-controlled walk-in chamber (5±2°C) during cold exposure. Relative humidity was controlled automatically at 45±5% in the chamber. All animals were housed individually in stainless steel wire mesh cages without bedding. The temperature inside the cage has been confirmed to be the same as that of the chamber. Standard rat chow (Purina Laboratory) and tap water were provided for all animals ad libitum. Lights were on from 0700 to 1900 h in both chambers.

Four groups of rats were used (6 rats/group): Two groups were pre-treated with HSP co-inducer (arimoclomol, IP, daily) 1 hour before exposure to cold and were treated daily during the whole period of experiment (7 days or 10 days). The other two groups were pre-treated with vehicle and were treated daily throughout the experiment. Following the first injection, 1 drug-treated and 1 vehicle group were exposed to cold continuously while the remaining groups were kept at room temperature (warm) and served as controls.

IV.2.2. Parameters

The parameters measured in this study included: mortality rate, body weight, and neurological functions. At the end of a 7-day chronic cold exposure, the animals were euthanized and perfused through the heart with heparinized saline and paraformaldehyde (4%). The tissue samples of the brain, liver, heart, and kidneys were prepared for immunocytochemistry and immunoblot analyses of HSP, superoxide production, tumor necrosis factor alpha receptor (TNFR-1), interleukin 6 (IL-6), and intercellular adhesion molecule (ICAM-1).

Neurological Tests

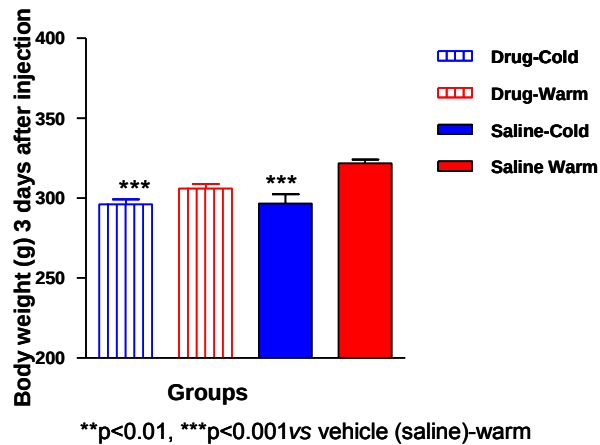
Four different tests were utilized over the 5-day recovery period following heat stress to assess potential behavioral changes associated with the challenge and/or drug intervention. On days 1, 3 and 5 following heat stress, rats in all four groups performed inclined screen and adhesive tape tests to evaluate motor function. The **inclined screen test** measured the time it took a rat to climb to the top (or fall to the bottom) of a wire screen that was placed at 60° and 90° inclines (several trials per rat). For the **adhesive tape test**, small circular stickers were placed on the forepaws of each rat, and the time it took for the animal to remove the sticker was measured (several trials per rat). Cognitive assessments were made on days 2 and 4 utilizing a **T-maze** test (several trials per rat) and an **object recognition test**.

For detailed description of these methods, please refer to section II.2.3.

IV. 3. RESULTS

IV.3.1. Body Weight

Similar to our previous results, cold exposure significantly decreased body weight gain compared with the animals maintained at room temperature (Fig. 19). The fact that the Arimoclomol[®] treatment didn't cause significant weight loss demonstrates the drug's non-toxicity.



No significant difference between treated and non-treated cold exposed animals

Figure 19. Body weight changes in rats treated with vehicle or Arimoclomol[®] and exposed to cold stress.

IV.3.2. Protein Analysis

IV.3.2.1. HSP 70

While Arimoclomol[®] did not alter HSP70 expression in rats maintained at room temperature (warm), it significantly increased HSP70 protein expression in the liver of cold-exposed rats (Fig. 20) compared to vehicle-treated cold-exposed animals. These results suggest that Arimoclomol[®] may induce HSP70 expression only under stress conditions. Similar results have also been observed in heart and kidney samples (results not shown).

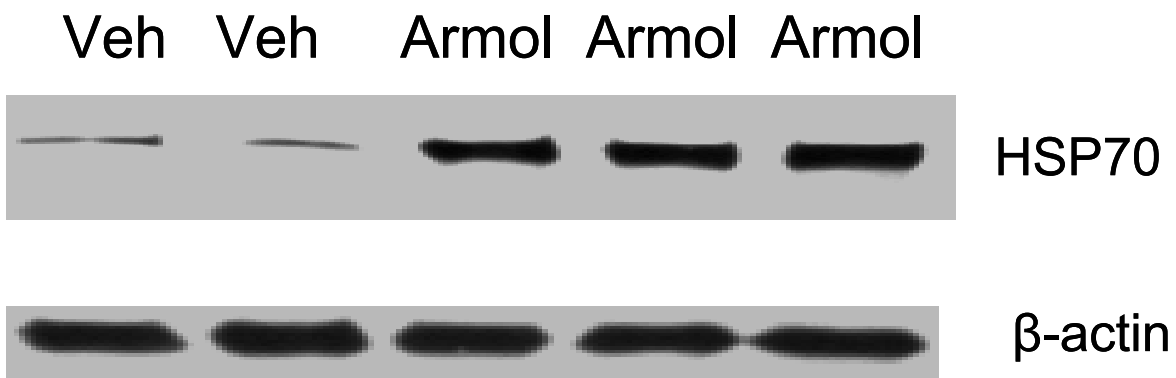
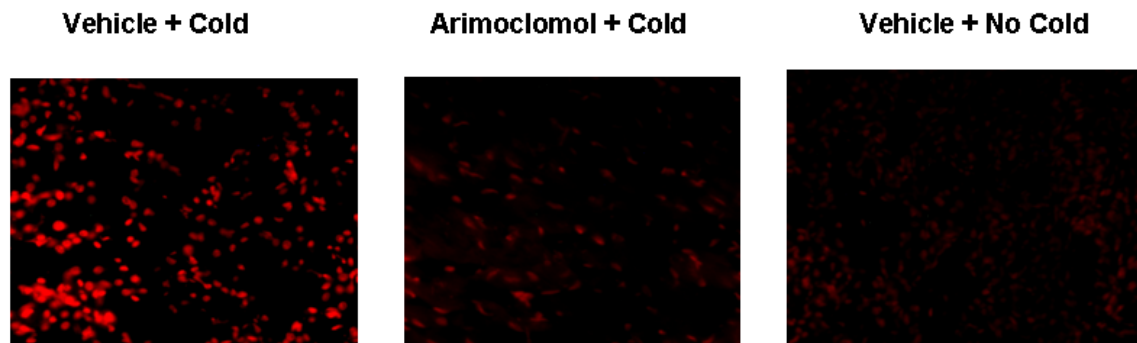


Figure 20. Representative immunoblots of HSP 70 expression in the liver of animals exposed to cold and treated with vehicle (Veh) or Arimoclomol[®]. B-actin was used as an internal quality control.

IV.3.2.2. Superoxide Production

Increased production of reactive oxygen species (ROS) has been implicated in the pathogenesis of numerous diseases ^{6 7}. Enzymatic systems such as the mitochondrial respiratory chain, vascular NAD(P)H oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS) produce superoxide anion (O₂^{•-}) in vascular cells as a response to a broad variety of toxic factors ⁸.

Similar to our previous studies ⁹, increased superoxide production has been found in the liver, kidneys (not shown) and heart of cold-exposed vehicle-treated animals (Fig. 21). Interestingly, Arimoclomol[®] decreased the cold-induced superoxide production. Taking into account literature data and our previous studies, this result suggests that there is a possible causal relationship between the increased HSP70 expression and reduction of the cold-induced oxidative stress. Future studies are needed to determine whether the anti-oxidative effect of the drug is mediated via NADPH oxidase-dependent mechanisms or anti-oxidant enzymes.



***In situ* superoxide production in heart (DHE staining)**

Figure 21. *In situ* superoxide production in the heart of rats exposed to cold and treated with vehicle (saline), exposed to cold and treated with Arimoclomol[®], and those kept at room temperature and treated with vehicle. The well-expressed positive staining shows increased production in the heart of vehicle treated cold-stressed animals. The treatment with Arimoclomol[®] significantly reduced the cold-induced superoxide production.

IV.3.2.3. TNFR-1

TNFR-1 has been shown to induce apoptotic pathways in multiple organs after a broad variety of pathological stimuli ¹⁰⁻¹³. Our immunoblot results show that cold exposure significantly increased TNFR-1 expression in kidneys of vehicle treated animals, whereas Arimoclomol[®] significantly decreased such an expression (Fig. 22). Consistently, immunocytochemistry staining confirmed a cold exposure-induced increase in TNFR-1 staining in renal glomerulus of vehicle-treated animals, and showed a decrease TNFR-1 staining in cold-exposed animals treated with Arimoclomol[®] (Fig. 23). These finding suggest that induction of HSP70 potentially inhibits cold-induced inflammation in kidneys.

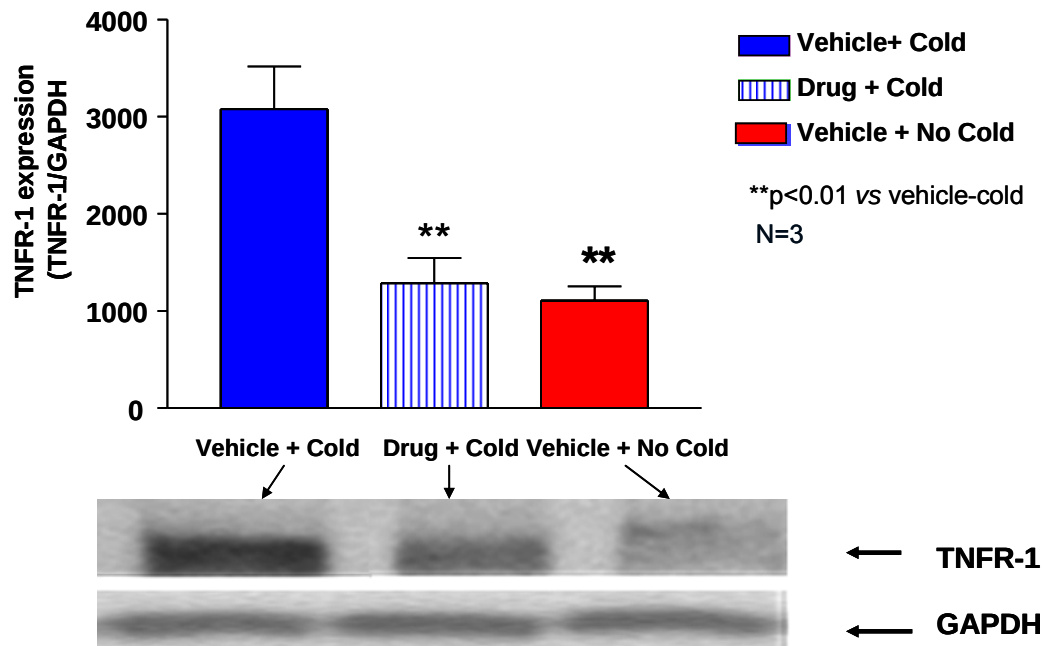
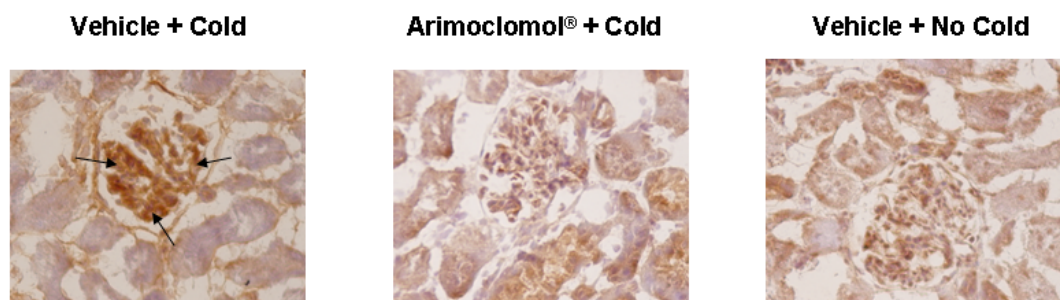


Figure 22. Representative immunoblot results showing TNFR-1 expression in the kidneys of rats exposed to normal temperature and treated with vehicle (Vehicle + No Cold), or animals exposed to cold and treated with vehicle (Vehicle + Cold) or treated with Arimoclomol®. The TNFR-1 expression is shown as a ratio of the immunoblot signal for TFNR-1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a protein for internal quality control.



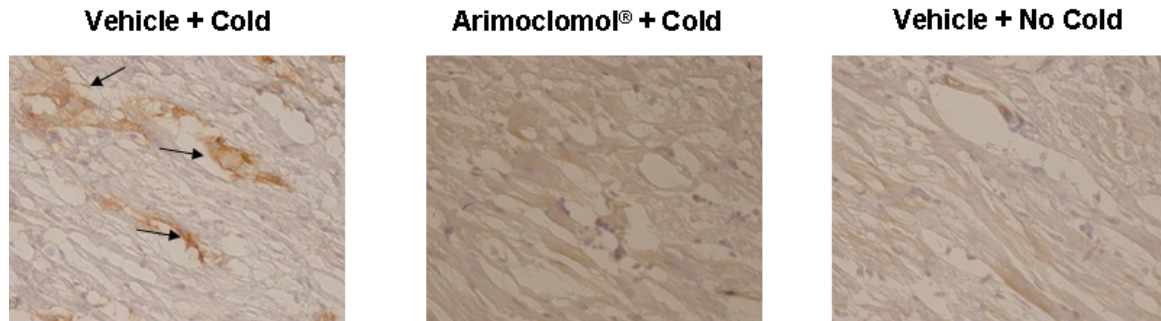
Strong TNFR-1 staining was found in kidneys (glomeruli) of vehicle-treated cold-exposed animal, whereas Arimoclomol® significantly decreased the TNFR-1 staining in cold-exposed rat.

Figure 23. Representative immunocytochemistry staining for TNFR-1 showing significant positive staining (arrows) in the kidney of cold-exposed and vehicle-treated (Vehicle + Cold) rat, whereas the TNFR-1 staining showed similar results in both Arimoclomol® treated cold-exposed animals and rats treated with vehicle and kept at room temperature (Vehicle + No Cold).

IV.3.2.4 ICAM-1

Intercellular adhesion molecule-1 (ICAM-1), an inducible cell adhesion glycoprotein of the immunoglobulin supergene family, is overexpressed by proinflammatory mediators in a wide variety of cell types¹⁴. These stimuli increase ICAM-1 expression primarily through activation of ICAM-1¹⁵. As such, adhesion molecules play a role in the inflammation and pathogenesis of a broad variety of vascular diseases^{16 14}.

In our study, cold exposure increased ICAM-1 expression in the heart of vehicle-treated animals; this cold-induced increase in ICAM-1 expression was attenuated by HSP co-inducer, (Fig. 24). These preliminary results indicate potential beneficial role of Arimoclomol® in cardiac remodeling.



Brown staining indicates ICAM-1 expression

Figure 24. Representative immunocytochemistry staining for ICAM-1 in the heart of rats treated with vehicle and kept at room temperature, or animals exposed to cold and treated with vehicle (Vehicle + Cold) or treated with Arimoclomol®. The arrows indicate the positive ICAM-1 staining in the heart of vehicle-treated cold-exposed rat.

IV.3.3. Neurological Functions

IV.3.3.1. T-Test

As mentioned previously, the T-maze function has been used to detect hippocampal damage, as well as to establish memory deficits and memory improvement induced by drugs. A high alternation rate suggests a sustained cognition as the animals must remember which arm was entered last and not re-enter it. Arimoclomol® significantly improved the cognitive performance of drug-treated rats exposed to cold versus vehicle treated cold-exposed animals (Fig. 25).

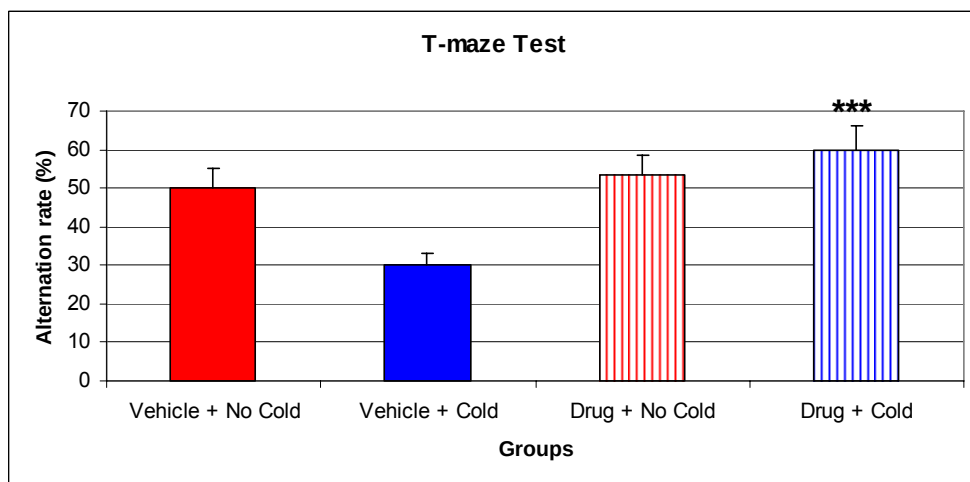


Figure 25. T-maze test performance of treated and non-treated rats exposed to cold. *** $p < 0.001$ compared to animals treated with vehicle and exposed to cold (Vehicle + Cold)

IV.3.3.2. Inclined Screen Test

This test was used to measure balance, muscle strength and coordination. The test was carried out at 60- and 90-degree incline levels for all groups at 1, 3, and 5 days exposure. Latency to climb to the top of the screen and/or latency to fall was recorded, and an overall score was calculated. For the cold-stressed rats, there was significant increase in overall score in the drug-treated group on day 3 of exposure (Figure 26), and a trend toward improved motor function during the entire heat stress period.

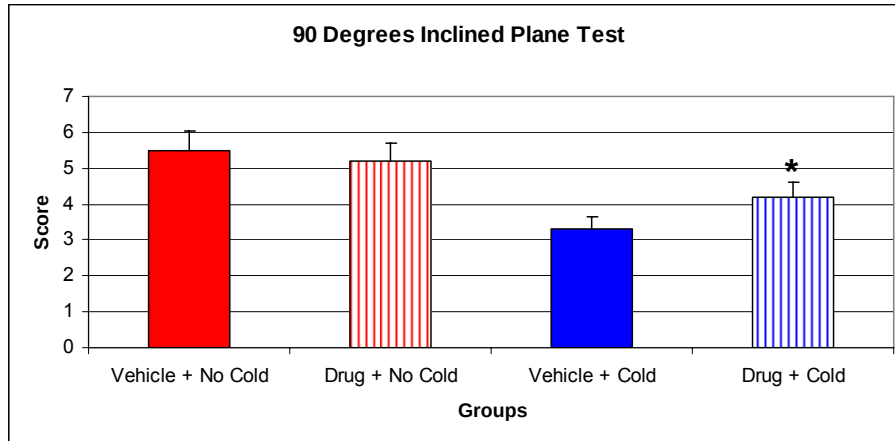


Figure 26: The score of the 90-degree inclined screen test in rats exposed to cold. Values are expressed as mean ± SD, n = 6 in each group. Scoring system: 2 = climb to the top successfully; 1 = fail to climb to the top but stay on the screen; and 0 = fall. The results are averaged for 3 trials per rat; 1 minute per trial.

IV.3.3.3. Adhesive Tape Removal Test

This test was used to measure somatosensory function: the faster to remove the tapes, the better the function. The performance of drug-treated cold-exposed animals was significantly improved compared to the vehicle-treated cold-exposed animals on day 5 of cold exposure (Fig. 27).

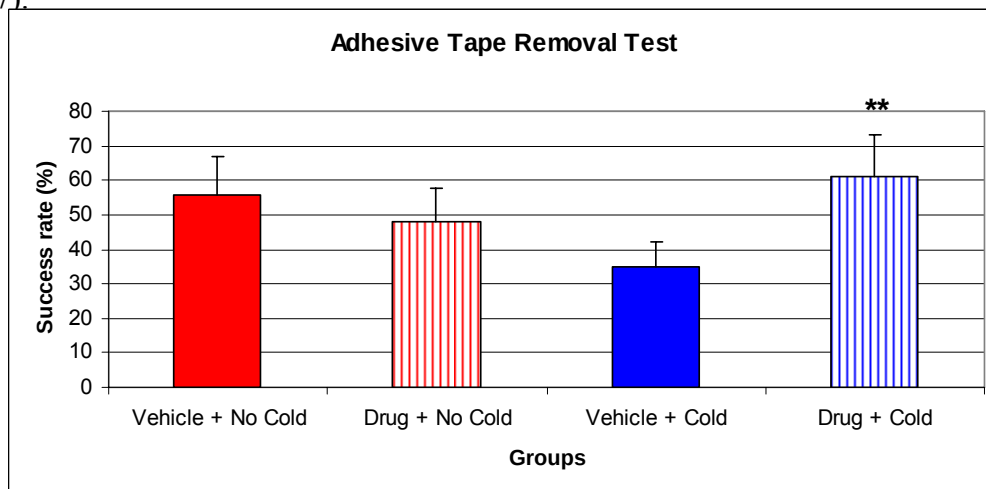


Figure 27: The success rate (%) in adhesive tape removal task in rats exposed to cold. Values are expressed as mean ± SD, n = 6 in each group. ** p<0.01 compared to Vehicle+Cold group.

IV. 4. CONCLUSION

Our preliminary results suggest that Arimoclomol[®] induces HSP70 protein expression in liver, heart, and kidneys resulting in reduction of cold-induced oxidative stress (superoxide), inflammation (TNFR-1), and increase in the heart remodeling factor (ICAM-1). These results indicate Arimoclomol[®] as potential protective treatment against cold exposure-induced damage of liver, heart, and kidneys. We suggest further studies to fully elucidate the mechanisms of these beneficial effects of Arimoclomol[®].

IV.5. FUTURE DIRECTIONS – COLD STRESS STUDIES

IV.5.1. Rationale

Cold temperatures have significant adverse effects on human health, increasing mortality and morbidity rates, and decreasing physical and cognitive performance. Thus, preventing cold-induced morbidity and functional decline is of essential value for Warfighters often exposed to extreme cold environment..

Our preliminary data confirmed that increase in heat-shock protein protects vital organs such as heart, kidneys, brain, and liver under cold stress conditions. Indeed, treatment with Arimoclomol[®], a heat-shock protein co-inducer, significantly increased HSP70 protein expression in the liver of cold-exposed rats. Moreover, Arimoclomol[®] attenuated the cold-induced superoxide production in the heart, thus reducing cardiac oxidative stress. In addition, Arimoclomol[®] attenuated cold-induced inflammation in the heart by reducing ICAM-1 expression. This treatment also decreased TNFR-1 expression in kidneys of cold-exposed rats, suggesting that induction of HSP70 may inhibit cold-induced inflammation in kidneys.

IV.5.2. Approach& Specific Aims

Our long-term goal is to understand how cold temperatures cause medical complications in humans, and develop preventive and therapeutic strategies targeting vital pathological mechanisms. The preliminary study showed that the Arimoclomol[®]-induced increase in HSP70 protein expression attenuated cold-induced oxidative stress, the cold-induced increase inflammation, and the cold-induced increase in the heart-remodeling factor. We propose validation of our preliminary findings in humans (Aim 1), and extend these findings in animals (Aim 2).

IV.5.2.1. Aim 1: Human Studies

The objective of this study is to determine whether pre-treatment with heat-shock protein co-inducer, Arimoclomol[®], can protect the liver, cardiovascular system, and kidneys from cold-stress in humans. We hypothesize that pre-treatment with Arimoclomol[®] will attenuate cold-induced oxidative stress and inflammation, and improve cardiovascular and renal functions in human subjects exposed to chronic cold environment (4°C/39°F) or -2 °C/28 °F) for varying time periods. Young healthy human subjects (both male and female) will be treated with

Arimoclomol[®] or vehicle prior to exposure to cold. The study design will include both continuous exposure to cold of limited duration and intermittent cold exposure. A variety of functional and biochemical parameters will be measured to evaluate whether induction of Hsp70 has beneficial effects on liver, cardiovascular system, and kidneys. These clinically relevant parameters include body temperature, heart rate, and blood pressure, serum markers of oxidative stress, hepatic enzyme functions, and renal excretory functions. In addition, we will evaluate the effects of Arimoclomol[®] on behavioral and cognitive responses to cold exposure.

IV.5.2.2. Aim 2: Animal Studies and Dynamical Feedback with Human Studies

We will continue to fine-tune our animal studies aiming to develop models capable of reproducing graded chronic cold exposure with or without cold water immersion – a situation the members of the Navy SEALs frequently face with. It has been shown that there is a cross-talk between different visceral organs during certain pathological conditions. Namely, the pathology of one visceral organ is known to affect the physiology and functioning of other organs, which not only enhances the discomfort and reduces proper functions but also makes treatment regimens more complex¹⁷. It has been demonstrated that in tropical men who were subjected to Antarctica cold, exposure to cold for various duration caused increased excretion of urinary epinephrine, norepinephrine, and salivary cortisol levels, associated with significant autonomic changes in heart rate and blood pressure¹⁸. Experimental results have shown that acute (+4 °C for 8 hrs) or chronic (+4 °C, 4 hr/day for 21 days) cold exposures caused significant bladder injury with significant alterations in cardiovascular functions¹⁹. Thus, one of our aims is to evaluate the pathological mechanisms leading to bladder injury and related cardiovascular dysfunction due to the cross-talk between visceral organs, using the rat model of acute or chronic cold exposure, with or without immersion of the lower part of the body into ice-cold water for varying time periods. The experimental findings will be compared to those obtained in human studies.

Additionally, we intend to study the effects of acute or chronic, continuing or intermittent cold exposures on hematological responses. Namely, it has been shown that cold exposure causes hemo-concentration, decrease in neutrophil count, and increased adhesion of the neutrophils to the endothelial wall^{20 21}. These hematological alterations and pro-inflammatory mechanisms could significantly change the Warfighters' response to injury and reduce the level of their physical endurance. Our experimental results will be compared with findings of human studies.

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V. FINAL DATA ANALYSIS

V.1. Statistical Analysis

For each of the performed functional tests, the data from the three laboratories (Case Western University, University of Iowa, and University of Oklahoma Health Science Center) were compiled into one database, and analyzed with the JMP Version 7 Statistical Discovery software¹. The measured data were initially examined for pair-wise correlation using the multivariate platform in JMP. After eliminating the non-independent cross-correlated measured values, since these will not result in additional information, analysis of variance (ANOVA) was performed on the fit model platform. The models used were resolution V models, which included main effects as well as two-factor interaction effects. Finally, the effects of any factor are reported as significant only when the F ratio gave a "p" value of less than 0.05.

Here we demonstrate our analytical approach by using the data sets for representative motor (inclined plane) and cognitive (T-maze) tests.

V. 1.1. Inclined Plane Test

This test was used to test balance, muscle strength and coordination. The test was carried out at a 60° and 90° incline (slope) levels. Latency to climb to the top of the screen and/or latency to fall were recorded, and an overall score was calculated.

Test Plan:

The test design is a $2 \times 2 \times 3 \times 2 \times 3 = 72$ full factorial design with factors and their levels:

- | | |
|--------------------------|------------------------|
| 1. Stress level: | Normal vs Stressed |
| 2. Performance Enhancer: | Vehicle or Drug |
| 3. Stress Type: | Heat, Hypoxia, or Cold |
| 4. Slope (degree): | 60° vs 90° |
| 5. Day: | 1, 2 or 3 |

There were many reported measurements for each run. However, Latency to Top (seconds), Latency to Fall (seconds) and % Success appear to be the only truly independent response variables. All other reported data on Standard Deviations (SD) and Standard Error of the Measurements (SEM) are derived values from these three independent measurements. In addition, the Score is derived from the % Success (or vice versa) and only one needs to be evaluated during the ANOVA technique. The three independent measurements are used to perform Analysis of Variance (ANOVA) with the test variables being the five factors enumerated above. For simplicity, results are reported in terms of the stress levels and performance enhancers. Initially the regression model used for ANOVA included two-factor interactions,

$$\begin{aligned}
 Y = & I + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_5X_5 \\
 & + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{15}X_1X_5 + a_{23}X_2X_3 + a_{24}X_2X_4 \\
 & + a_{25}X_2X_5 + a_{34}X_3X_4 + a_{35}X_3X_5 + a_{45}X_4X_5 + \text{error}
 \end{aligned}$$

Where (except for the intercept I and the error terms) the first five terms are the main effects of the five factors and the last ten terms are the two-factor interactions terms.

Our analyses address the following questions:

A. What affects the Latency (seconds) to Top?

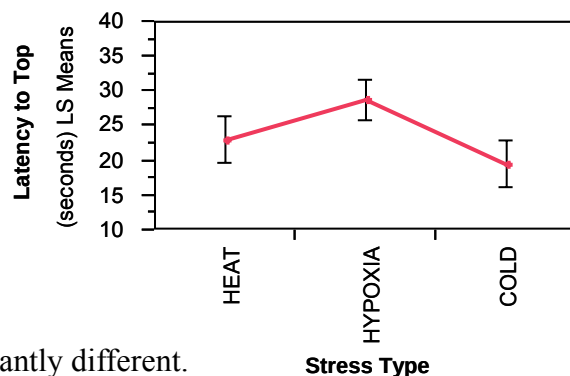
This test estimates the animals' ability to climb to the top on inclined plane by measuring the time necessary to achieve this task; thus, the shorter time demonstrates better motor function.

1. Stress type has the strongest effect: hypoxia is the most powerful stressor, which affects this motor task, compared to heat stress or cold stress. In average, the animals exposed to hypoxia needed about 5 seconds longer to reach the top compared to heat-exposed rats, and about 9 seconds longer compared to cold exposed animals.

LSMeans Differences Student's t

$\alpha=0.050$; $t=2.001$

Level		Least Sq Mean
Hypoxia	A	28.602800
Heat	B	22.956250
Cold	B	19.403981



Levels not connected by same letter are significantly different.

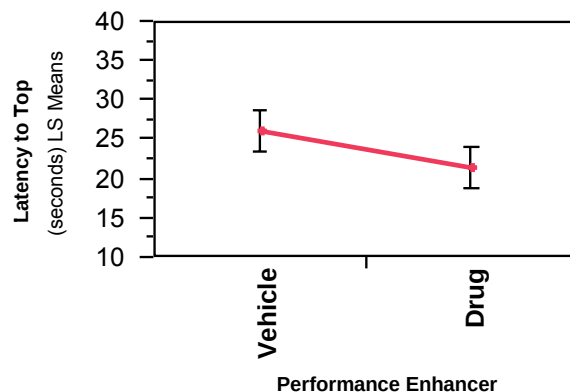
2. The Performance Enhancer, i.e., drug treatment, is a significant predictor of the Latency to Top.

In average, the drug reduced the time (Latency to Top) necessary to reach the top of the inclined plane by about 4.4 seconds, which represents a performance improvement by 17 %. Nevertheless, in the case of animals exposed to hypoxia, the drug treatment improved their performance by 31.4 %.

LSMeans Differences Student's t

$\alpha=0.050$; $t=2.001$

Level		Least Sq Mean
Vehicle	A	25.889735
Drug	B	21.418953

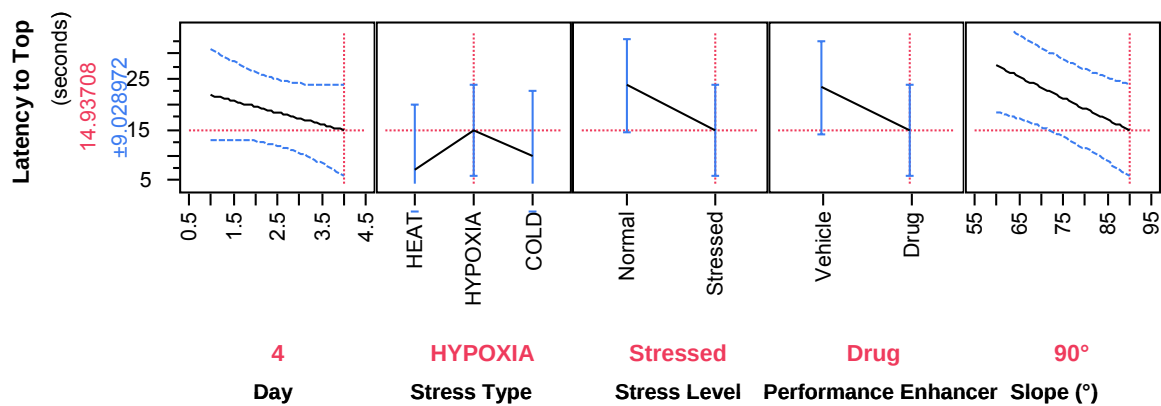


Levels not connected by same letter are significantly different.

3. What are the Conditions for Maximum Reduction in Latency to Top for Each Stress Type?

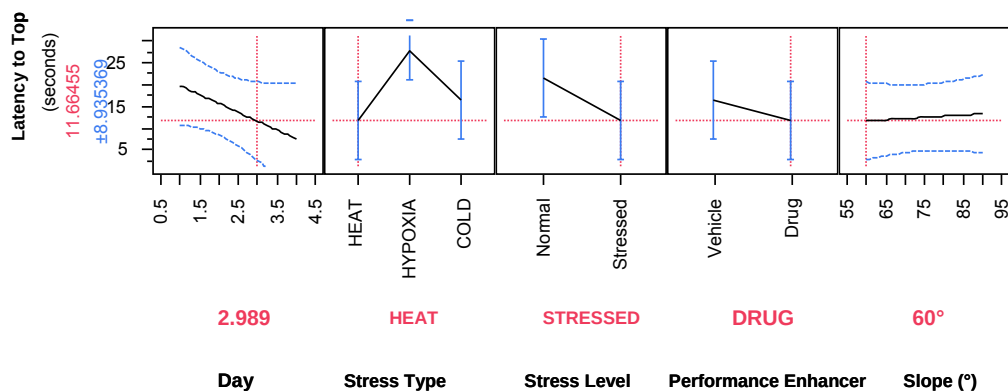
Hypoxia: Day 4, with drug at 90° results in Latency to top of 14.9 seconds.

Prediction Profile



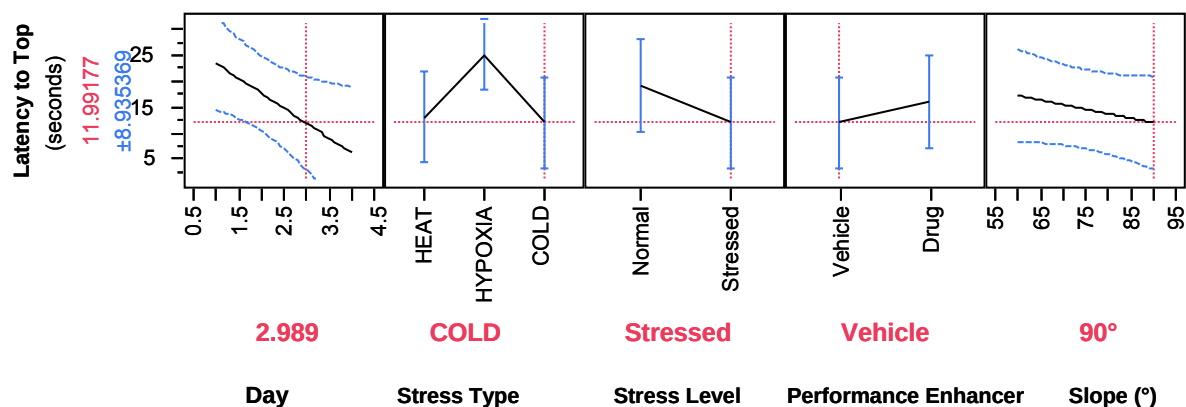
Heat: Day 3, with drug, 60° results in Latency to Top of 11.7 seconds.

Prediction Profile



Cold: Day 3, without drug, at 90° results in Latency to Top of 12 seconds.

Prediction Profile



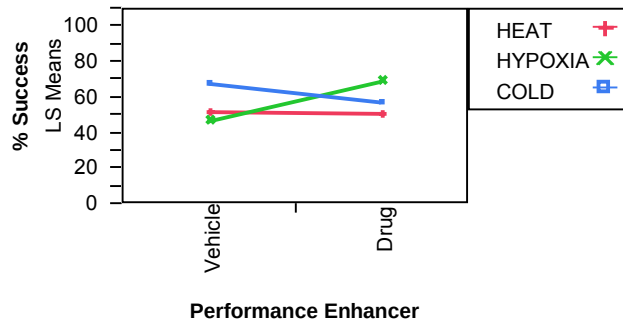
B. What Affects % Success?

1. The two factor interaction of Day and Slope. At high slope, the % success increases with days suggesting a learning ability.
2. Treatment (Performance Enhancer) significantly improves the % success of the task.

LS Means Differences Student's t

$\alpha=0.050$; $t=2.001$

Level		Least Sq Mean
Hypoxia, Drug	A	68.089472
Cold, Vehicle	A	66.991065
Cold, Drug	A B	56.573750
Heat, Vehicle	B	51.642315
Heat, Drug	B	50.035185
Hypoxia, Vehicle	B	45.885028



Levels not connected by same letter are significantly different.

V.1.2. T-Maze Tests

Design:

A $2 \times 3 \times 2 \times 2 = 24$ full factorial design, with the following factors and their levels:

1. Day: 1 v 2
2. Stress Type: heat stress; high-altitude hypoxia; and cold stress
3. Stress Level: Normal vs Stressed
4. Performance Enhancer: Vehicle vs Drug

Total = 24×4

Note: this is a small sample size.

Conclusions with T-Maze data:

A. Time (seconds):

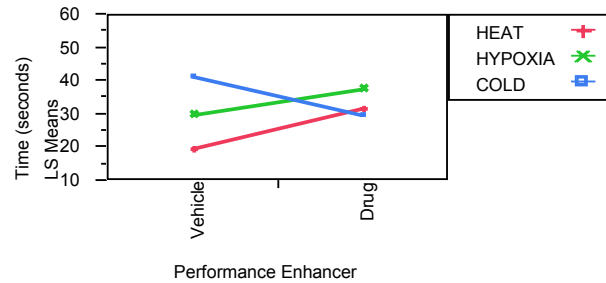
1. Stress Type and Treatment, i.e., Performance Enhancer Relation

For Stress type, the Performance Enhancer, i.e., the presence or absence of treatment has the strongest significant effect. With T-Maze, the drug appears to reduce the time for cold stress but not for hypoxia or heat stress.

LSMeans Differences Student's t

$\alpha = 0.050$; $t = 2.16037$

Level		Least Sq Mean
Cold, Vehicle	A	40.903199
Hypoxia, Drug	A B	37.281448
Heat, Drug	A B	31.558656
Cold, Drug	A B C	29.357118
Hypoxia, Vehicle	B C	29.300562
Heat, Vehicle	C	19.137376



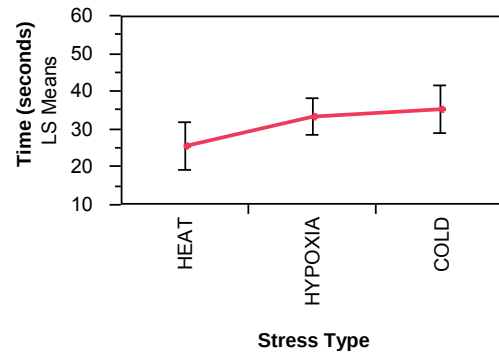
Levels not connected by same letter are significantly different.

Moreover, the stress type influences the time necessary for the T-maze task. For example, cold increases this time more than exposure to heat.

LSMeans Differences Student's t

$\alpha = 0.050$; $t = 2.16037$

Level		Least Sq Mean
Cold	A	35.130159
Hypoxia	A B	33.291005
Heat	B	25.348016



Levels not connected by same letter are significantly different.

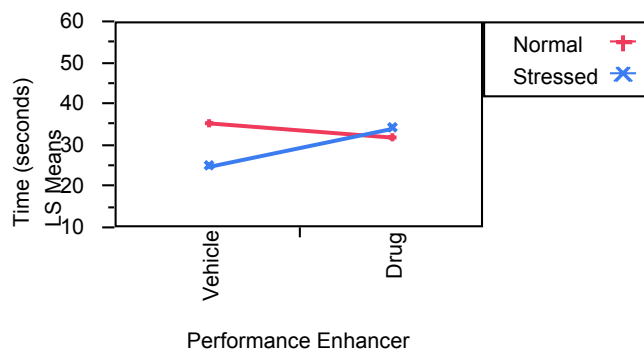
2. Stress Level (Normal or Stress) and Treatment, i.e., Performance Enhancer Relation

For the Stress Level, the Performance Enhancer (i.e., absence or presence of the drug) has the second strongest effect when the Time (sec) necessary to complete the task is analyzed. The drug appears to lower the time with normal stress but increases it when stress is already present.

LSMeans Differences Student's t

$\alpha = 0.050$ $t = 2.16037$

Level		Least Sq Mean
Normal, Vehicle	A	35.225805
Stressed, Drug	A	33.859799
Normal, Drug	A B	31.605015
Stressed, Vehicle	B	24.334954



Levels not connected by same letter are significantly different.

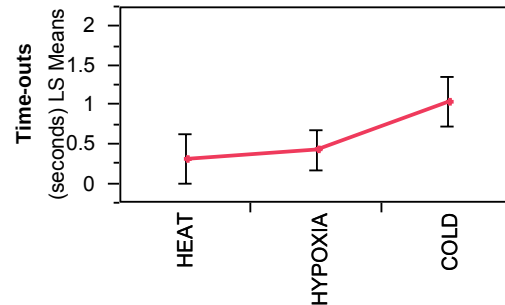
B. Time-outs (seconds):

The Stress Type is the only factor significantly affecting the Time-out time: cold exposure causes longer Time-out than either hypoxia or heat stress.

LSMeans Differences Student's t

$\alpha=0.050$; $t=2.16037$

Level		Least Sq Mean
Cold	A	1.0434524
Hypoxia	B	0.4218254
Heat	B	0.3065476



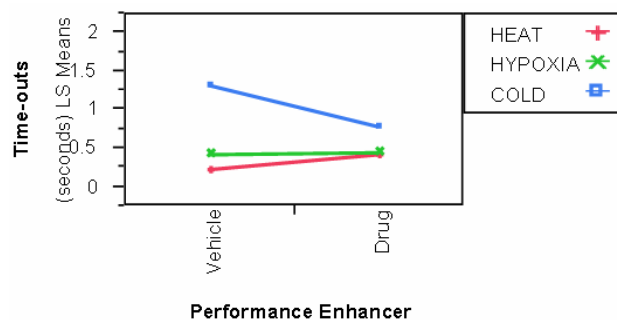
Levels not connected by same letter are significantly different.

The drug treatment significantly reduced the duration of Time-outs for cold stress.

LSMeans Differences Student's t

$\alpha=0.050$; $t=2.16037$

Level		Least Sq Mean
Cold, Vehicle	A	1.3145833
Cold, Drug	A B	0.7723214
Hypoxia, Drug	B	0.4416667
Hypoxia, Vehicle	B	0.4019841
Heat, Drug	B	0.3979167
Heat, Vehicle	B	0.2151786



Levels not connected by same letter are significantly different.

V.2. References

1. Sall J, Creighton L, Lehman A. *JMP Start Statistics: A Guide to Statistics and Data Analysis Using JMP*. 4th ed. Cary, North Carolina: SAS Institute, Inc., 2007.

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