# Heat shock increases survival in rats exposed to hyperbaric pressure

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**Key words**
Decompression, bubbles, trauma & stress, Doppler, hyperbaric research

**Abstract**

(Medby C, Bye A, Wisløff U, Brubakk AO. Heat shock increases survival in rats exposed to hyperbaric pressure. *Diving and Hyperbaric Medicine*. 2008; 38: 189-193.)
It has been shown that a single bout of exercise performed 20 hours prior to hyperbaric exposure reduces bubble formation and increases survival in rats. Heat shock proteins (HSPs) are stress proteins expressed in cells that are exposed to different stressors. HSPs are known to protect cells, by binding to proteins and stabilizing them. As it is known that a single bout of exercise induces HSPs, and that HSPs exert their protective effects 20–24 hours after the stimulus for induction, we hypothesized that HSPs might be one mechanism behind the observed exercise-induced protection. We hypothesized that rats that expressed HSPs would develop fewer bubbles and have a lower mortality than their non-stressed control group. Twenty-four female Sprague-Dawley rats (300–330 g) were divided into a heat-shock group and a control group and anaesthetized. The rats in the heat-shock group were heated to 42 ± 0.5$^{O}$C for 15 min. The following day, all rats were compressed to 700 kPa for 45 min in a hyperbaric chamber. The right ventricles were insonated and bubbles were identified and graded. Six of 12 rats in the heat-shock group survived, while 1 of 12 control rats survived ($\chi^2 = 5.042$, P = 0.034). There was no difference in bubble grade between the groups. The study suggests that the effect of heat shock on survival is not the same as observed after exercise, as the heat-shocked rats developed bubbles. However, heat shock appears to protect rats against the effects of bubbles by an independent mechanism.

## Introduction

We have shown previously that a single bout of exercise 20 hours prior to a simulated dive reduces bubble formation and increases survival in rats exposed to hyperbaric pressure.[1] The precise mechanisms are not known, but nitric oxide (NO) seems to be involved, as NO synthetase (NOS) blockade promotes bubble formation in sedentary rats and NO donors protect against bubble formation.[2,3] It is known that NO plays an important role in adaptive defence of the cardiovascular system, in particular as a result of induction of heat shock protein-70 (HSP70) synthesis.[4] NO production is also increased with increased expression of HSP90.[5] Thus, a link between NO and HSPs has been established in the literature.

HSPs are stress proteins that are induced when cells are exposed to different stressors, such as hyperthermia, hypoxia, hyperbaric stress or exercise.[6] Increased expression of these proteins is associated with cell protection, probably by acting as molecular chaperones and rescuing denatured proteins. The protection is not only against the original stressor, but also against other stressors (cross-tolerance).[7]

A non-significant 23% reduction has been observed in the incidence of decompression sickness in rats after preconditioning with heat shock, whereas a significant protection occurred against the effects of venous air infusion.[8] In either case, symptoms or death are caused by gas bubbles in the circulation, obstructing and injuring the vessels. This leads to reduced blood flow and consequent ischaemia.

Preconditioning with whole body hyperthermia ('heat shock') has been shown not only to protect rats against subsequent hyperthermia that is otherwise fatal, but also against ischaemic injury to the heart and central nervous system. The protection is thought to be mediated through increased expression of HSPs, and the optimal interval between the preconditioning and the insult seems to be about 24 hours.[9] This is similar to our training-induced protection against bubble formation and death. Acute severe exercise also increases HSP expression in rats, and this increased expression protects rats against ischaemia.[7,10,11] It is tempting to speculate whether HSPs could be responsible for one of the mechanisms behind the exercise-induced protection against decompression, as the time frame for the exercise-induced protection and the expression of HSPs is similar.[12]

Thus the aim of the present study was to determine whether rats exposed to heat shock are protected against hyperbaric exposure similarly to exercised rats. The rats were exposed to the same hyperbaric stress as in the exercise studies, and would be expected to behave similarly if the underlying mechanisms of protection were the same. We hypothesized that rats exposed to heat shock would develop fewer bubbles and have a lower mortality than the control group.

For protein analysis, HSP70 was chosen on the basis of

being the uppermost inducible protein of all the HSPs. HSP90 was analyzed due to its capability of inducing NO-production, in addition to its chaperon effect. Both proteins are considered markers of HSP induction. We did not aim to show any causation.

## Methods

The study protocol was reviewed and approved by the Norwegian Animal Research Authority and all procedures were conducted in accordance with the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes*.

### MATERIAL

A total of 26 adult female Sprague-Dawley rats (300–330 g) (Kirkeby, Sweden) were acclimatized for a minimum of five days. Prior to the experiments, the rats were kept in cages with a volume of 46 L in groups of four to six. The room was artificially lit from 1800 to 0600 h. Temperature was maintained at 25.2 ± 1.7$^{O}$C, and humidity at 49 ± 4%. The rats were fed a pelleted rodent diet (rat and mouse standard diet, B&K Universal, UK) and water *ad libitum*; the rats were not fasted at any time.

### METHODS

Twenty-four rats were divided into a heat-shock or a control group. Rats from both groups were anaesthetized using a mixture of midazolam (1 mg.kg$^{-1}$), fentanyl (0.07 mg.kg$^{-1}$) and fluanisone (2 mg.kg$^{-1}$) as subcutaneous injections. The rats in the heat-shock group were placed in a custom-built heating chamber and heated for 31.2 ± 5.8 min until rectal temperature reached 42.0 ± 0.5$^{O}$C, maintained for 15 min. The cylindrical heating chamber was open at both ends, allowing room air to circulate freely. The temperature was regulated using electrical heating elements built into the wall. The rats were placed on a grate, in no direct contact with the heating elements. After the heat shock procedure, the rats were returned to the cage with free access to food and water and allowed to recover from anaesthesia.

Twenty-four hours later the rats did a simulated dry air dive in a hyperbaric chamber. Rats were compressed in pairs at 200 kPa.min$^{-1}$ to 700 kPa. After 45 min, the rats were returned to surface pressure at 50 kPa.min$^{-1}$ and immediately anaesthetized. The right ventricle was insonated using a GE Vingmed Vivid 5™ scanner, with a 10 MHz transducer. Bubbles were identified as bright spots in the ventricle or in the pulmonary artery, and graded on a scale from zero to five as described previously.[13] Doppler and slow-motion playback were used to aid grading. The rats were observed for 60 min, before surviving rats were sacrificed by decapitation. The hearts and the great vessels were examined for gas bubbles. The left ventricle of the heart and the aorta were dissected free, frozen in liquid nitrogen and stored at –80$^{O}$C for subsequent analysis of HSP70, HSP90 and endothelial NOS (eNOS) expression.

To investigate the relative contribution of the hyperbaric exposure to the expression of HSPs, two rats served as non-diving controls. The rats were treated like the control group, but were sacrificed without undergoing the compression–decompression procedure.

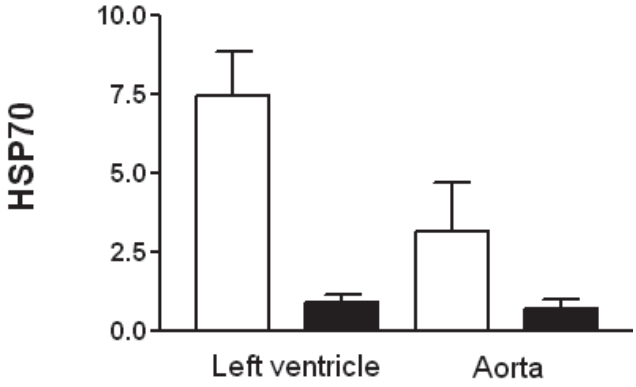
### WESTERN BLOT ANALYSIS

The expression of HSP70 (inducible), HSP90 and eNOS was determined by Western immunoblotting. Tissue samples (20 mg) from both the aortas and the left ventricles of the hearts were homogenized in cold lysis buffer (100 µL) using a Mixer Mill MM301 at 20–25 Hz. The lysate was centrifuged at 12,000 *g* for 5 min at 4$^{O}$C. The protein concentration in supernatant was quantified by using a Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) and was diluted with BupH Tris-HEPES-SDS Running Buffer (Pierce, Rockford, IL, USA) to a concentration of 1µg.µl$^{-1}$. Equal amounts of sample and sample buffer (12.5% Tris buffer (0.5 M), 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol and 0.05% bromophenol blue) were mixed together, and then denatured in boiling water for 5 min and stored on ice. 10 µg of total protein per sample was loaded on a 10% polyacrylamide gel (Pierce, Rockford, IL, US). Two standards were used on all gels: Magic Mark Western Standard (Pierce, Rockford, IL, US) and the pre-stained SeeBlue standard (Invitrogen, Carlsbad, CA, USA). The electrophoresis was performed under constant voltage (150 V) for 39 min. The proteins were then blotted onto a PVDF-membrane (BioRad, Hercules, CA, USA) under constant voltage (30 V) for 1 hour, using NU-PAGE Transfer Buffer (Invitrogen, Carlsbad, CA, USA). Nonspecific binding to the membrane was blocked by 5% bovine serum albumin in Tris-buffered saline (TBS) overnight at 4$^{O}$C. After two washes with TBS-T (containing Tween20) the blots were cut into appropriate pieces depending on the migration of the proteins on the gel. In this way, one might separate the bands of eNOS, HSP90, HSP70 and actin (housekeeping protein). Each piece of the membrane was incubated in its appropriate antibody for one hour at room temperature. The membrane pieces were subjected to four washes with TBS-T and incubated with their appropriate secondary antibody for one hour at room temperature. The membrane pieces were once again subjected to four washes with TBS-T and developed with a chemiluminescence detection system (Supersignal, WestFemto, Pierce, Rockford, IL, USA) for five min and exposed to film (Amersham ECL, Sweden). The results were quantified using VersaDoc Imaging system and QuantityOne software (BioRad, Hercules, CA, USA).

### STATISTICS

The Chi-square test was used to evaluate differences in survival between groups. Fisher's Exact test (1-sided)

**Figure 1**
**Semi-quantitative levels of heat shock protein-70 (HSP70) in left ventricular and aortic tissue; mean density ratios ± SD (arbitrary units)**
**□ Heat shock group ■ Control group**




was used to calculate *P* values. Western immunoblotting is a semi-quantitative method in which it is possible to detect relative differences in concentration. Differences in protein expression were evaluated using the Mann-Whitney test (two-tailed). A value of $P < 0.05$ was considered significant.

## Results

In the heat-shock group, six of 12 rats survived the observation period, while one of 12 control rats survived ($\chi^2 = 5.04$, df = 1, $P = 0.034$).

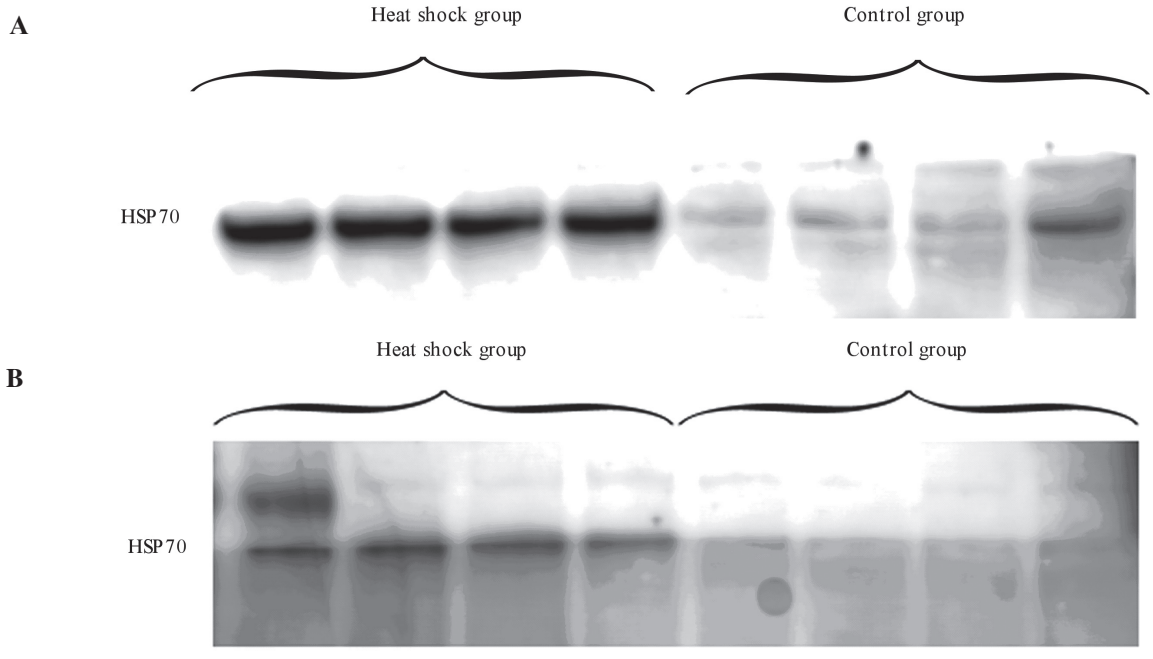
Eleven rats in each group developed severe bubbling, either grade 4 or fatal before they could be graded. In the control group all rats with bubbles died, while five rats in the heat shock group with bubbles survived ($\chi^2 = 6.47$, df = 1, P = 0.018). One rat in each group had bubble grade 0 during the entire observation period, and survived. At autopsy all rats that died before the end of the observation period had their hearts filled with blood foam. There was less gas in the hearts and vasculature of surviving rats, but bubbles could be identified in all rats, except the rats with a grade 0 bubble score.

Pretreatment with heat shock and exposure to hyperbaric pressure caused an eight-fold increase in HSP70 expression in left ventricular tissue ($P = 0.021$) and over a four-fold increase in aortic tissue ($P = 0.021$; Figure 1). Representative blots are shown in Figure 2. The eNOS and HSP90 levels were not affected by the pretreatment with heat shock (blots not shown). The non-diving controls expressed HSPs and eNOS at the same levels as the diving controls (blots not shown).

## Discussion

This study demonstrates that preconditioning with heat shock protects rats from the effects of bubbles following decompression from a dive. However, the pretreatment does not affect the number of bubbles formed. Earlier, we reported that acute severe exercise performed 20 hours prior to a dive increased survival in rats,[1] but this protective mechanism appears different from the one revealed in the present study, since the exercise-induced protection also involves reduced bubble formation. In the present study, half the rats in the

**Figure 2**
**Western immunoblotting for expression of heat shock protein 70 (HSP70); representative blots are shown**
**A – left ventricular tissue; B – aortic tissue**



heat-shock group survived in spite of the bubble formation. The protective effect of exercise has been shown to be related to NO production.[2,3,14] Exercised rats also express HSPs and would be expected not only to be protected against forming bubbles, but also against the effects of the bubbles.

The dive profile in this study was chosen to make comparison with the exercise studies possible, and the results clearly show that HSPs alone cannot explain the protective effect of exercise. The animal model used is, however, too crude to draw any clear conclusions about the mechanisms behind the observed heat-shock-induced protection.

Western blot analysis showed a significant increase in HSP70 levels in both left ventricular and aortic tissues after thermal preconditioning, but the causality between HSP70 and increased survival after decompression remains speculative. Since increased expression of HSPs also appears to protect rats against myocardial ischaemia in infarct models, the increased survival in our study could be due to a better tolerance for ischaemia caused by hypotension and gas embolism.[15,16] It is plausible that the elevated amounts of HSP70 are involved in the protection against effects of myocardial ischaemia, such as arrhythmias. Increased tolerance for ischaemia means that cells would be able to withstand otherwise damaging effects of bubbles. HSP70 is known to stabilise different proteins responsible for maintaining homeostatic conditions.[17] Since preconditioning with heat shock causes elevated levels of HSP70, cells can initiate the protective HSP70 mechanisms immediately when subsequent cellular trauma, such as ischaemia, occurs. At the same time, we should consider other circulatory effects of heat shock, for instance that vasodilatation could theoretically lead to a wash-out of nuclei and hence reduced bubble load. This should, however, lead to fewer bubbles, which is not the case in this study.

A similar study, in which rats were exposed to the same heat treatment as in our study, but without a following dive, showed an increase in HSP70, HSP90 and eNOS in heat-shocked animals compared to controls.[5] These results are inconsistent with our results, which imply no change in eNOS and HSP90 after a dive compared to non-diving controls, with or without the pretreatment with heat. A possible explanation is that the differences between preheated rats and controls were too small to be observed.

Massive bubbling, as observed in this study, is known to cause extensive endothelial injury.[18] If HSPs and eNOS are mainly expressed in the endothelial cells of the vessels, the explanation of our findings of no changes in HSP90 and eNOS could be that the tissue samples are stripped of endothelium.[19] The bubbles could cause disruption of the endothelial cell membranes, allowing proteins of the cytoplasm to be washed out into the blood stream, leaving only parenchymal cells to be analyzed in the tissue samples. It is conceivable that the dive itself is capable of inducing a stress response causing increased expression of HSPs and eNOS, but in this study the rats in the diving control group showed no increase in either compared to the two non-diving control rats. Again, this could be due to endothelial injury, masking a possible stress response in the diving controls.

## Conclusion

We conclude that heat shock induced a stress response as evidenced by the expression of HSP70. Prior heat shock reduced mortality but through a different mechanism to that of exercise, as exercise has been shown to also decrease bubble formation. Although we cannot conclude that the protective effect is caused by HSP70 we believe that HSP70 is part of the protective mechanism, either as a signal transduction factor, or as a more central component.

## Acknowledgements

This study was supported by the Norwegian Petroleum Directorate, Norsk Hydro, Esso Norge and Statoil under the “dive contingency contract” (No 4600002328) with Norwegian Underwater Intervention (NUI), as well as the MD/PhD program of the Medical Faculty at the University of Science and Technology, Trondheim, Norway.

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Submitted: 26 June 2008
Accepted: 05 October 2008

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