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ROLE OF GRAM-NEGATIVE BACTERIA AND THEIR ENDOTOXINS IN RAT DEATH--ETC(U)  
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the experiments were repeated in rats found to be free of extra-intestinal gram-negative bacteria (group II). After heat stress, short (<10h) and intermediate (>10h but <72h) survivors of group I had a significantly increased incidence of duodenal invasion (gram-negative bacterial count  $\geq 1.5 \times 10^4$ /g), as compared to controls. Intermediate survivors also had extra-intestinal invasion (a significantly increased incidence of gram-negative bacteria or endotoxins in liver, spleen or blood). These signs of invasion were generally associated with low bacterial count and LAL activity. Short and intermediate survivors of group II had duodenal, but not extra-intestinal invasion. No significant differences in the mean survival times of groups I and II were found. Duodenal invasion did occur after heat stress. This did not result in extra-intestinal invasion in group II and even the extra-intestinal invasion noted in group I had no significant impact on length of survival. Signs of extra-intestinal invasion in group I were likely the result of factors present prior to heating. Therefore, extra-intestinal invasion of gram-negative bacteria and their endotoxins did not appear to play a mediating role in rat death after heat stress.

Role of Gram-Negative Bacteria and their Endotoxins  
in Rat Death after Heat Stress.

Running title: Endotoxemia after Rat Heat Stress.

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## ABSTRACT

Stress which induces an increase in the gram-negative bacterial count of the upper intestinal tract can be associated with extra-intestinal invasion of gram-negative bacteria and their endotoxins. The Limulus amoebocyte lysate (LAL) test and standard microbiological procedures were used to determine if duodenal and extra-intestinal invasion occurred in rat heat stress. Because gram-negative bacteria were found in extra-intestinal tissues (lung, liver and spleen) of some non-heated controls of the first rat group studied, the experiments were repeated in rats found to be free of extra-intestinal gram-negative bacteria (group II). After heat stress, short ( $< 10h$ ) and intermediate ( $> 10h$  but  $< 72h$ ) survivors of group I had a significantly increased incidence of duodenal invasion (gram-negative bacterial count  $\geq 1.5 \times 10^4/g$ ) as compared to controls. Intermediate survivors also had extra-intestinal invasion (a significantly increased incidence of gram-negative bacteria or endotoxins in liver, spleen or blood). These signs of invasion were generally associated with low bacterial count and LAL activity. Short and intermediate survivors of group II had duodenal, but not extra-intestinal invasion. No significant differences in the mean survival times of groups I and II were found. Duodenal invasion did occur after heat stress. This did not result in extra-intestinal invasion in group II and even the extra-intestinal invasion noted in group I had no significant impact on length of survival. Signs of extra-intestinal invasion in group I were likely the result of factors present prior to heating. Therefore, extra-intestinal invasion of gram-negative bacteria and their endotoxins did not appear to play a mediating role in rat death after heat stress.

### Introduction:

Endotoxins or lipopolysaccharides are complex substances associated with the cell walls of gram-negative microorganisms. The release of endotoxins into the circulating blood may ultimately lead to a state of shock (5). A natural source or pool of gram-negative bacteria and their endotoxins is the intestinal tract. Increased number of gram-negative bacteria in the upper intestinal tract is associated with extra-intestinal invasion in cases of enteritis (14) and irradiation stress (15). The intestinal tract is also thought to be the source of the extra-intestinal invasion noted in other body stresses (8, 16, 19). There are a few isolated reports associating plasma endotoxins with the circulatory collapse and coagulative disorders noted in human heatstroke (2, 9). Furthermore, treatment to reduce gut flora increases length of survival in experimental dog heatstroke (1). To determine if gram-negative bacteria and their endotoxins were released in heat stress, the present study examined the incidence of *duodenal and extra-intestinal invasion in the rat model of experimental heatstroke* (11,12). Gram-negative bacterial invasion of the duodenum was found to occur after heat stress. The extra-intestinal invasion noted had little impact, for length of survival was not significantly different in rat groups with and without a significantly increased incidence of extra-intestinal invasion. Therefore, extra-intestinal invasion of gram-negative bacteria and their endotoxins did not appear to play a significant role in rat death due to heat stress.

### Materials and Methods:

Male Sprague-Dawley rats (Charles River, Wilmington, MA) were individually caged and given food and water *ad libitum*. When weight was between 498-515g, rats were placed in an environmental chamber maintained at

26°C and approximately 50 ± 20% relative humidity. After 5 days in this chamber, rats were fasted 18-24 hours and then either subjected to heat stress or examined as non-heated controls.

Rat heat stress followed the procedures of Hubbard (11, 12). Animals were first placed in small individual restrainer cages. A thermocouple of copper/constantan was inserted 6.5 cm into the rectum to measure core temperature using a Leeds and Northrup (North Wales, PA) multipoint temperature scanning system. Rats were then placed in the heat stress chamber (Napco incubator, Portland, OR) which was maintained at 41.5 ± 1°C. Core temperatures were determined at 1 minute intervals and thermal area (12) calculated when core temperature exceeded 40.4°C. Restrained rats were subjected to heat stress until obtaining a desired core temperature or thermal area. At this time, rats were removed from the heat stress chamber and restrainer cages and allowed to cool passively at an ambient temperature of 26°C.

Heated and non-heated rats were examined for the presence of endotoxins and gram-negative bacteria in blood, liver, spleen and lung samples. In addition, gram-negative bacterial counts and endotoxin activity in the duodenum were determined.

Sample collections: All blood samples were heparinized using heparin derived from beef lung (Upjohn Co., Kalamazoo, MI). Lateral tail vein blood samples were drawn before heating and after removal from the heat stress chamber. Agonal blood samples were collected from the heart. Tissue samples were obtained at death using sterile surgical techniques and endotoxin-free surgical instruments. Non-heated controls or those rats surviving 72 hours after heat stress were anesthetized by injection (0.3 ml/500g body weight) of sodium

pentobarbital (Abbott Labs, North Chicago, IL) into the lateral tail vein before sample collection.

Sample preparation: Blood samples for endotoxin assay were centrifuged (20x g) for 10 minutes to obtain platelet rich plasma (PRP). PRP was then diluted 1:3 in sterile endotoxin-free water (Travenol Labs, Mansfield, MA). A laminar flow, biohazard safety hood (Baker Co., Sanford, ME) was used to provide a suitable sterile environment for the mincing of tissue samples. Liver (1:2), spleen (1:6), lung (1:6) and duodenum (1:5) samples were then diluted with sterile, pyrogen-free saline (Travenol Labs). In the biohazard hood, liver, spleen and lung samples were homogenized using endotoxin-free teflon tip grinders and glass grinding vessels. Dilutions of 1:6, 1:12 and 1:24 of these tissue homogenates were placed in endotoxin-free screw cap glass tubes for extraction before endotoxin testing. Minced and diluted duodenal samples were blended in a vortex mixer for one minute and then centrifuged for one minute at 20x g to remove debris. Serial 1:2 and 1:10 dilutions of the supernatant were then made.

Endotoxin assay: The Limulus amoebocyte lysate (LAL) test (17) was used to detect the presence and activity of endotoxins in the blood, tissue and duodenal samples. The dilution + heating extraction procedure (3, 4) was employed to remove protein LAL inhibitors from the samples before testing. All samples for LAL testing were heat extracted except duodenal samples which required no extraction due to their high level of dilution. Pyrotell (Associates of Cape Cod, Woodshole, MA) LAL was used in a 0.1 ml. volume with 0.1 ml. of test sample. All readings were made after 1 h. of incubation in a 37° C waterbath. A test was considered positive by formation of a firm gel or a change in the viscosity of the reactants. Endotoxin positive samples were diluted serially 1:2 and LAL

tested to determine the end-point of endotoxin activity. The end-point dilution factor (highest dilution of sample in which a firm gel could be obtained by LAL testing) was then multiplied by the lysate sensitivity to obtain the endotoxin activity in the sample. Lysate sensitivity was determined by testing serial dilutions of Escherichia coli endotoxin (Associates of Cape Cod). Appropriate positive and negative controls were employed.

Gram-negative bacterial analysis: Both aerobic and anaerobic blood cultures were prepared from agonal blood samples (1-5 ml) using trypticase soy and thioglycolate broth (Becton-Dickinson, Rutherford, NJ). These were incubated at 37°C and tested over a 21 day period for the presence of gram-negative bacteria. Both direct and indirect methods for isolation of aerobic gram-negative bacteria from lung, liver and spleen homogenates were employed. Direct isolation involved inoculation of a small volume (0.05 ml) of the homogenate on selective (MacConkey, Difco, Detroit, MI) and non-selective (5% sheep blood agar) plating media. In indirect isolation, a large volume (1-2 ml) of the homogenate was first incubated in Brain heart infusion broth (Difco) and then inoculated on plating media. Using both methods, an estimate of the level of aerobic gram-negative bacterial invasion could be made. A pre-reduced anaerobically sterilized chopped meat broth (Carr-Scarborough, Inc., Stone Mountain, GA) was used to isolate anaerobic gram-negative bacteria from lung, liver and spleen homogenates. Gram-negative bacterial count per gram of duodenal sample was determined from colony counts made from 5 replicate MacConkey plates prepared from each dilution of the duodenal sample.

Though all rats appeared asymptomatic, gram-negative bacteria could be isolated from extra-intestinal tissues (lung, liver and spleen) of some non-heated controls of the first rat group studied. To ensure that the gut was the only

source of possible gram-negative bacterial extra-intestinal invasion, the study was repeated in a second group of rats in which all non-heated controls were found to be free of extra-intestinal gram-negative bacteria. With this group an additional LAL, Pyrotest (Difco) was used to confirm the endotoxin testing results obtained by the Pyrotell lysate.

The data from rat groups I and II were analyzed to determine the incidence of invasion of endotoxins and gram-negative bacteria in duodenal and extra-intestinal samples after heat stress. The incidence of invasion, length of survival and heat treatment of the two rat groups were compared. Statistical analysis employed chi square and student t- tests. Probability level for significance was  $< 0.05$ .

## RESULTS

The data from group I rats can be found in Table 1. An examination of the non-heated controls revealed that the duodenum had both low gram-negative bacterial count and LAL activity. It also indicated that gram-negative bacteria and positive LAL tests could be found in tissues of apparently healthy asymptomatic rats. This was especially true for lung tissue. After heat stress, both short and intermediate survivors had a significantly increased incidence of duodenal invasion (gram-negative bacteria count  $\geq 1.5 \times 10^4$ /gram or LAL activity  $\geq 50$  ng of E coli endotoxin/gram). The incidence of duodenal invasion in rats sacrificed 72 hours after heat stress did not differ significantly from control values. Extra-intestinal invasion (a significantly increased incidence of gram-negative bacteria or endotoxins in the blood, liver or spleen) occurred only in the intermediate survival group. The incidence of gram-negative bacteria or endotoxins in the lung did not significantly change after heat stress.

Most tissue isolates found in group I rats after heat stress were detected by indirect isolation (Table 1). Isolates found by the direct method were generally too few in number for accurate counting. Mean LAL activity in tissue and blood samples after heat stress (2.87 ng/g) was not significantly different from the activity noted in non-heated control rats (3.15 ng/g).

Figure 1 illustrates the relationship between length of survival and incidence of duodenal and extra-intestinal invasion after heat stress in group I rats. Lung invasion was not included in this comparison since it did not significantly change from control values (Table 1). The incidence of both duodenal and extra-intestinal invasion increased as length of survival increased. These indices of invasion decreased in rats sacrificed 72 hours after heat stress. Only the intermediate survivors had a significantly increased incidence for duodenal and any sign of extra-intestinal invasion, as compared to controls. The incidence for multiple signs of extra-intestinal invasion was never significantly different from control values.

Invasion in the second group of rats is reported in Table 2. No gram-negative bacteria were isolated from tissues or blood in non-heated controls, though some LAL activity was found. After heat stress, both short and intermediate survivors had a significantly increased incidence of duodenal invasion. Short survivors also had a significantly increased incidence of gram-negative bacteria in the lungs. No significantly increased incidence of extra-intestinal invasion was noted in group II. No significant changes in the endotoxin test results obtained by Pyrotell lysate (Table 2) were noted in group II rats when samples were retested with Pyrotest lysate.

Table 3 compares the control and intermediate survival data of the two rat groups. Controls from group I had a significantly higher incidence of gram-negative bacteria and LAL activity in the lung. No significant differences

in the incidence of high gram-negative bacterial count or LAL activity in the duodenum were noted between the two control groups. A comparison of the intermediate survivors indicated that there was no significant difference in the incidence of duodenal invasion, but group I had a significantly higher incidence of gram-negative bacteria in tissues and LAL activity in the spleen. The incidence of duodenal and extra-intestinal invasion in short and 72 hour survivors of groups I and II were not significantly different.

A comparison of heat treatments and mean survival times of groups I and II can be found in Table 4. Heat treatments were similar except for a significantly greater total thermal area of group I intermediate survivors. Repeated attempts to obtain group II intermediate survivors with increased thermal areas resulted in short survival. No significant differences in survival times were noted between the two groups.

#### DISCUSSION

Non-heated controls of group I were found to have gram-negative bacteria in extra-intestinal tissues. After heat stress, there appeared to be an association among duodenal invasion, length of survival and extra-intestinal invasion. However, the incidence for multiple signs of extra-intestinal invasion was not significantly different from control values (Figure 1). Furthermore, the number of gram-negative bacteria in liver and spleen samples was considered low, since most isolates were found by indirect isolation and low counts were associated with most direct isolations. Significant endotoxemia did not appear likely because extra-intestinal LAL activity after heat stress was not significantly different than the activity noted in non-heated controls. In addition, the increased incidence of invasion in group I appeared to have little impact on survival length (Table 4). Thus, it is likely that this invasion did not originate

from the gut but represented multiplication or spread of extra-intestinal bacteria and endotoxins present prior to heating.

Group II rats were free of extra-intestinal gram-negative bacteria prior to heating (Table 2). After heating, there was no significantly increased incidence of extra-intestinal invasion. This was confirmed by the retesting of samples with the Pyrotest lysate. This lysate is reported to be more sensitive to endotoxins of an impure state (18). Thus, if gut-derived endotoxins were in samples from group II rats, then this lysate would likely indicate their presence. Testing for endotoxemia was limited in this study by the low number of blood samples which could be collected. It has however, been reported that the existence of an ante-mortem endotoxemia can be determined by the finding of positive LAL tests in liver samples (6). Using both lysate sources, group II rats were not found to have a significant number with positive liver assays. Therefore, the number of group II rats experiencing a state of endotoxemia was insignificant.

Group II did have an increased incidence of invasion in the duodenum (short and intermediate survivors) and lung (short survivors). The consistency between the two rat groups for signs of duodenal invasion after heat stress may be due to multiplication of the low numbers of gram-negative bacteria normally present in this area or to the depositing of lower bowel gram-negative bacteria into the upper bowel. Isolates in the lungs of short survivors of group II rats may reflect the rat's coprophagic nature and represent spread of gram-negative bacteria from the oral cavity into the lung during extreme heat stress.

In conclusion, heat stressed rats resulting in both short and intermediate survival had a significantly increased incidence of duodenal invasion. In children with enteritis (14) and irradiated rats (15) increased levels of gram-negative bacteria in the upper intestinal tract are reported to result in endotoxemia. Similar findings occurred only in intermediate survivors of group I rats, but

appear to be associated with the animals' health status prior to heating. The group I findings were significant in that even rats of a suspect health status did not suffer levels of gram-negative bacteria and endotoxins in extra-intestinal test sites which had significant impact on length of survival. Therefore, extra-intestinal invasion did not appear to have a major role in death in the rat model for experimental heatstroke.

Why heat stress induced duodenal invasion did not result in extra-intestinal invasion as previously noted in other forms of stress is not known. Perhaps, the level of duodenal invasion was not sufficiently high enough to result in extra-intestinal invasion or the rats died before extra-intestinal invasion could occur. Thus, the lack of extra-intestinal invasion in heat stress may just reflect differences in the pathophysiology of different forms of stress.

These findings also do not explain why heat stressed dogs pre-treated to reduce gut flora experienced a significantly increased incidence of 18 hour survival (1). Perhaps, this is due to species differences and reflects the rat's natural resistance to endotoxin. However, Grun has previously used a rat model and the LAL test to elucidate a role for gut-derived endotoxins in galactosamine-hepatitis (10). Therefore, it has been established that the rat and the LAL test is suitable for the study of endotoxin invasion originating from the gut. Since thermal area was not determined in the dog study (1), it is possible that differences in length of survival are due to significant differences in the heat treatment experienced by the pre-treated and non-treated dog groups. This may not be a complete explanation, for rat groups with similar mean rectal temperatures and significantly different mean total thermal areas did not experience significantly different mean lengths of survival (Table 4). Thus, the treatment to reduce gut flora (antibiotics, cathartics and enemas) may increase 18 hour survival by increasing hydration or protecting gut tissue from damage

during heat stress. The possible reduction in damage may be due to the reduced exposure of gut tissue to endotoxins after heat stress. This might indicate a possible role in the pathophysiology of heat stress death for the duodenal invasion noted in rats.

The lack of support for a significant role for extra-intestinal invasion in rat heat stress death may indicate that the isolated reports of positive LAL tests from human heatstroke victims (2, 9) represent possible complications that can occur after heat stress or reflect the patient's health status prior to heatstroke. Thus, endotoxin may not be the mediator of the shock-like state associated with heat stress death. This conclusion conforms with other shock models in which significant evidence of extra-intestinal invasion associated with the shock syndrom is lacking (13, 20). Though invasion by gut-derived endotoxins is an attractive hypothesis as a possible common mediator of shock after stress (7), the present results indicate that this may not be true for all forms of shock associated with stress.

Table 1. ENDOTOXIN AND GRAM-NEGATIVE BACTERIAL INVASION IN GROUP I RATS AFTER HEAT STRESS

	Duodenal <sup>A</sup> Bacterial Invasion		Gram-Negative Bacterial Isolates In:				% with B		Duodenal <sup>C</sup> Endotoxin Invasion		% with Positive LAL Tests In:	
	Lung D	Lung I	Liver D	Liver I	Spleen D	Spleen I	Blood	Blood	Lung	Liver	Spleen	Blood
Short Survivors	38.5	64.3	18.4	37.1	14.3	30.0	7.7	47.1	35.7	13.2	15.0	6.3
< 10 h	N=20	N=14	N=38		N=21		N=13		N=17	N=14	N=20	N=32
Intermediate Survivors	83.3*	70.0	50.0*	61.5*	36.4	63.6*	40.0*	70.0*	50.0	28.6	36.4	45.6*
> 10 h but < 72 h	N=12	N=10	N=14		N=11		N=10		N=10	N=10	N=11	N=11
72 h Survivors	0.0	40.0	10.0	10.0	10.0	20.0	10.0	62.5	50.0	11.1	22.2	20.0
	N=8	N=5	N=10	N=10	N=10	N=10	N=10	N=8	N=4	N=9	N=9	N=10
Non-Heated Controls	7.1	20.0	40.0	4.7	20.0	6.6	13.3	0.0	30.0	42.9	12.5	13.3
	N=14	N=15	N=15	N=21	N=15	N=15	N=15	N=10	N=14	N=24	N=15	N=89

A = % with aerobic gram-negative bacterial count  $\geq 1.5 \times 10^6$  /g

B = includes both aerobic and anaerobic tests results

C = % with LAL activity  $\geq 50$ ng of *E. coli* endotoxin/g

D = Direct isolation

I = Indirect isolation

LAL = Limulus Amebocyte Lysate

N = Number tested

\* = differs significantly from control value (p < 0.05)

Figure 1. Relationship between length of survival and the incidence of duodenal and extra-intestinal invasion after heat stress in group I rats. Duodenal invasion: gram-negative bacterial count  $\geq 1.5 \times 10^4$  / gram or LAL activity  $\geq 50$  ng of *E. coli*/gram. Any sign of extra-intestinal invasion: gram-negative bacterial isolates or positive LAL tests in any test site (blood, liver and spleen). Multiple signs of extra-intestinal invasion: gram-negative bacterial isolates or positive LAL tests in more than one test site.

▨ DUODENAL INVASION

□ ANY SIGN OF EXTRA-INTESTINAL INVASION

■ MULTIPLE SIGNS OF EXTRA-INTESTINAL INVASION

\* DIFFERS SIGNIFICANTLY FROM CONTROL VALUE  
( $p < 0.05$ )

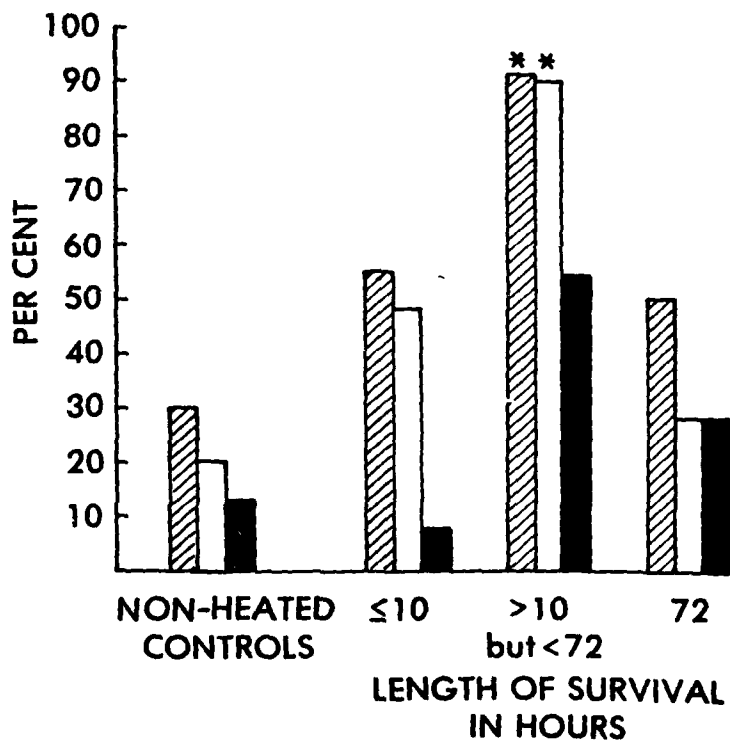


Table 2. ENDOTOXIN AND GRAM-NEGATIVE BACTERIAL INVASION IN GROUP II RATS AFTER HEAT STRESS

	Duodenal <sup>A</sup> Microbial Invasion		Gram-Negative Microbial Isolates In:				Duodenal <sup>C</sup> Endotoxin Invasion		% with $\bar{f}$ Positive LAL Tests In:			
	D	I	D	I	D	I	Blood	Lung	Liver	Spleen	Blood	
Short Survivors	91.7*	33.3*	33.3*	0.0	0.0	0.0	14.3	33.3	33.3	0.0	0.0	16.7
≤ 10 h	N=12	N=12	N=12	N=12	N=12	N=12	N=7	N=12	N=12	N=12	N=12	N=12
Intermediate Survivors	40.0*	6.7	13.3	0.0	0.0	0.0	6.7	66.7	13.3	6.7	0.0	20.0
> 10 h but < 72 h	N=15	N=15	N=15	N=15	N=15	N=15	N=15	N=15	N=15	N=15	N=15	N=15
72 h Survivors	14.3	0.0	0.0	14.3	14.3	14.3	0.0	28.6	14.3	28.6	14.3	14.3
	N=7	N=7	N=7	N=7	N=7	N=7	N=7	N=7	N=7	N=7	N=7	N=7
Non-Heated Controls	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.3	11.1	11.1	0.0	3.9
	N=18	N=56	N=18	N=18	N=18	N=18	N=18	N=18	N=18	N=18	N=18	N=18

Abbreviations and symbols as in Table 1.

$\bar{f}$  = shows results from Pyrotell testing only, Pyrotell results were not significantly different.

Table 3. COMPARISON OF ENDOTOXIN AND GRAM-NEGATIVE BACTERIAL INVASION IN INTERMEDIATE SURVIVORS AND CONTROLS OF RAT GROUPS I & II

Intermediate Survivors	Duodenal <sup>A</sup> Bacterial Invasion		Lung		Gram-Negative Bacterial Isolates In:		% with <sup>B</sup> Gram-Negative Bacterial Isolates In:		Duodenal <sup>C</sup> Endotoxin Invasion	% with Positive LAL Tests In:			
	Group I	Group II	D	I	D	I	D	I		Lung	Liver	Spleen	Blood
Group I	81.8	30.0	70.0*	50.0*	61.5*	36.4*	63.6*	40.0	70.0	50.0	28.6	36.4*	45.6
Group II	40.0	6.7	13.3	0.0	0.0	0.0	0.0	6.7	66.7	13.3	6.7	0.0	20.0
Controls													
Group I	7.1	20.0*	40.0*	4.8	20.0	6.6	13.3	0.0	30.0	42.8*	12.5	13.3	3.4
Group II	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.0	7.1	11.1	0.0	3.9

Abbreviations and symbols as in Table 1.  
\*significant difference between groups I and II (p < 0.05)

TABLE 4. COMPARISON OF HEAT TREATMENTS AND MEAN SURVIVAL TIMES IN RAT GROUPS I & II

	Mean Max <sup>f</sup> Temp. in Degrees C	Mean Total <sup>f</sup> Thermal Area in Degree- Mins.	Mean Survival Length In Hours
<b>Short Survivors</b>			
Group I	42.42 ± 0.28	82.78 ± 24.56	2.88 ± 2.38
Group II	42.41 ± 0.14	78.81 ± 18.85	2.25 ± 1.48
<b>Intermediate Survivors</b>			
Group I	42.31 ± 0.20	54.71* ± 10.80	19.00 ± 4.24
Group II	42.26 ± 0.13	37.92 ± 5.52	20.66 ± 8.21
<b>72 Hour Survivors</b>			
Group I	42.14 ± 0.27	52.85 ± 6.40	* *
Group II	42.00 ± 0.27	47.04 ± 18.24	* *

<sup>f</sup> = values are means ± standard deviation

\* = significant difference between groups I and II (p < 0.05)

\* \* = animals sacrificed 72 hours after heat stress

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