

Original article

70 kDa heat-shock proteins in surgical stress: thoracotomy vs herniorrhaphy[☆]

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Introduction: During and after surgical procedures, there is an oxidative stress response that releases cytokines and reactive oxygen species. This can activate Heat Shock Response (HSR), leading to an increase in Heat Shock Proteins (HSPs) expression proportional to the intensity of the stimulus.

Objective: This study examined the biology of leukocyte Hsps70 and IL-6 as potential biomarkers of post-operative inflammatory stress, and a potential antiHsp70 autoimmune reaction in patients undergoing two surgical procedures of different severity.

Material and methods: Longitudinal cohort study including a group of patients undergoing thoracotomy under general anaesthesia (n=11), a group of patients undergoing inguinal hernia repair under regional anaesthesia (n=10), and a group of healthy controls (n=6). Leukocyte Hsps70, antiHsp70 antibodies and IL-6 were analysed, just before and 24h after surgery.

Results: Patients undergoing thoracotomy showed a significant decrease in leukocyte Hsp70 and antiHsp70 antibodies in the early post-operative period; patients with the greatest Hsp70 decreases after surgery showed the lowest pre-surgical Hsp70 levels and these patients also experienced various post-operative complications. A significant post-operative increase in IL-6 levels in both groups was observed.

Conclusions: Patients undergoing a more aggressive surgery showed a significant Hsp70 reduction in the post-operative period. Patients with the lowest values of Hsp70 in the immediate post-operative period had the worst clinical course, which has led to propose use of Hsp70 as a prognostic post-surgical marker. The post-operative decrease in intracellular Hsp70 is parallel to the decrease in circulating autoantibodies. The different response of both groups to surgical stress is not due to systemic inflammatory response, but to HSR.

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Proteínas de choque térmico 70 kDa en estrés quirúrgico: toracotomía vs. herniorrafia

R E S U M E N

Palabras clave:

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Introducción: Como consecuencia de los procedimientos quirúrgicos, se produce una respuesta de estrés oxidativo con liberación de citoquinas y especies reactivas de oxígeno que activan la Heat Shock Response (HSR, 'respuesta de choque térmico') o respuesta al estrés, con un incremento en la síntesis de Heat Shock Proteins (HSP, 'proteínas de choque térmico').

Objetivo: Estudiar la biología de las Hsps70 intraleucocitarias y la IL-6 como posibles biomarcadores de la inflamación postquirúrgica, y una potencial respuesta anti-Hsp70, en pacientes sometidos a 2 situaciones quirúrgicas de distinta intensidad.

Material y métodos: Estudio longitudinal de cohortes, con un grupo de pacientes sometidos a toracotomía bajo anestesia general (n = 11), un grupo de pacientes sometidos a herniorrafia inguinal bajo anestesia locorregional (n = 10) y un grupo de donantes voluntarios sanos (n = 6). Se analizaron Hsps70 intraleucocitarias, Ac anti-Hsp70 e IL-6 inmediatamente antes y a las 24 h de la cirugía.

Resultados: Los pacientes toracotomizados mostraron una disminución significativa de Hsp70 intraleucocitaria y de Ac anti-Hsp70 en el postoperatorio inmediato. Los pacientes con mayores descensos de Hsp70 postoperatoria presentaron diversas complicaciones postquirúrgicas. Ambos grupos presentaron un significativo aumento postoperatorio de los niveles de IL-6.

Conclusiones: Cuanto más agresiva es la cirugía, mayor reducción de Hsp70 se produce en el postoperatorio, especialmente en pacientes con peor evolución, lo que ha llevado a proponer a la Hsp70 como marcador pronóstico postquirúrgico. El significativo incremento de IL-6 en ambos grupos permite concluir que la dispar respuesta al estrés quirúrgico entre ambos grupos se debe no a la respuesta inflamatoria sistémica, sino a la HSR.

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Introduction

During surgical procedures and after their finalisation, an oxidative stress response is produced, comprising the activation of inflammatory, metabolic, endocrine, and immunological mediators, including Interleukin-6 (IL-6)¹ and the so-called "Heat Shock Response" (HSR), or stress response²; this produces an increase in the synthesis of Heat Shock Proteins (HSP), which is proportional to the intensity of the stimulus.³ HSP are classified into families according to their molecular weight, among which the HSP70 family is the most widely studied, containing proteins that are expressed constitutively, Hsc70 (HSPA8), and proteins that are induced, Hsp70 (HSPA1A). The cytoprotective role of Hsp70 has been broadly documented in human diseases.⁴ Although these have traditionally been conceptualised as intracellular proteins, recent studies have described their release into the blood stream, whether by passive mechanisms from necrotic cells such as in surgery,⁵ or by active mechanisms from viable cells. Circulating Hsps are immunomodulatory molecules that act as potent autoantigens.⁶ Recognition of these molecules by lymphocyte T-cells triggers an autoimmune

response that has been correlated to some pathologies such as atherosclerosis.⁷

Different anaesthetic techniques modulate this stress response: in techniques with nervous transmission blocking (local anaesthesia), the neuroimmunoendocrine connection is interrupted without inhibiting the cerebral cortex; in general anaesthesia, cortical activity is silenced, while the indicated connection remains operational.⁸

Polymorphonuclear neutrophil leukocytes (PMN) belong to the first line of recognition and defence against external aggression thanks to Toll receptors that involve them in the innate immune response.⁹ Oxidative stress activates the PMN and induces the synthesis of HSP within them. These proteins will play an important role in the self-protection of these cells that produce oxygen free radicals.^{10,11}

The objective of this paper was to study the biology of intraleukocyte Hsps70 and IL-6 as possible post-operative inflammation biomarkers in patients subjected to two surgical situations of different severity: 1) thoracotomy (major surgery with general anaesthesia), and 2) herniorrhaphy (minor surgery with local anaesthesia). We also analysed the possible antiHsp70 autoimmune reaction in response to the 2 different levels of surgical damage.

Material and methods

Study design

We performed a longitudinal cohort study with a group of patients subjected to thoracotomy under general anaesthesia, a group of patients subjected to inguinal hernia repair under local anaesthesia, and a control group of healthy subjects. The sample size was calculated in terms of statistical robustness by the epidemiology department at the Hospital General Universitario Gregorio Marañón of Madrid (HGUGM) according to the results previously obtained in a pilot study.¹²

Twenty-one male patients were included in the study, which were admitted in the surgery and anaesthesia and reanimation departments at the HGUGM. Six control healthy male subjects paired by age provided the reference values. The study was approved by the research and ethics commissions for clinical research. The patients and volunteers were assigned to one of the following groups: group I, voluntary healthy controls (n=6); group II, patients subjected to thoracotomy in order to perform lobectomies under general anaesthesia (n=11); group III: patients subjected to inguinal hernia repair under local anaesthesia (n=10). Physical examination and laboratory tests were performed on all participants in order to ensure that all study subjects were free of infectious and autoimmune diseases, along with a review of clinical histories and pre-operative reports. Immediately before surgery, 5 ml of blood were extracted into non-heparinised tubes for obtaining serum, and 20 ml of blood into tubes with EDTA for polymorphonuclear neutrophil leukocyte isolation. Twenty-four hours after the surgery, we obtained a second sample with similar characteristics in all study groups.

Analytical methods

1. Obtaining serum

Following blood extraction, the tubes were left for 15 min at room temperature in order to allow clot formation. This was then centrifuged for 15 min at 3,500 rpm and 4 °C, and the supernatant was removed. The serum samples were stored at -70 °C until processing.

2. Isolating Polymorphonuclear neutrophil leukocytes (PMN)

Blood was obtained by vein puncture in venoject™ vacuum tubes containing EDTA at 0.47 mM/l, 21% w/v (other anticoagulants that activate the PMN were excluded, such as citrate and heparin). PMN isolation was performed by gradient, centrifuging the blood at 450-500 g for 30 min in Polymorphprep™ (Nycomed, Oslo, Norway). The PMN band was removed and added to 10ml of phosphate buffer solution, PBS (17.11 mM NaCl, 0.335 mM KCl, 0.44 mM KH₂PO₄, 1.016 mM Na₂HPO₄); this was then centrifuged for 5 min at 450 g. In order to avoid possible erythrocyte contamination, the cells were subjected to osmotic shock by adding cold sterile distilled water. This solution was then centrifuged for 5 min at 450-500 g and the supernatant was decanted. The precipitate with the PMN was re-suspended

in 100 µl of RIPA lysis buffer supplemented with protease and phosphatase inhibitors: 1 mM PMSF, 1mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin. Cell lysates were homogenised by 40 rounds in a Kontes homogeniser and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was stored at -70 °C until later processing.

3. Protein content assessment of cell lysates

The protein content of the soluble fraction was assessed using the Lowry microassay method with a Bio-Rad protein assessment kit compared against an albumin calibration curve (0-10 µg/ml) (bovine serum albumin, BSA, Bio-Rad).

4. Immunoblotting

After performing a one-dimensional electrophoresis on a polyacrylamide gel under denaturing conditions (SDS-PAGE), we identified intracellular Hsc70 and Hsp70 using a western blot. Briefly, the polyacrylamide gels are transferred to a PVDF membrane (Immobilon, Millipore), where non-specific unions are blocked by incubation with powdered skimmed milk at 3% (w/v) in PBS-Tween 20 (Bio-Rad, 0.05% (v/v)). The samples are then washed three times with PBS-Tween 20 at 0.05% (v/v) and then incubated with monoclonal Ab (Stressgen, SPA 815-Hsc70-, SPA 810-Hsp70) diluted 1:1,000 in 0.05% PBS-Tween 20 (v/v)-0.5% BSA (w/v). These are then washed three times and incubated with a second mouse anti-IgG Ab conjugated with biotin (Amersham). The signal is then amplified by incubation with HPR (horseradish peroxidase streptavidin) (Amersham). These are shown with 4-chloro-1-naphtol (Sigma) as a substrate. Protein identification in the study was performed by molecular weight, using coloured pattern bands as a reference, which are run parallel to the samples (Rainbow Markers Amersham).

The band images were digitalised with a SCANJET-II CX scanner (Hewlett-Packard) and processed using Scil-Image software. The total gray of the test samples were normalised against the mean gray levels of the control samples, and the quantification of Hsc70 and Hsp70 content was expressed in Arbitrary Units (AU).

5. AntiHsp70 AB quantification

AntiHsp70 Ab were quantified in serum diluted to 1:1,000 using an ELISA commercial kit following the manufacturer's instructions (EKS-750, Anti Human Hsp70 (IgG/IgM/IgA) ELISA Kit, Stressgen). The results obtained are expressed in micro µg/ml. Linearity was 31.25-1,000 µg/ml, and sensitivity: 6.79 µg/ml. Intra and inter-assay variation coefficients were <10%.

6. Interleukin 6 (IL-6) quantification

The serum IL-6 concentration was measured using a commercial ELISA kit (MedSystems Diagnostics GMBH, BMS213/2®). The IL-6 concentration of each sample was calculated by interpolation of a standard curve and expressed in pg/ml. Normal serum values ranged from 0 to 14.1 pg/ml, with a mean of 1.3 pg/ml and a standard deviation of 3.2 pg/ml.

7. Statistical analysis

All procedures were performed at least in duplicate. Results were expressed as a mean±mean standard error

(MSE), and compared using Kolmogorov-Smirnov and Student's t-tests for paired data, and ANOVA for data adjusted for normality, and Wilcoxon, Mann-Whitney, and Kruskal Wallis tests for non-parametric data. We deemed values of $P < .05$ to be statistically significant. We used the SPSS 12.0 statistical package for Windows.

Results

The 3 groups were homogeneous in sociodemographic variables (age, weight, height), as in laboratory analysis parameters (glycaemia, urea, creatinine, aspartate aminotransferase, lactate dehydrogenase, sodium, potassium, and haemoglobin). Basal Hsc70 and Hsp70 levels were also homogeneous (Table 1).

In the immediate post-operative stages we observed the tendency for lower intraleukocyte Hsps70 concentrations, which was statistically significant for Hsp70 in patients who underwent thoracotomies, while these levels remained stable in patients who underwent inguinal herniorrhaphies (Table 2). The four patients in group II with the greatest reductions in Hsps70 levels following the operation had the lowest pre-

operative Hsps70 concentrations (Figure 1) and had presented previous inflammatory pathologies (TB, ulcerative colitis, and chemotherapy). These were also the patients with various post-operative complications.

We also observed stable levels of antiHsp70 Ab in patients who undergone inguinal hernia operations before and after the procedure, while there was a significant decrease in concentrations following the operation in patients who underwent thoracotomies (Table 3, Figure 2). Similarly, the patients from this group with previous inflammatory pathologies presented the highest pre-operative Ab levels.

The analysis for interleukin 6 showed a significant increase in post-operative concentrations in both groups without significant differences between groups (Table 4).

Discussion

The response to surgical and anaesthetic stress includes both a response to oxidative stress and a response to thermal shock with hsp gene activation, especially hsp70,¹³ whose intensity is in accordance with the severity of the surgical damage.¹⁴ In spite of this, the present study showed a significant

Table 1 – Demographic and lab analysis values and Hsps 70 levels before surgery (mean±standard deviation)

	Control	Herniorrhaphy	Thoracotomy	*P
Age, years	59±12	63±17	63±15	**NS
Haemoglobin, g/dl	14.7±0.8	14.6±1.7	14.3±1.3	**NS
Glycaemia, mg/dl	99±10	87±14	124±49	**NS
Urea, mg/dl	41±11	42±13	42±18	**NS
Creatinine, mg/dl	1.15±0.3	1.08±0.2	1.02±0.4	**NS
AST, UI/l	18±6	22.6±7	20±12	**NS
LDH, UI/l	280±84	311±36	261±10	**NS
Sodium, meq/l	140±2	138±2.4	137±2.5	**NS
Potassium, meq/l	4.2±0.4	4.2±0.4	4.0±0.2	**NS
Hsc70, AU	1.10±0.11	1.01±0.31	0.8±0.26	**NS
Hsp70, AU	0.95±0.07	0.72±0.4	0.74±0.24	**NS

AU indicates arbitrary units taken from the normalisation of total gray scale from the immunoblot bands yielded by the test samples with respect to the control samples.

*P: referring to the differences in means between groups according to the analysis of variance (one-way ANOVA).

**NS: not significant.

Table 2 – Hsc70 and Hsp70 levels before and after the surgical intervention in patients who underwent a thoracotomy (n=11) and in patients who underwent an inguinal herniorrhaphy (n=10)

		Thoracotomy (n=11)	*P	Herniorrhaphy (n=10)	*P
Hsc70, AU	Pre-operative	0.8±0.1	NS	1.01±0.1	NS
	Post-operative	0.70±0.1		0.88±0.1	
Hsp70, AU	Pre-operative	0.74±0.1	<.05	0.72±0.1	NS
	Post-operative	0.57±0.0		0.73±0.1	

AU indicates arbitrary units taken from the normalisation of total gray scale from the immunoblot bands yielded by the test samples with respect to the control samples; NS, not significant. Results expressed as mean±MSE.

*Referring to the effect of the treatment according to the Student's T-test for paired samples.

post-operative decrease in Hsps70 in the group of patients subjected to the highest surgical damage. This could be explained by the intense and prolonged surgical anaesthetic stress of the procedure, which could have triggered a rapid stress response that would induce the immediate synthesis of large quantities of Hsp70. The over-expression of Hsp70 inhibits the activation of the Heat Shock Factor (HSF), and, as a consequence, the transcription of this gene, with a subsequent decrease in protein translation.¹⁵ The post-operative reduction in Hsp70 levels detected in this group of patients could also be due to an excessive consumption given the specific role of Hsp70 in the PMN in the neutralisation of reactive oxygen species.¹⁶

One of the differential characteristics in the study of the biology of human heat shock proteins is the high inter-individual variability in the basal protein synthesis.¹⁷ The antioxidant status of the patient, which modulates the activation of HSF through the levels of oxygen free radicals, is among the factors that could contribute to the differential expression of basal Hsps.¹⁸ Patients that have lower Hsps70 levels before the operation had previous inflammatory diseases, which could determine a lower pro-oxidative state due to over-stimulation of the system.¹⁹ The level of basal Hsps70 synthesis is correlated with the percentage of Hsps70 synthesis induction following an *ex vivo* or *in vivo* stimulus, which explains the different levels of stress tolerance and

uneven individual susceptibility to disease.²⁰ In the present study, patients with lower pre-operative Hsps70 levels also presented the lowest immediate post-operative values, and were also the patients who had the worst clinical evolution. Hsp70 has anti-inflammatory properties due to its inhibition of the expression of pro-inflammatory cytokines²¹ and pro-inflammatory transcription factors such as β nuclear factor,²²

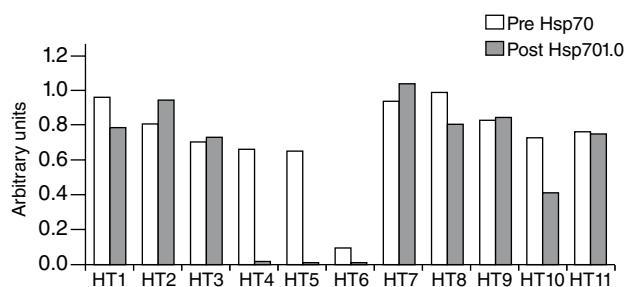


Figure 1 – Individual Hsp70 (HSPA1A) levels before and after the thoracotomy. Patients with lower pre-operative Hsp70 levels had previous inflammatory pathologies, and presented the greatest drops in post-operative levels and post-surgical complications.

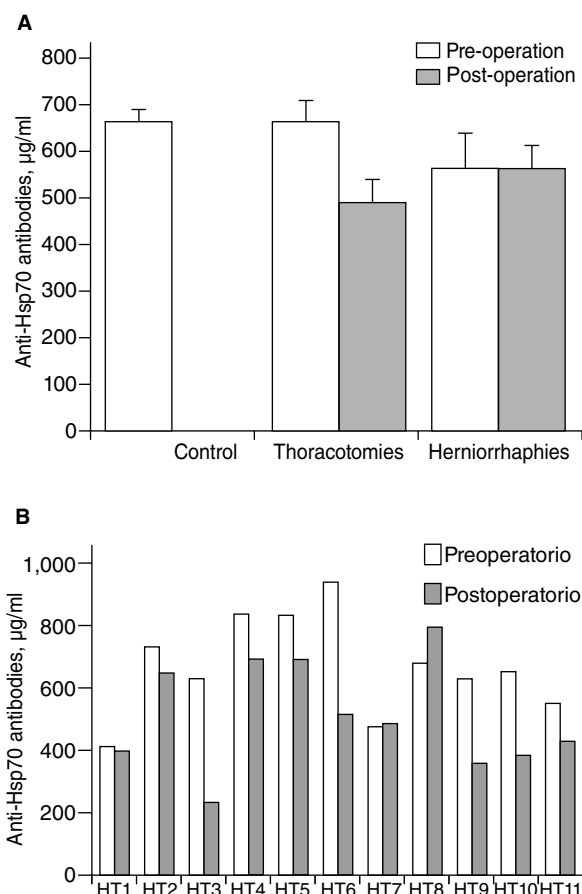


Figure 2 – A) Quantification of antiHsp70 Ab before and after the surgery. Results are expressed in µg/ml (mean±MSE). * P<.05 refers to the effect of the treatment according to the Student's T-test for paired data. B) Individual quantification of anti-Hsp70 Ab levels before and after the thoracotomy.

Table 3 – Quantification of antiHsp70 Ab before and after the surgical intervention in patients who underwent a thoracotomy (n=11) and in patients who underwent an inguinal herniorrhaphy (n=10)

Anti-Hsp70, µg/ml	Pre-operative	Post-operative	*P
Thoracotomies (n=11)	662±45.2	490±47.9	<.05
Herniorrhaphies (n=10)	561±76.2	559±50.9	NS
Controls (n=6)	660±26.9		

NS indicates not significant.
Results expressed as mean±MSE (µg/ml).
*Referring to the effect of the treatment according to the Student's T-test for paired samples.

Table 4 – IL-6 quantification before and after the surgical procedure in patients who underwent thoracotomies (n=11) and patients who underwent inguinal herniorrhaphies (n=10)

IL-6, pg/ml	Pre-operative	Post-operative	*P
Thoracotomies (n=11)	2.91±2.05	20.03±5.01	<.001
Herniorrhaphies (n=10)	4.99±3.54	18.3±5.79	<.001
Controls (n=6)	2.74±2.12		
Results expressed as mean±MSE (pg/ml).			
*Referring to the effect of the treatment according to the Mann-Whitney Test.			

which would protect the cells from the deleterious effects of the inflammatory response, and low levels of Hsp70 would promote a pro-inflammatory state. This has supported the use of Hsp70²⁰ as a prognostic marker for the development of post-operative complications in high-risk patients. Monitoring these values could allow surgeons and anaesthesiologists to modify surgical procedures, and even increase protein levels before the operation in order to improve post-operative evolution. Current research has shown that small changes in lifestyle, such as increasing physical exercise, have proven effects on increasing Hsp70²³ levels, and the treatment of certain tumours with Hsp70-vaccines has already shown results.²⁴

Hsp70 has also been described as participating in the innate immune response, interacting with toll-like receptors,²⁵ as well as in autoimmune processes. This is explained by a double mechanism, in which Hsps are released by necrotic cells,²⁶ and are also transported from the cytosol of viable cells to the cellular membrane for later excretion into the extracellular matrix.²⁷ The group of patients who underwent inguinal herniorrhaphies under spinal anaesthesia maintained stable antiHsp70 Ab levels before and after the surgical intervention. This is explained by 2 different but simultaneous processes: on the one hand, epidural anaesthesia reduces intraoperative stress and thus the immune response attenuates the post-operative worsening.²⁸ On the other hand, the less invasive nature of the inguinal herniorrhaphy does not alter the metabolic endocrine response to surgical stress and reduces the inflammatory response and immune suppression.²⁹ In the group of patients who underwent thoracotomies under general anaesthesia, we observed a significant decrease in the antiHsp70 autoimmune response, with a simultaneous decrease in intracellular Hsp70 levels. An immediate HSR was produced in this group of patients exposed to greater surgical damage, with synthesis of large quantities of intracellular Hsps70 and release of these proteins into the blood stream with the consequent production of antiHsp7 antibodies. The Abs tend to concentrate in the area of surgical damage with a decrease in plasma concentrations.²⁶ Meanwhile, the increase in serum Hsp70 levels would increase the formation of antigen-antibody immunocomplexes, decreasing the detectable concentrations of circulating antiHsp70i Ab³⁰ as has been evidenced in this study. This decrease could also reflect a given immunosuppressant state following a severe damage.³¹

Serum IL-6 levels were significantly higher 24 hrs after the surgical interventions, independently of the anaesthetic technique used, which coincides with results from other studies.³² Given that IL-6 plays a major role in acute-phase response and it was similar in both groups, and given that in patients who underwent inguinal herniorrhaphy, a minimally invasive surgery, we have not been able to demonstrate the presence of an HSR, we suspect that the reduction in post-operative intracellular Hsps70 levels and circulating anti-Hsp70 autoAb in patients who underwent thoracotomies responds to a cellular stress signal that is modulated by the level and severity of the lesion, and not by a systemic inflammatory response.

In summary, the greater the surgical damage, the greater the decrease in intracellular Hsp70 immediately after the operation. This decrease is even more important in patients with worse post-operative evolution, which has made Hsp70 a possible post-surgical prognostic marker. The reduction in intracellular Hsp70 levels is parallel to the decrease in antiHsp70 Ab, whether by the formation of circulating immunocomplexes or by their concentration in the damaged tissue, which could reflect an immunosuppression condition following a severe damage. The significant increase in IL-6 in both groups of patients, both those subjected to major and minor surgeries, allows us to conclude that the different response to surgical stress is not due to the systemic inflammatory response, but instead due to thermal shock.

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Conflict of interest

The authors affirm that they have no conflicts of interest.

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