J Anesth (1999) 13:144–149

Journal of
Anesthesia

Expression of heat shock protein 70 mRNA in polymorphonuclear cells responding to surgical stress

Katsuhiro Ohse[1], Shinji Yashiki [2], Toshinobu Fujiyoshi[2], Shunro Sonoda[2], and Nozomu Yoshimura[1]

Departments of [1] Anesthesiology and Critical Care Medicine, and [2] Virology, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Abstract
Purpose. This study was performed to investigate the expression of heat shock protein (HSP) 70 mRNA in polymorphonuclear neustrophils (PMN) as a possible new biomarker for surgical stress.
Methods. The HSP70 mRNA in PMN of 10 patients who underwent lobectomy was evaluated by Northern blot analysis. Their leukocyte counts, including white blood cells (WBC) and PMN, plasma cortisol levels, and plasma interleukin-6 (IL-6) levels, were obtained by cell counting, radioimmunoassay, and enzyme-linked immunosorbent assay, respectively.
Results. The level of HSP70 mRNA in PMN slightly increased at the end of surgery and showed a significant increase 6 h after surgery. It promptly decreased at 24 h postoperatively and returned to the basal preanesthetic level 48 h after surgery. On the other hand, WBC/PMN counts, plasma cortisol, and IL-6 significantly increased at the end of surgery. WBC/PMN counts remained at increased levels until 48 h postoperatively. Cortisol peaked at 6 h postoperatively and gradually decreased. IL-6 reached a maximum at 1 h postoperatively, then tapered down to its basal level at 48 h postoperatively.
Conclusion. Expression of HSP70 mRNA in PMN that is induced after thoracic surgery appears to be a promising candidate as a marker for evaluating surgical stress.

Key words: Heat shock protein, Surgical stress, Interleukin-6, Cortisol

Introduction

Surgical stress induces various physiopathological responses in patients after surgery. The most common response to surgical stress is an increased count and activation of polymorphonuclear neutrophils (PMN) [1]. To evaluate surgical stress and prognosis of the patient, PMN functions have been widely investigated. It has been found that excessive activation of PMN causes deleterious complications such as adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF) [2,3], whereas insufficient function of PMN results in serious bacterial infection with sepsis [4].

Heat shock protein (HSP) 70 is one of the family of HSP that has an important function in protecting hosts from various biohazardous effects [5]. HSP70 is induced in human host cells by thermal stress, hypoxia, ischemia, oxidative injury, heavy metal intoxication, and infections [6,7]. Recently, it was shown that HSP70 was induced in PMN in patients with sepsis or ARDS [8,9]. The induction of HSP70 has also been reported in the adrenal cortex and the greater vessels in rats in response to surgical stress. However, the expression of HSP70 in human PMN after surgery has not been studied [10].

In the present study, we investigated the significance of HSP70 as a new biomarker for surgical stress by analyzing the expression of HSP70 mRNA in PMN and comparing it with some widely accepted biomarkers of surgical stress: cortisol and interleukin-6 (IL-6) plasma levels and cell counts of PMN in the circulation.

Materials and methods

Patients

Ten patients with lung cancer aged 62–77 years, ASA I-II, who were scheduled for lobectomy were enrolled in the study. This study was approved by the Institutional Ethical Committee, and informed patient consent was obtained. Patients with metabolic or hormonal diseases were excluded. No patients were taking corticosteroids. All patients received oral premedication with 5 mg of

Address correspondence to: K. Ohse
Received for publication on August 31, 1998; accepted on March 10, 1999

diazepam 1 h before arrival in the operating room. After arrival in the operating room, an intravenous cannula was inserted and an arterial cannula was placed into the radial artery for continuous measurement of arterial pressure and blood sampling. Oxygen saturation, end-tidal CO_2, and inspired and end-tidal concentrations of anesthetics were monitored by a respiratory gas monitor (Ohmeda RGM 5250, Ohmeda, Louisville, CO, USA). After placement of a thoracic epidural catheter into the epidural space at the T4-7 intervertebral level, anesthesia was induced with 5 mg·kg^{-1} thiamylal sodium, 0.1 mg fentanyl, and 0.1 mg·kg^{-1} vecuronium. The trachea was intubated with a double-lumen endobronchial tube (Broncho-Cath, Mallinckrodt, St. Louis, MO, USA) under fiberoptic control. Anesthesia was maintained by inhaled isoflurane with either a mixture of O_2 and air or O_2 and N_2O, and epidural anesthesia with continuous infusion of 1.0% mepivacaine 4–6 ml·h^{-1}. Vecuronium was administered as required as an intraoperative muscle relaxant. All patients were extubated before transfer to the intensive care unit. No patients had complications during or after surgery.

Blood Samples

Five-milliliter samples of arterial blood were collected for WBC, PMN, cortisol, and IL-6 analysis at the following perioperative times: before induction of anesthesia (prearesthetia), immediately after skin incision (operation), at the end of the operation, and 1, 2, 4, 6, 24, and 48 h after the end of the operation. Twenty milliliters of arterial blood was necessary for HSP70 mRNA analysis, and blood samples were collected from the patients at five intervals: prearesthetic, end of operation, and 6, 24, and 48 h after the operation.

RNA extraction and Northern blot analysis of HSP70 mRNA expression

PMN for RNA extraction was obtained from 20 ml of heparinized arterial blood using the single-step centrifugal technique with Polymorphprep (NYCOMED, Oslo, Norway) [11]. Arterial blood sedimentation in Polymorphprep was performed at room temperature at 450 *g* for 30 min. After centrifugation, the PMN were harvested and diluted with 0.45% NaCl solution to restore normal osmolarity. The PMN were resuspended in phosphate-buffered saline (PBS) and counted. The purity and viability of PMN were >95% and >98%, respectively, as determined by trypan blue staining.

Total RNA of PMN was extracted by the acid guanidium thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan). RNA was quantitated by absorbance at $\lambda = 260$ nm. Northern blot analysis was performed according to standard procedures [12]. In brief, 15 μg of total RNA was analyzed by electrophoresis through 1% agarose/formaldehyde gels and transferred to a nylon membrane (Hybond-N$^+$; Amersham, Oakville, ON, Canada) overnight. The membranes were prehybridized with buffer (5× Denhardt's reagent, 1% sodium dodecyl sulfate [SDS], 100 μl·ml^{-1} salmon sperm DNA) for 5 h at 48°C. Hybridization was run overnight at 48°C with the same buffer, including the specific ^{32}P-labeled 741-bp probe (StressGen Biotechnologies, Sidney, BC, Canada), encoding the HSP70 cDNA [13]. After hybridization, the membrane was washed with 2 × SSPE (2 × saline-sodium phosphate-EDTA) for 15 min at room temperature and then with high stringency buffer (2 × SSPE, 0.1% SDS) for 15 min at 65°C, followed by 0.5 × SSPE for 15 min at 65°C. The membranes were autoradiographed with Amersham Hyperfilm-MP for 48 h at −80°C in an x-ray film cassette and quantitated by computerized planimetry (NIH Image 1.6, public domain software). Expression of β-actin mRNA in PMN before and after surgery was used as an internal control for HSP70 gene expression. All samples exhibited a constant relative density as compared with the β-actin expressed at preanesthesia.

Cell counting and assays for plasma cortisol and IL-6

Two milliliters of arterial blood was used for counting the number of WBC and PMN. Three milliliters of arterial blood was centrifuged to extract plasma samples for cortisol and IL-6 analysis. Plasma samples were stocked at −80°C until use. Cortisol concentration was measured in duplicate using the Amerlex Cortisol Kit (Kodak Japan Diagnostics, Tokyo, Japan), also known as the ^{125}I-direct radioimmunoassay (RIA) kit. This assay kit uses a cortisol antiserum that has negligible cross-reactivity with other endogenous corticosteroids and cortisol metabolites. The intra- and interassay coefficients of variation were 5.7% at 1.98 μg·dl^{-1} and 8.9% at 2.18 μg·dl^{-1}, respectively.

The plasma concentration of IL-6 was measured in duplicate by an in vitro enzyme-linked immunosorbent assay (Endogen Interleukin-6 ELISA, Endogen, Cambridge, MA, USA). The lower sensitivity of the assay was 1 pg·ml^{-1}.

Data analysis

All data were analyzed using two-way analysis of variance (ANOVA) with Dunnett's test for comparison versus preanesthetia. The difference was considered significant if the *P* value was less than 0.05. Values are expressed as means ± SEM.

Table 1. Patient data ($n = 10$)

Patient characteristic	Median (range)
Age (yr)	71.2 (62–77)
Weight (kg)	50.0 (37.5–66)
Sex (M/F)	7/3
Duration of anesthesia (min)	663.9 (315–910)
Duration of surgery (min)	511.7 (300–805)
Blood loss (ml)	333.7 (103–465)

Results

We investigated 10 patients undergoing thoracic surgery (Table 1). All the patients underwent lobectomy for lung cancer. The outcome of the patients was fair, without deleterious complications such as sepsis, MOF, and ARDS, although some of them temporarily had fevers and exhibited increases in C-reactive protein (CRP). All patients left the hospital within one month.

HSP70 mRNA in PMN was measured using the patient's PMN before and after surgery (Fig. 1). The upper panel shows the basal level of HSP70 gene expression and successive changes of HSP70 mRNA from the end of operation to 48 h postoperatively. The relative densities of HSP70 and β-actin were measured by autoradiogram (Fig. 1). HSP70 mRNA slightly increased at the end of operation as compared with preanesthesia, and then significantly increased at 6 h postoperatively. The expression of HSP70 mRNA decreased at 24 h postoperatively and returned to the basal level at 48 h postoperatively. On the other hand, β-actin mRNA maintained constant levels throughout the period of preanesthesia to 48 h postoperatively.

In order to evaluate the correspondence between HSP70 mRNA expression and surgical stress, we compared WBC and PMN counts and plasma levels of cortisol and IL-6 in the period of preanesthesia, beginning and end of operation, and postoperation (Fig. 2). The WBC counts significantly increased from preanesthesia ($6.5 \pm 0.5 \times 1000 \cdot \mu l^{-1}$) to the end of operation ($9.7 \pm 1.1 \times 1000 \cdot \mu l^{-1}$) ($P < 0.05$). The PMN counts also significantly increased from preanesthesia ($4.6 \pm 0.5 \times 1000 \cdot \mu l^{-1}$) to the end of operation ($8.3 \pm 1.0 \times 1000 \cdot \mu l^{-1}$) ($p < 0.05$). Both WBC and PMN counts remained high during the period from the end of operation to 48 h postoperatively (Fig. 2A).

The plasma cortisol level significantly increased from preanesthesia ($17.2 \pm 3.0\,\mu g \cdot dl^{-1}$) to the end of operation ($31.3 \pm 2.9\,\mu g \cdot dl^{-1}$) ($P < 0.05$) and reached its peak at 4 h postoperatively. The cortisol concentration remained significantly increased for 6 h postoperatively and then decreased from 24 h to 48 h postoperatively (Fig. 2B). Plasma IL-6 levels significantly increased at the end of operation ($163.3 \pm 22.6\,pg \cdot ml^{-1}$) as compared with preanesthesia ($41.1 \pm 10.6\,pg \cdot ml^{-1}$) ($P < 0.05$). The elevation of plasma IL-6 reached a maximum level 1 h postoperatively ($225\,pg \pm 10.6\,pg \cdot ml^{-1}$). After reaching the maximum value, the plasma IL-6 levels gradually decreased to the basal preanesthesia level at 48 h postoperatively (Fig. 2C). The kinetics of WBC, PMN, cortisol, and IL-6 were different from HSP70 mRNA expression in the initial response to surgical stress at the end of operation and after 24 to 48 h postoperatively.

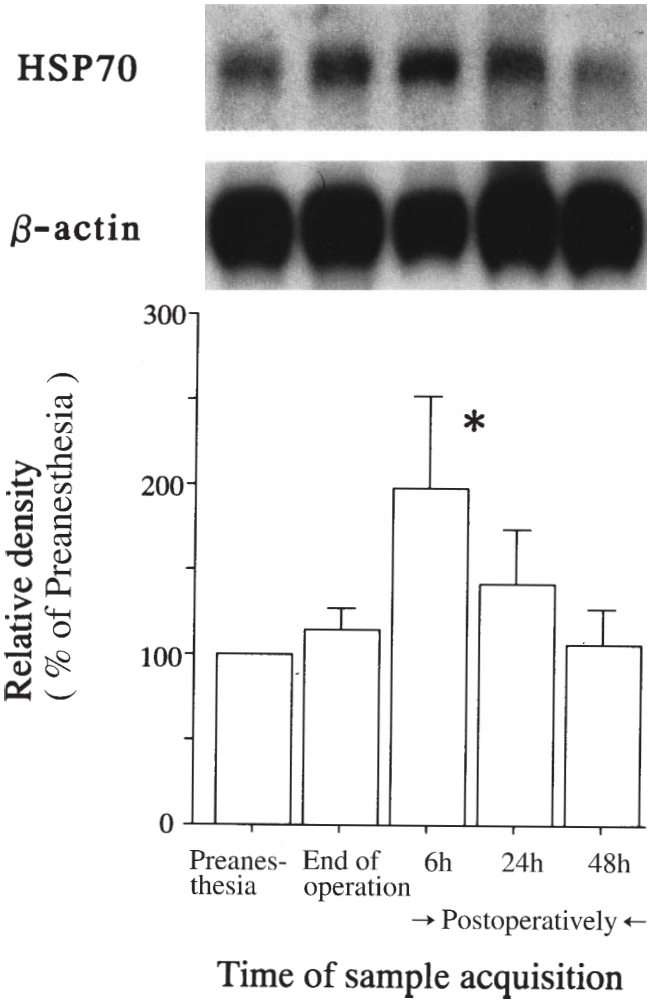

Fig. 1. Time course of neutrophil HSP70 mRNA expression during and after operation. Northern blot analysis of HSP70 (**upper panel**) and β-actin (**lower panel**). The level of HSP70 mRNA was normalized with the level of β-actin in each specimen and was compared by relative density (*graph*). Data are shown as means ± SEM. $*P < 0.05$ compared with preanesthesia values

Discussion

HSP has a cytoprotective effect against harmful conditions, but the response and the role of HSP in surgical

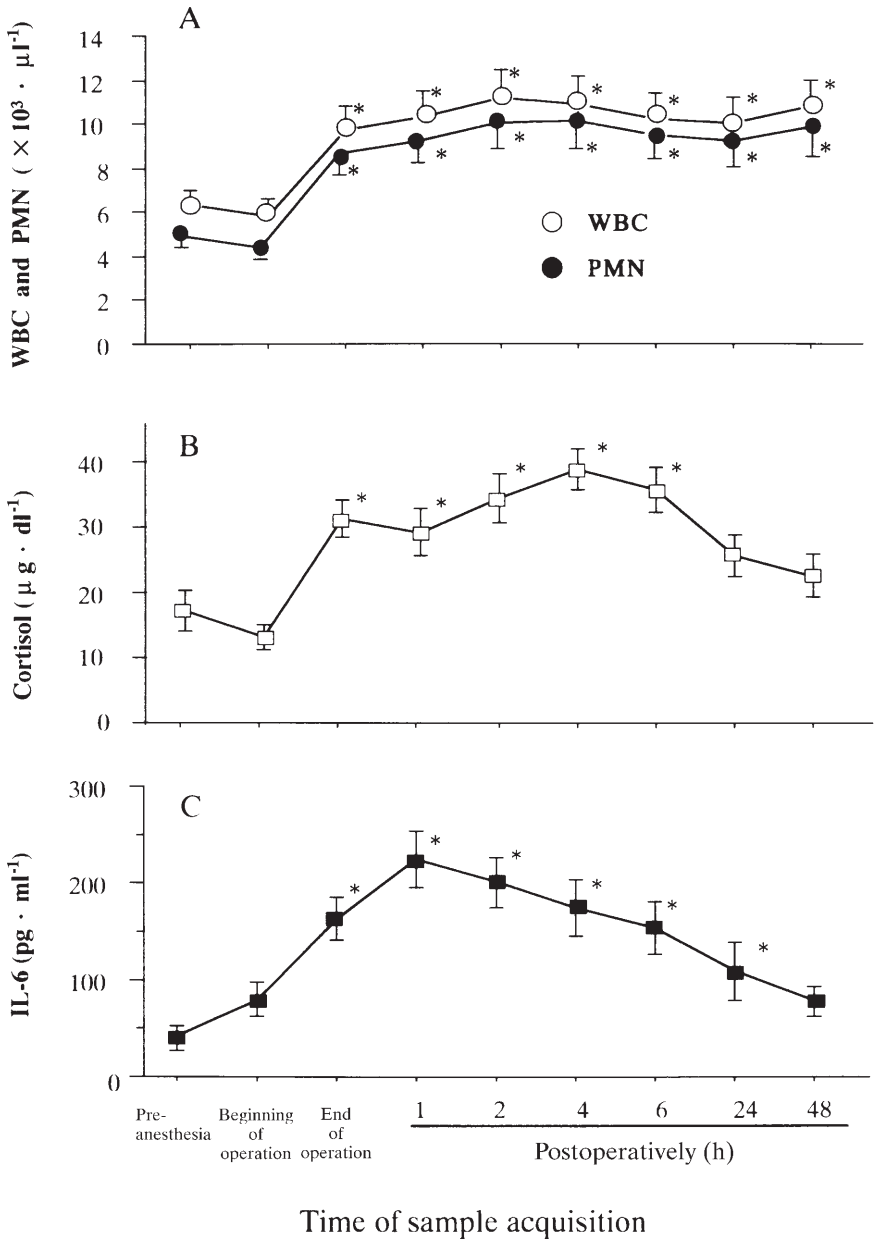

Fig. 2. **A** Changes in WBC counts (*open circles*) and PMN counts (*closed circles*), **B** plasma levels of cortisol (*open squares*), and IL-6 **C** (*closed squares*) during and after operation. Data are shown as means ± SEM. $*P < 0.05$ compared with preanesthesia values

stress have not yet been fully elucidated. Recently, several investigators reported expression of HSP by peripheral blood mononuclear cells and by PMN in critically ill patients. Delogu et al. showed that peripheral blood mononuclear cells from patients with sepsis contained significantly higher levels of HSP70 than those of a healthy control group [8]. Kindas-Mügge et al. also observed that spontaneously expressed HSP of the 70-kDa family in PMN was found in critically ill patients but not in healthy donors [14]. Furthermore, they reported that a high expression of HSP mRNA in alveolar macrophages from patients with ARDS was correlated with clinical situations of ARDS [9]. This suggests that a high level of HSP mRNA in alveolar macrophages is a marker for the severity of disease in patients who are developing or at risk for development of ARDS. In our study, we demonstrated that the induction of HSP70 mRNA in PMN increased dramatically 6 h after thoracic surgery. The increase in HSP mRNA was specific with respect to surgical stress, with the phenomenon returning promptly to the basal level 24 h after surgery. If we consider that this phenomenon reflects the severity of surgical stress in the same way that HSP70 in macrophages in the patients with ARDS seems to, then the temporal increase in HSP70 observed in our case strongly suggests that our patients suffered slight surgi-

cal damage. IL-6 and cortisol are also well known to be markers that reflect the severity of surgical stress [15–18]. In our study, levels of IL-6 and cortisol increased significantly at the end of surgery before returning to baseline levels within 48 h after surgery, indicating that our patients had suffered slight surgical stress. Furthermore, none of the patients had harmful complications after surgery, thus indicating a lack of interference in the levels of HSP70 due to postoperative pathologies.

The increase in HSP70 mRNA was different from that of other markers, although the sampling time was restricted in the initial postoperative phase. Indeed, only a slight increase in HSP70 was observed at the end of surgery, despite marked increases in other markers. The reason that HSP70 mRNA did not increase by the same magnitude as the other markers at the end of surgery may be due to a different response time in HSP70 or to a response mechanism different from the other markers. Bratton et al. reported that the level of HSP mRNA in peripheral blood mononuclear cells was increased 1 and 2 h after heat stress and that the level decreased after 4 to 6 h [19]. Udelsman et al. reported that HSP70 mRNA was prominently increased in the aorta and the vena cava 30 min after surgical stress in Wistar rat models. They also suggested a functional relationship between HSP70 induction and activation of the hypothalamic-pituitary-adrenal axis [10]. Thus, different responses at the end of surgery may be due not to the slow response of HSP70 but rather to other factors. One factor to account for the delayed reaction of HSP70 as compared with the reactions of cortisol and IL-6 at the end of surgery may be the fact that the PMN must first be recruited from bone marrow. Thus, it takes time for the PMN to enter the blood circulation. It is this time which is most likely responsible for the delayed appearance of HSP70 observed at the end of surgery. However, the delayed expression of HSP70 does not hinder its use as a marker for surgical stress, since the time required for the response is a matter of hours and not days.

Surgical stress is composed of many kinds of factors, including surgical injury, anesthesia, hemorrhage, transfusion, ischemia, hypoxia, and so on. These factors complicate the interpretation of markers used to examine the effects of surgical stress. Many investigators have tried to evaluate surgical stress and to determine the prognosis of a patient by using several kinds of biological markers, such as PMN function, cortisol and IL-6, as means to quantitate surgical stress itself [15–18]. In addition to these humoral factors, this study revealed that HSP70 mRNA might be considered a candidate molecular marker to evaluate surgical stress because of its distinct increase after surgery. HSP70 mRNA expression as a marker for surgical stress is also supported by an animal model study [10]. To our knowledge, this is the first report of human HSP70 gene expression under surgical stress. Further study of HSP70 mRNA expression in cases with postsurgical complications will provide us with greater understanding of HSP70 mRNA response to surgical stress and prompt us to explore appropriate strategies for preventing disease after surgery. Advances in molecular biology will allow us to develop a more rapid and simple method for HSP70 mRNA analysis.

In conclusion, HSP70 mRNA is induced in PMN after thoracic surgery and is feasible as a new marker, similar to cortisol and IL-6, to evaluate surgical stress.

Acknowledgment. The authors thank Mr. Chris Genge for his helpful advice on this manuscript.

References

1. Cullen BF, van-Belle G (1975) Lymphocyte transformation and changes in leukocyte count: effects of anesthesia and operation. Anesthesiology 43:563–569
2. Powe JE, Short A, Sibbald WJ, Driedger AA (1982) Pulmonary accumulation of polymorphonuclear leukocytes in the adult respiratory distress syndrome. Crit Care Med 10:712–718
3. Goya T, Morisaki T, Torisu M (1994) Immunologic assessment of host defense impairment in patients with septic multiple organ failure: relationship between complement activation and changes in neutrophil function. Surgery 115:145–155
4. Buffone V, Meakins JL, Christou NV (1984) Neutrophil function in surgical patients. Relationship to adequate bacterial defenses. Arch Surg 119:39–43
5. Lindquist S (1988) The heat-shock proteins. Annu Rev Genet 22:631–677
6. Polla BS (1988) A role for heat shock proteins in inflammation? Immunol Today 9:134–137
7. Yung RA, Elliott TJ (1989) Stress proteins, infection, and immune Surveillance. Cell 59:5–8
8. Delogu G, Bosco LL, Marandola M, Famularo G, Lents L, Ippoliti F, Signore L (1997) Heat shock protein (HSP70) expression in septic patients. J Crit Care 12:188–192
9. Kindas-Mügge I, Pohl WR, Zavadova E, Kohn HD, Fitzal S, Kummer F, Micksche M (1996) Alveolar macrophages of patients with adult respiratory distress syndrome express high levels of heat shock protein 72 mRNA. Shock 5:184–189
10. Udelsman R, Blake MJ, Holbrook NJ (1991) Molecular response to surgical stress: specific and simultaneous heat shock protein induction in the adrenal cortex, aorta, and vena cava. Surgery 110:1125–1131
11. Ferrante A, Thong YH (1980) Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. J Immunol Meth 36:109–117
12. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 7.39–7.52
13. Voellmy R, Ahmed A, Schiller P, Bromley P, Rungger D (1985) Isolation and functional analysis of a human 70,000-dalton heat shock protein gene segment. Proc Natl Acad Sci USA 82:4949–4953
14. Kindas-Mügge I, Hammerle AH, Frohlich I, Oismuller C, Micksche M, Trautinger F (1993) Granulocytes of critically ill

patients spontaneously express the 72kD heat shock protein. Circul Shock 39:247–252
15. Naito Y, Tamai S, Shingu K, Shindo K, Matsui T, Segawa H, Nakai Y, Kenjiro M (1992) Response of plasma adrenocorticotropic hormone, cortisol, and cytokines during and after upper abdominal surgery. Anesthesiology 77:426–431
16. Tang GJ, Kuo C-D, Yen T-C, Kuo HS, Chan K-H, Yien H-W, Lee T-Y (1996) Perioperative plasma concentrations of tumor necrosis factor-α and interleukin-6 in infected patients. Crit Care Med 24:423–429
17. Kato M, Suzuki H, Murakami M, Akama M, Matsukawa S, Hashimoto Y (1997) Elevated plasma levels of interleukin-6, interleukin-8, and granulocyte colony stimulating factor during and after major abdominal surgery. J Clin Anesth 9:293–298
18. Cruickshank AM, Fraser WD, Burns HJG, Damme JV, Shenkin A (1990) Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. Clin Sci Colch 79(2):161–165
19. Bratton SL, Jardine DS, Mirkes PE (1997) Constitutive synthesis of heat shock protein (72kD) in human peripheral blood mononuclear cells: implications for use as a clinical test of recent thermal stress. Int J Hyperthermia 13:157–168