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COLD INJURY - PHARMACOLOGIC PREVENTION

FINAL REPORT

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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The objective of this study was:

1. To develop an animal experimental model that would allow quantitative evaluation of tissue loss secondary to local cold exposure (frostbite), such that measures of possibly protective value could be evaluated.

2. To determine whether such frostbite injury is a rewarming injury rather than tissue damage due solely to cold or freezing.

3. To determine the pathogenesis of the rewarming injury, specifically, whether associated with:

a. Superoxide release

b. Complement activation

c. Leukoembolus

d. Prostaglandin or prostacyclin activity

e. Other non-specific aspects of the inflammatory response blocked by steroids or non-steroidal anti-inflammatory agents.

f. Platelet aggregation.

4. To evaluate the timing of administration of any agent found to be protective to determine whether they are effective if given prior to, during, or after cold exposure. (K-1) ←

Adult New Zealand white rabbits weighing 1500-2000 grams were anesthetized with sodium pentothal. Ringers lactate was administered I.V. at 0.5 ml/min into a vein in the right ear.

The left ear was shaved and a thermoprobe connected to a Yellow Springs recorder inserted near the midline of the ear 2.5 cm from the top.

Marks were made with a colored skin pencil at centimeter intervals from the top of the ear to assure subsequent accurate levels of immersion and cold exposure.

The 7 cm diameter chamber, for immersion of the ear, was placed within a 20 cm diameter outer chamber filled with dry ice and alcohol.

The inner immersion chamber was filled with a 50% alcohol solution in water. When water alone was used in preliminary experiments, it formed ice crystals on the ear which obscured a precise end point for cooling and rewarming.

With the anesthetized animal lying on its side, the left ear was immersed to the 5 cm mark for a period of 15-20 minutes, as required to produce a reading of -5oC. In preliminary experiments, temperatures were plotted every 2 minutes, but they were so repetitive that they subsequently were omitted and only checked randomly.

When the thermoprobe recorded a $-5^{\circ}C$ tissue temperature, the dry ice and alcohol solution in the outer chamber was quickly removed and the outer container promptly filled with warm water at $62^{\circ}C$. Characteristically, it required 2-3 minutes for the ear temperature probe to register 37oC at which time the ear was removed from the immersion unit.

The animals were returned to their cages when awake and the ears inspected daily.

This animal model is a modification of the technique originally designed in this laboratory by Weatherly-White, Paton and Knize (4-5).

Quantitation of Tissue Damage. Prior to cold immersion, a silhouette of the distal 5 cm of the left ear was traced on a piece of heavy cardboard. A cutout of this template was weighed as the control.

Seven days following cold exposure, the animal was sacrificed and the black gangrenous top of the ear excised. The silhouette of the remaining viable part of the ear was traced on carboard using the 5 cm marked as the baseline. The truncated silhouette was cut out and weighed.

The percent of ear tip loss caused by cold was expressed as:

$$\frac{\text{Weight of Template 7 Days Following Cold}}{\text{Weight of Template Prior to Freezing}}$$

The mean percent loss in this cold immersion model was 71% of the distal 5 cm of the ear with a 28% standard deviation in the untreated animals. The cold exposure was designed to be sufficiently mild to permit detection and quantitation of protective techniques.

In addition to more than 30 preliminary experiments to establish the mean level of tissue loss in untreated animals, randomized simultaneous controls were used in at least 1 in 10 animals in each drug study to confirm that other factors such as

possible cold adaptation during the winter months or variation in the animal sources did not skew the model.

TEST GROUP

A. Saline

1.5 ggt/min was started 15 minutes prior to cooling and continued for 3 hours (2 1/2 hours after rewarming).

B. Dimethyl Sulfoxide (DMSO)

DMSO is a free oxygen radical scavenger used extensively as a cryobiologic preservative (5).

1. Started Before Cooling (15 animals)

Fifteen minutes before cold immersion a DMSO solution (4 grams of DMSO in 24 ml physiologic saline) was infused intravenously at a rate of 750 mgm DMSO/hour to a 1500 gram animal into the right ear of a rabbit. The infusion continued during and for 2 1/2 hours after rewarming -- a total of 3 hours.

2. Started Upon Rewarming (10 animals)

The identical protocol was used except that the DMSO solution was begun only after the tissue temperature had reached $-5^{o}C$ and rewarming begun.

3. Local DMSO (14 animals)

When the tissue temperature reached $-5^{o}C$ the alcohol/saline solution in which the ear was immersed was quickly replaced with a 100% DMSO solution warmed to $37^{o}C$ and the ear kept in contact with DMSO for 1 hour.

C. Dimethyl Thiourea (10 animals)

Dimethyl thiourea was given intravenously at a dose of 0-5 mgm/kg B wt/hour starting 15 minutes prior to cooling and continued for three hours in a manner equivalent to use of DMSO in A-1 above.

D. Allopurinol (AP)

Allopurinol in its role as a xanthine peroxidase inhibitor blocks formation of hydroxyl ion radicals (6).

1. Oral AP for 3 Days Before Cold Exposure (9 animals)

Oral AP 50 mgm/Kg B wt was given 48, 14 hours and immediately before cold exposure to each animal (50 mgm/Kg B wt.

2. I.V. AP Before and After Cold (16 animals)

The sodium salt of AP was provided by Dr. S. Winston Singleton of Burroughs Wellcome, Research Triangle Park, NC, for intravenous administration. Fifty mgm/Kg B wt was given intravenously over a period of 5 minutes immediately before cooling in the opposite ear. Four hours after rewarming the I.V. AP was repeated.

3. I.V. AP When Cold and Repeated 4 Hours Later (22 animals)

The same protocol as in C-2 was used except that the intravenous AP was started only when the ear had reached $-5^{\circ}C$. The drug was repeated 4 hours later.

4. Oral AP Plus DMTU (12 animals)

Oral AP was given for 3 days when DMTU was begun intravenously 15 minutes prior to cooling and continued for 3 hr.

E. Combinations of Allopurinol (AP) and DMSO

1. Oral AP 2 Days and I.V. DMSO Pre-Cold Exposure (39 animals)

The animals were given oral AP 50 mgm/Kg B wt 48 and 24 hours prior to cold exposure and DMSO 750 mgm/Kg B wt started intravenously starting 15 minutes prior to cold exposure and continued 3 hours.

2. Oral AP 2 Days and IV. DMSO Started When Cold (13 animals)

The protocol was as above, the DMSO was not begun until the tissue temperature had reached -5°C.

3. I.V. AP and DMSO Both Started 15 Minutes Prior to Cooling (9 animals)

Continued for 3 hours.

4. I.V. AP and DMSO Both Started 15 Minutes Prior to Cooling (23 animals)

Continued for 3 hours plus AP daily x 2 thereafter.

F. Nitrogen Mustard (NM_2)

If the cytotoxic oxygen-free radical arises primarily from leukocytes in cold injury, it was reasoned that leukopenia might be protective.

1. Nitrogen Mustard for 3 Days Prior to Cold Exposure (26 animals)

Three days prior to cold exposure the animals were given 1.75 mgm/Kg B wt oral Nitrogen Mustard which reduced the circulating leukocyte count from 7,000-8,000 to 300-500/ml. Less

than 1% of the circulating cells were polymorphonuclear. The peripheral white blood cells were counted on each animal to confirm leukopenia prior to cold exposure.

2. Intravenous NM_2 for 3 Days Plus DMSO Prior to Cooling (12 animals)

Intravenous NM_2 1.75 mgm/Kg B wt was administered daily starting 3 days prior to cooling. In addition DMSO was started 15 minutes prior to cooling and continued for 3 hours through and after the cooling period.

This drug regimen was so rigorous that half of the animals did not survive the immediate test period. Only the 12 animals surviving 7 days were available for inclusion in the study.

G. Imidazole I.V. (9 animals)

The thromboxane synthenase inhibitor (7) Imidazole was administered intravenously at a rate of 25 mgm/Kg/hour starting 30 minutes before cooling and continuing for 1 1/2 hours thereafter.

H. N-acetyl Cysteine (11 animals)

This free radical scavenger (8) was given intravenously at a rate of 150 mgm/Kg starting 15 minutes prior to cooling and continued at a rate of 10 mgm/Kg/hour for 3 hours. At the end of this time a final bolus of 100 mgm/kg was given intravenously.

I. Persantine (12 animals)

The anti-platelet drug Persantine (0-25 mgm/Kg) was administered I.V. starting 15 minutes prior to cooling and repeated when the ear temperature registered -5°C.

J. Motrin Prior to Cooling (34 animals)

The prostaglandin inhibitor (9) Motrin was given as a bolus (10 mgm/Kg) 15 minutes prior to cooling and repeated 4 hours later -- 3 1/2 hours following rewarming.

K. Colchicine (8 animals)

Colchicine immobilized leukocytes (10). Dosage was 0.1 mgm I.V. 24 hours prior to cooling and daily x 3 thereafter.

L. Verapamill Before Cooling (30 animals)

This calcium channel blocker (11) was given (0.2 mgm/Kg) 15 minutes prior to cooling and repeated 3 hours later.

M. Solumedrol

1. Prior to Cooling (24 animals)

Solumedrol 30 mgm/Kg was given I.V. 15 minutes prior to cooling and repeated 4 hours later.

2. When Cold (35 animals)

The 30 mgm/Kg steroid was administered when the ear temperature registered -5°C and was repeated 4 hours later.

N. Solumedrol, Allopurinal and Motrin (13 animals)

Each of these three drugs was given simultaneously when the tissue temperature reached -5°C and was repeated 4 hours later.

Solumedrol I.V. 30 mgm/Kg

Allopurinal I.V. 50 mgm/Kg

Motrin I.V. 10 mgm/Kg

O. Superoxide Dismutase (SOD)

1. Prior to Cooling (26 animals)

Superoxide Dismutase (Sigma Chemical Co.) was dissolved in physiologic saline and given I.V. 15 minutes prior to cooling. The dose was 25 mgm/Kg.

2. Continued 2 Hours After Cooling (10 animals)

SOD 2.5 mgm/Kg was given as a bolus when the tissue temperature reached $-5^{\circ}C$ and continued at a rate of 2.0 mg/Kg for 2 hours.

GRP.	AGENT	DOSE SCHEDULE	NO. ANIMALS	% TISSUE LOSS MEAN	S.D.	P VALUE
A.	Saline	I.V. 15" pre-freeze contd. for three hours.	100	71	±28	--
B.	DMSO	1) I.V. 15" pre-freeze contd. for three hours.	15	46	±30	<0.001
		2) I.V. on rewarming.	10	53	±22	<0.05
		3) Local to ear on rewarming	14	55	±38	<0.05
C.	Dimethyl Thiourea (DMTU)	IV 15" pre-freeze contd. three hours.	10	92	±18	<0.5
D.	Allopurinol (AP)	1) Oral 48 and 24 hours pre-freeze.	9	45	±29	<0.005
		2) I.V. 15" prior to cold and repeated four hours later.	16	18	±39	<0.0005
		3) I.V. when cold; repeated four hours later.	22	32	±18	<0.0005
		4) AP and DMTU. P.O. AP 48 and 24 hours pre-freeze. I.V. DMTU 15" pre-freeze contd. three hours.	12	69	±20	<0.35
E.	Allopurinol (AP) and DMSO	1) Oral AP 48 and 24 hours pre-freeze. I.V. DMSO 15" pre-freeze contd. three hours	39	24	±27	<0.05
		2) Oral AP 48 and 24 hours pre-freeze. I.V. DMSO started when cold contd. three hours	13	54	±35	<0.35
		3) I.V. AP 15" pre-freeze. I.V. DMSO pre-freeze contd. three hours.	9	48	±40	<0.01
		4) I.V. AP 15" pre-freeze. I.V. DMSO 15" pre-freeze contd. three hours. Oral AP Day 1 and 2 after freezing.	23	44	±38	<0.0005

	AGENT	DOSE SCHEDULE	NO. ANIMALS	% TISSUE LOSS MEAN	S.D.	P VALUE'
F.	Nitrogen Mustard (NM_2)	1) I.V. 72 hours pre-freeze	26	40	±35	<0.0005
		2) NM_2 and DMSO. I.V. NM_2 72 hours pre-freeze I.V. DMSO 15" pre-freeze contd. three hours. (This killed 50% of animals.)	12	41	±31	<0.0005
G.	Imidazole	I.V. 15" pre-freeze contd. 1.5 hours.	9	82	±23	<0.5
H.	N-Acetyl Cysteine	I.V. 15" pre-freeze contd. three hours.	11	76	±33	<0.5
I.	Persantine	I.V. 15" pre-freeze repeated when cold	12	34	±34	<0.0005
J.	Motrin	1) I.V. 15" pre-freeze repeated four hours later.	34	26	±18	<0.0005
		2) I.V. started when cold repeated four hours later.	19	28	±12	<0.0005
K.	Colchicine	I.V. bolus 24 hours pre-freeze 15" pre-freeze, on rewarming and daily x 3	8	65	±28	<0.3
L.	Verapamil	I.V. 15" pre-freeze repeated two hours later.	30	34	±27	<0.0005
M.	Solumedrol	1) I.V. 15" pre-freeze repeated four hours later.	24	23	±18	<0.0005
		2) I.V. started when cold repeated four hours later.	35	34	±26	<0.0005
N.	Solumedrol Allopurinol Motrin	All I.V. started when cold repeated four hours later.	13	24	±19	<0.0005
O.	Superoxide Dismutase (SOD)	1) I.V. is prior to cooling	26	29	±25	<0.0005
		2) I.V. when cold and contd. for two hours	10	42	±11	<0.0005

DISCUSSION

At the date of Navy contract expiration (September 1983), this was the status of our data. There was a 3 month hiatus before the Army assumed the financial responsibility for the project.

At the time of Navy contract termination on the basis of this data we drew the following conclusions:

1. An effective accurate animal model had been developed.

2. The following were shown NOT to have protective effect against tissue damage by cold in this model.

a.	Dimethyl Thiourea	(group C)
b.	AP and DMTU	(group D 4)
c.	AP and DMSO	(group E 2)
d.	Imidazole	(group G)
e.	N-acetyl Cysteine	(group H)
f.	Colchicine	(group K)

3. Suggestive of protective effect when given before rewarming were:

a.	DMSO	(group B-1)
b.	Allopurinol	(group D 1-3 and E4)
c.	Nitrogen Mustard	(group f)
d.	Persantine	(group 1)
e.	Motrin	(group J)
f.	Verapamil	(group L)
g.	Solumedrol	(group M & N)
h.	Superoxide Dismutase	(group C)

Taken in sum at this stage of the study, it was hypothesized that tissue loss from frostbite had a significant factor of rewarming injury and that at least a part of such damage could be blocked by pharmacologic methods.

It would seem from these data that rewarming damage must be multifactorial, for these agents work at various parts of the inflammatory response. The most reasonable hypothesis is that the fundamental course is a release of super oxide at the time of rewarming. This hypothesis is supported by the protective value of superoxide dismutase (SOD). There is no other reason why SOD is protective and heat inactivated SOD in a double blind test not protective.

Although dimethyl sulfoxide (DMSO) has other pharmacologic actions, its protective effect could be due to its role as an oxygen scavenger.

Allopurinal's protection also supports the key role of superoxide as the offending agent since allopurinal as a xanthine oxidase inhibitor blocks superoxide production.

The protection provided by leukopenia produced by nitrogen mustard suggests that the offending superoxide comes from circulating leukocytes. Leukopenia would decrease the availability of superoxide from this origin.

The protective effect of corticosteroids and motrin could be explained by their non-specific effect in modulating the immune response.

How or why the calcium blocker verapamil is protective is purely hypothetical.

The anti-platelet drug persantin might be protective by minimizing platelet trapping in the rewarmed ear and therefore decreasing the amount of released thromboxane.

This discussion is an attempt to fit the observed data into a single hypothesis as to the mechanism of rewarming injury following local cold exposure.

Practical Implications. The obvious question is how these data can be of practical benefit to the armed forces. If, as we have shown, frostbite is primarily a rewarming injury, clinical benefit will only result if proper pharmacologic treatment is begun before rewarming. Obviously, this seldom will be possible under combat conditions. However, in both military and civilian practice there may be occasional clinical scenarios where this can be accomplished. It is therefore thought advisable to find the optimal pharmacologic approach to prevention of local tissue injury by drug administration prior to rewarming.

CONCLUSIONS

1. A reproducible and quantifiable animal model of frostbite damage has been developed. It consists of acutely lowering tissue temperature of a rabbit's ear to $-5^{\circ}C$ with rapid rewarming. Tissue damage is measured by the percent of ear lost by gangrene 7 days after cold exposure.

2. The following agents administered prior to rewarming are significantly protective against tissue loss:

   Dimethyl sulfoxide

   Allopurinol

   Nitrogen mustard

   Persantine

   Motrin

   Verapamil

   Solamedrol

   Superoxide dismutase

3. It is hypothesized that these drugs variously act by blocking cellular toxicity of superoxide released upon rewarming or by a non-specific blocking action of the inflammatory response caused by high-energy oxygen radicals.

4. Frostbite is primarily a rewarming injury. This provides a challenge to minimize or prevent tissue loss if treated prior to rewarming.

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