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Research Article

THE EFFICACY OF 200 µG/KGBW/IP HEAT SHOCK PROTEIN-70 IN REDUCTION OF CYT C, BAX, AND CASPASE 3 EXPRESSION, AND MORTALITY IN MICE MODEL WITH MULTIPLE ORGAN DYSFUNCTION SYNDROME

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ABSTRACT

Objective: Heat shock protein 70 (HSP70) decreases Cyt expression c, Bax, and Caspase 3 in apoptosis multiple organ dysfunction syndrome (MODS), thus inhibiting death. This study aimed to analyze the efficacy of HSP70 200 µg/KgBB/ip to decrease Cyt c, Bax, and Caspase 3 expression, to reduce mortality, and to increase survival rate, in the MOD alveolar lung epithelial of 78-h sepsis model.

Methods: This was a post-test only quasi-experiment conducted at Inter-University Central Laboratory of Gadjah Mada University, Yogyakarta, and the Anatomy Pathology Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta. The study used a type of Balb/c mice, male, aged of 6–8 weeks, body weight of 25–33 g. Sepsis induction used LIG SIGMA L2880-10MG Lot #025M4040V from *Escherichia coli* 055:B5 purified by phenol extraction. Medication to reduce mortality used HSP70 Lot #L16020515 and then continued with 400× immunohistochemistry (IHC) examination. A sample of 30 mice were divided into three groups: (1) Control group without 78 h treatment, (2) lipopolysaccharide (LPS) group with a dose of 0.25 mg/kgBW/ip 78 h, and (3) HSP70 group with a dose of 200 µg/kgBB/ip after LPS injection 0.25 mg/kgBW/ip 78 h. The outcome variables included expression of Cyt, Bax, Caspase 3, and mortality in mice model with multiple organ dysfunction syndrome. The data were analyzed by Kruskal–Wallis and continued by Mann–Whitney U-test.

Results: Administration of HSP70 200 mg/KgBW/ip after LPS 0.25 mg/kgBW/ip significantly decreased Cyt c expression (p=0.014), Bax (p=0.004), and Caspase 3 (p=0.015) in 78 h pulmonary alveolar cells, reduced mortality rate, and increased the number of survivors. Expressions of Cyt c, Bax, and Caspase 3 of IHC 400× magnification had a near-normal image change.

Conclusion: There is a decrease of Cyt c, Bax, and Caspase 3 expression in the MOD alveolar lung epithelial cells of the 78-h sepsis model mice, a decrease of mortality rate, and an increase of survival rate, and the image of IHC is almost normal.

Keywords: Sepsis, Multiple organ dysfunction syndrome, Apoptosis, Heat shock protein 70.

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INTRODUCTION

Sepsis is a serious and complex problem because metabolic changes can lead to apoptotic tissue, multiple organ dysfunction syndrome (MODS), sepsis shock, and death [1-4]. Apoptosis, the term introduced by Kerr in 1972, is the process of programmed cell death occurring in multicellular organisms which causes changes in certain morphological and biochemical characteristics and cell death [3,5]. Apoptosis consists of two main pathways: The extrinsic or receptor death and the intrinsic or mitochondrial [6]. There is now an additional pathway involving T-cell-mediated cytotoxicity perforin-granzyme [7,8].

Heat shock protein 70 (HSP70) has been shown to have antiapoptotic function, but the mechanism is unclear [9,10]. Cytoprotective effects of HSP70 and HSP27 are related to their ability to deactivate apoptosis [10]. In mitochondrial apoptosis, HSP interacts closely with various components both upstream and downstream. In the mechanism of HSP70 in upstream apoptosis as well as in the outer mitochondrial membrane, the interaction between cardiolipin and Cyt c becomes weaker, allowing Cyt c in the intermembrane space to escape into the cytosol, as initiation of apoptosis [10]. In cytosol Cyt c allosteric, apaf-1 is activated to an apoptosome complex for proteolytic maturation of caspase 9 and caspase 3. Activation of caspase eventually leads to the removal of apoptotic cells [11]. Afterward, caspase 9 cuts and activates caspase 3 and 7 and then splits the number of subintracellulars for execution of apoptosis [12]. In the upstream, mitochondrial HSP70 prevents releasing mitochondrial apoptosis-inducing factor (AIF) and prevents nuclear translocation of AIF so that apoptosis does not occur [9]. Whereas in the downstream apoptosis mechanism, HSP70 binds apaf-1 and blocks apoptosome and preventing caspase 3 activation so that apoptosis does not occur [13-14]. In mitochondrial apoptosis, HSP70 placement either in upstream or downstream in stress-induced apoptosis pathways shows a mechanism to ensure preventable death [15]. Further, research is needed on the importance of optimal doses of exogenous HSP70 using ip injection, resulting in improved balance of homeostasis and rapid and effective treatment, or at least, to reduce very high MODS mortality rates [9,13,16].

Kustanova *et al*. suggested that injecting HSP70 exogenous at a dose of 266 µg/kg at 10 min before and 10 min after administering lipopolysaccharide (LPS) at 2 mg/kg iv can reduce mortality and modify some hemostasis and hemodynamic parameters [17]. Aschkenasy *et al*. suggested that HSP70 doses of 266 µg/kg exogenous in mice increase B-cell lymphoma 2 (BCL2) which inhibit apoptosis [18]. Choudhoury *et al*. stated that exogenous HSP70 administration inhibits apoptosis through Cyt c complex release prevention pathways, apaf 1, and pro-

caspase 9 and 3 [11]. Sharma and Masison state that HSP70 can affect apoptosis through the interaction of complementary proteins and antiapoptotic proteins BCL 2. Increased HSP70 protects cytotoxicity cells from apoptotic, radiation, and chemotherapeutic agents. HSP70 binds directly to apaf 1 protease and prevents apoptosome formation [19]. Results of Mazzei *et al*. show that HSP70 inhibits apoptosis and inflammation, repairs damaged proteins, prevents folded protein aggregation, and targets damaged proteins for degradation and cytoskeletal stabilization [20].

This study aimed to determine the effect of HSP70 200 mg/KgBW/i.p. post-LPS 0.25 mg/KgBW/i.p. on the expression of Cyt c, Bax, and Caspase 3 in 78 h of lung alveolar cells, mortality, and the number of survivors.

METHODS

This study used laboratory experimental research designed post-test only group. Maintenance and treatment were carried out at the Pau Laboratory of Gadjah Mada University, Yogyakarta, from 1 to April 21, 2017, and then continued with immunohistochemistry (IHC) examination at the Anatomy Pathology Laboratory, the Faculty of Medicine, Universitas Sebelas Maret, Surakarta. Subjects were mice of Balb/c strain, male, aged 6–8 weeks with a body weight of ±25–30 g. The drug used to cause sepsis was LPS dose of LD_{75} 0.25 mg/KgBW from SIGMA L2880-10MG Lot #025M4040V LPS from *Escherichia coli* 055:B5. HSP70 Lot #L16020515 drug was used to reduce the expression of Cyt c, Bax, and Caspase 3, to reduce mortality, and to increase survival rates. Ethical reviews for this study were provided by the research ethics committee of Dr. Moewardi Hospital, Surakarta, Central Java, Indonesia, number 377/IV/HREC/2017.

A sample of 30 mice was divided into three groups of 10 mice for each.
P0: Normal control with sodium chloride.
P1: The treatment group received 0.25 mg/KgBW/i.p.
P2: The treatment group received HSP70 injection of 200 µg/KgBW/ip post-LPS 0.25 mg/KgBW/ip.

The obtained data were statistically analyzed by one-way ANOVA test of the SPSS for Windows Release 11.5, and $p<0.05$ was chosen as the minimum level of statistical significance. The one-way ANOVA test requirement is a numerical scale and the data distribution is normal and homogeneous. If the one-way ANOVA test showed a significant difference ($p<0.05$), the result would be proceeded with the LSD *post hoc* test. If the one-way ANOVA test requirement could not be fulfilled, the non-parametric alternative test of Kruskal–Wallis would be used. If the Kruskal–Wallis test showed significant differences ($p<0.05$), the *post hoc* test would be continued using the Mann–Whitney U-test.

RESULTS

There were a total of 30 mice of Balb/c strain, male, aged 6–8 weeks with a body weight of ±25–30 g. In the treatment group that received LPS injection of 0.25 mg/KgBW/ip, there was an increase in Cyt c expression (p=0.011), Bax (p=0.005), and Caspase 3 (p=0.011) while in the treatment group that received HSP70 of 200 µg/KgBW/ip injection after LPS 0.25 mg/kgBW/ip, there was a decrease in Cyt c expression (p=0.014), Bax (p=0.004), and Caspase 3 (p=0.015) as shown in Table 1.

Whereas, the description of Cyt c, Bax, and Caspase 3 expression in alveolar epithelial cells lung of 78-h sepsis can be seen in Diagram 1: (1) KN: Control group without treatment but only injected by 1 cc NaCl intraperitoneal, (2) treatment group that received 0.25 mg LPS injection/KgBW/ip to make sepsis, and (3) HSP70 group of 200 µg/KgBB/ip after LP 0.25 mg/kgBW/ip on the lung alveolar epithelium of 78-h MODS model sepsis can be seen in Diagram 1.

There was a significant increase in Cyt c expression (p=0.011), Bax (p=0.005), and Caspase-3 (p=0.011) in 78 h of pulmonary alveolar epithelial cells after LPS injection

HSP70 administration significantly decreased Cyt c expression (p=0.014), Bax (p=0.004), and Caspase-3 (p=0.015) in 78 h of pulmonary alveolar epithelial cells after LPS injection (Fig. 1).

The results of IHC alveolar epithelial of 78-h sepsis at 400× magnification in normal control (NC) where no treatment was performed show a basic brownish blurry appearance and Cyt c, Bax, and Caspase 3 expression in a less bright brown color with a blurry base. In those who receive LPS injection 0.25 mg/KgBW/i.p, the expression looks brighter, denser and the base looks brighter compared to that of NC. In the HSP70 200 ug/KgBW/ip injection, the expression appears to be a reduced brownish color and less dense and the base is less bright and almost the same as that of NC.

DISCUSSION

LPS injection of 0.25 mg/KgBW/ip was able to make sepsis in mice with increased expression of Cyt c, Bax, and Caspase 3. These results are almost in line with Guntur study on making sepsis with LPS 0.1 mg/Kg BW [1]. In contrast, the results of Markwart *et al*. show LPS injection of 9–11 mg/KgBW/ip injected once cause systemic inflammatory response syndrome and sepsis [21]. Zhao *et al*. suggested that low dose LPS ip injection of 5 mg/KgBw is used for studies of sepsis and organ injury while high doses of 15 mg/KgBW cause death [22]. Fodor *et al*. increased LPS injection of 3.5, 10 mg/KgBW/ip at 6 h resulting in a correlation in dose increase with the severity of hypoxemia [23].

At the injection of HSP70 200 µg/KgBW/ip, there was a decrease in the expression of Cyt c, Bax, and Caspase 3. However, in the previous studies of HSP70, the dose was generally HSP70 266 ug/KgBB/ip to

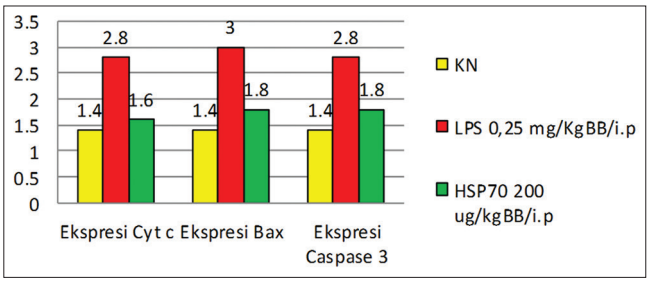

Diagram 1: Results of Cyt c, Bax, and Caspase 3 expressions in alveolar epithelial cells of 78-h sepsis. Range magnitude: Value 1: Range 1–30%, Value 2: Range 31–70%, Value 3: Range 71–100%

Table 1: Results of Chi-square test using expression of Cyt c, Bax, and Caspase 3 with LPS injection of 0.25 mg/KgBW/ip to make sepsis and to those receiving HSP70 injection of 200 µg/KgBW/ip after $LP_{0.25}$ mg/kgBW/ip at alveolar lung epithelium of 78-h MODS sepsis model

Organ	LPS			HSP70		
	Cyt c	Bax	Caspase 3	Cyt c	Bax	Caspase3
Epithelial alveolar lung 78 h	p=0.011	p=0.005	p=0.011	p=0.014	p=0.004	p=0.015

Pulmonary alveolar epithelium of 78 h=LPS has a significant increase in Cyt c, Bax, and Caspase 3. HSP 70 has a significant decrease in Cyt c, Bax, and Caspase 3.
LPS: Lipopolysaccharide, HSP70: Heat shock protein 70, MODS: Multiple organ dysfunction syndrome

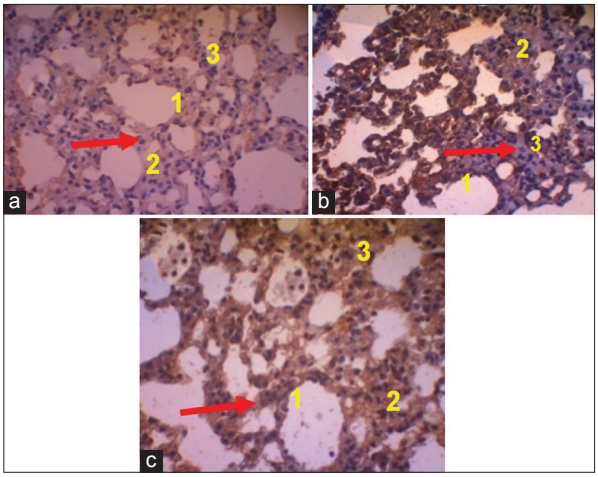

Fig. 1: Overview of immunohistochemistry expressions of Cyt C, Bax, and Caspase 3 in epithelial alveolar cells lung of 78-h 400× enlargement sepsis. (a) Normal control (NC), Cyt c, Bax, and Caspase 3 expressions (red arrow) are shown in brown less bright with an opaque base. (b) Administration of lipopolysaccharide 0.25 mg/kgBW/i.p, the expressions of Cyt c, Bax, and Caspase 3 (red arrow) shows brighter brown, denser, and brighter base. (c) Administration of Heat shock protein 70 200 g/kgBW/ip after lipopolysaccharide injection 0.25 mg/kgBW/ip, the expression of Cyt c, Bax, and Caspase 3 (red arrow) shows brownish and less dense in which the base is less bright and almost the same as that of NC. 1 = Lung alveolar epithelial cells, 2 = Lung cytosol, 3 = Nucleus of lung cells

reduce the expression of Cyt c, Bax, and Caspase 3. As Aschkenasy *et al.* suggest, exogenous HSP70 dose of 266 µg/kgBW in mice increases the number of BCL 2 which can inhibit apoptosis [18]. In his study, Kumar *et al.* used HSP70 injection tumor models, dosages varied from 1 µg to 100 µg to improve TNF-α production [24]. As Choudhoury *et al.* (2011) states, exogenous HSP70 administration inhibits apoptosis through the prevention of Cyt c complex release pathways, apoptotic protease-activating factor 1 (apaf 1), pro-Caspase 9 and 3. Other researchers mention HSP70 injection exogenous doses of 266 µg/kg at 10 min before and 10 min after LPS injection of 2 mg/kg iv can reduce mortality rates and modify some hemostasis and hemodynamic parameters [11].

CONCLUSION

Low dose LPS of 0.25 mg/KgBW/ip has been able to make sepsis while HSP70 200 ug/KgBW/ip injection has been able to reduce the expression of Cyt c, Bax, and Caspase 3 thus can be used for apoptosis therapy of MODS of 78-h sepsis model.

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CONTRIBUTION OF COAUTHORS

IGL Sukamto generated the initial research question, identified coauthors relevant to the study question, carried out some literature review, and wrote of the study proposal. Bambang Purwanto discussed and reviewed the biomolecular aspects relevant to address the research question. Ambar Mudigdo reviewed the immunohistochemistry pathological anatomy of Cyt c, Bax, and Caspase 3 expressions. Suroto discussed, reviewed, and critically questioned the sepsis aspects involved in this study.

CONFLICTS OF INTEREST

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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