Novel strategy for in-vitro validation of Babesia orientalis heat shock proteins chaperone activity and thermostability

Jinfang Hu (1057030377@qq.com)
State Key Laboratory of Agricultural Microbiology https://orcid.org/0000-0002-8563-8278
Lizhe Fan
State Key Laboratory of Agricultural Microbiology
Yuan Huang
State Key Laboratory of Agricultural Microbiology
Pei He
State Key Laboratory of Agricultural Microbiology
Lan He
State Key Laboratory of Agricultural Microbiology
Junlong Zhao
State Key Laboratory of Agricultural Microbiology

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Novel strategy for in-vitro validation of *Babesia orientalis* heat shock proteins chaperone activity and thermostability

Jinfang Hu[1, 3], Lizhe Fan[1, 2], Yuan Huang[1, 2], Pei He[1, 2], Lan He[1, 2], Junlong Zhao[1, 2*]

[1]State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, P. R. China

[2]Key Laboratory for Development of Veterinary Diagnostic Products, Ministry of Agriculture, Huazhong Agricultural University, Wuhan 430070, P. R. China

[3]Guangdong Laboratory Animals Monitoring Institute, Guangzhou 510633, China

Guangdong Key Laboratory of Laboratory Animals, Guangzhou, Guangdong

*Corresponding author at: State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, China

Tel.: +86 27 87281810; fax: + 86 27 8728 0408.

E-mail address: 910414153@qq.com(Junlong Zhao)

Jinfang Hu:1057030377@qq.com

Lizhe Fan: 375175369@qq.com

Yuan Huang: 543612282@qq.com

Pei He: 1415144593@qq.com

Lan He: helan@mail.hzau.edu.cn

Junlong Zhao: 910414153@qq.com

ABSTRACT:

Background: *Babesia orientalis,* an intracellular protozoan, which infects red blood cells and causes water buffalo babesiosis. The genome of *B. orientalis* has been reported and various genes have been accurately annotated, including heat shock proteins (HSP). Three *B. orientalis* HSPs (HSP90, HSP70 and HSP20) have been previously identified as potential antigenic targets. Here, a new validation strategy for the chaperone activities and cell protection characteristics of the three *B. orientalis* HSPs was developed in vitro.

Methods: BoHSP20, BoHSP70 and BoHSP90B were amplified from cDNA, followed by cloning them into the pEGFP-N1 vector and transfecting the vector plasmid separately into 293T and Hela mammalian cells. Their expression and localization were determined by fluorescence microscopy. The biological functions and protein stability were testified through an analysis of the fluorescence intensity duration. Their role in the protection of cell viability from heat-shock treatments was examined by MTT assay.

Results: Fusion proteins pEGFP-N1-BoHSP20, pEGFP-N1-BoHSP70, and pEGFP-N1-BoHSP90B (pBoHSPs: pBoHSP20; pBoHSP70 and pBoHSP90B) were identified as 47 kDa/97 kDa/118 kDa with a 27 kDa GFP tag, respectively. Prolonged fluorescent protein half-time was observed specifically in pBoHSPs under heat shock

treatment at 55 ℃, and BoHSP20 showed relatively better thermotolerance than BoHSP70 and BoHSP90B. Significant difference was found between pBoHSPs and controls in the cell survival curve after 2 h of 45 ℃ heat shock.

Conclusion: Significant biological properties of heat stress-associated genes of *B. orientalis* were identified in eukaryote by a new strategy. Fusion proteins pBoHSP20, pBoHSP70 and pBoHSP90B showed good chaperone activity and thermo-stability in this study, implying that BoHSPs played a key role in protecting *B. orientalis* against heat-stress environment during parasite life cycle. In a word, the in-vitro model explored in this study provides a new way to investigate the biological functions of *B. orientalis* proteins during the host -parasite interaction.

Keywords: *Babesia orientalis*; heat shock proteins; HSP20, HSP70, HSP90, protein thermostability; heat stress

1. Introduction

Babesia orientalis, a tick-borne hemo-protozoan parasite, was classified as a new species based on pathogenicity, transmission and morphology in 1997[1,2], and it is responsible for severe morbidity and mortality in water buffaloes, causing a significant impact on socio-economic development[3,4].

The complete genome of *B. orientalis* had been sequenced and characterized[5,6] and several stress-associated genes (HSP20, HSP70, HSP90B) were identified[7-9]. Heat shock proteins (HSPs) are known as the most abundant and highly conserved proteins

66 in the entire organism, and play key roles in many cellular processes[10]. Protozoa
67 have complex life cycles and often have to pass through multiple organisms including
68 vectors and mammalian hosts, the increasing expression of HSPs were reported in
69 many protozoan parasites encountering environmental cues and stresses, such as
70 nutritional deprivation, osmotic stress, oxidative stress and thermal stress[11,12].
71 In parasite, the critical roles of HSPs are involved in adaptation to the host new
72 environment and regulation as antigens[13,14]. HSPs are classified into 5 main
73 families according to their sequence homology and molecular weight, including HSP
74 110s, HSP 90s, HSP 70s, HSP 60s and small HSPs[15]. Most of heat shock proteins
75 are referred to as “molecular chaperones” due to their functions in assisting proteins
76 in the acquisition of native structures or degrading mis-folded proteins[16]. Under
77 normal conditions, HSPs serve as “housekeeping functional proteins” in multiple cells,
78 while under stress conditions, they play an important role in cell survival, protecting
79 and dis-aggregating stress-labile proteins[17]. As a main stressor, temperature
80 represents a challenging problem for survival of organisms when heat is above their
81 optimum growth. Heat-shock has deleterious effects on the internal organization of
82 cells, especially in eukaryotes, causing major damages such as defect of cytoskeleton,
83 malposition of organelles and dysregulation of intracellular transport processes[15].
84 The cooperation of HSP families is involved in remodeling cell structure and protein
85 homeostatics under stress conditions, which is known as a chaperone network, such as
86 HSP70/HSP40[18] and small HSPs /HSP70[19]. HSP70s are important components
87 of the cellular network of molecular chaperones and serve as the major transport

proteins with antigen properties in parasite[13,20-21]. HSP90s (two putative HSP90 proteins in *B. orientalis*: HSP90A and HSP90B) as major and conserved chaperones are an ATP-controlled machine and do not modulate client affinity, but they control the influx of substrates from HSP70s [9,22]. HSP20 forms large oligomers and possesses classical chaperone activity as one of the small HSPs (sHSP) and a molecular mini-chaperone with a signature alpha-crystallin domain[23-26].

*B. orien*talis caused serious anemia, fever, icterus and hemoglobinuria in infected buffalo, with reported cases of mortality in China. As chaperones with good immunogenicity and functions of protein trafficking and internal homeostasis of host cell, HSPs play an important protective role in the life cycle of parasites. The chaperone activities and cell protection characteristics of HSPs were achieved via protein quality control, refolding and degradation. In this study, the main characteristics of the three parasite proteins (HSP20, HSP70 and HSP90B) were demonstrated in eukaryotic expression, which may facilitate further research on the action mechanism of *B. orientalis* HSPs between parasites and host cells.

2. Materials and methods

2.1 Parasites, cDNA preparation

B. orientalis (Wuhan-strain) was preserved in liquid nitrogen in the State Key Laboratory of Agriculture Microbiology, Huazhong Agricultural University, China. Total RNA was extracted from *B. orientalis* using the E.Z.N.ATM Blood RNA Kit, reverse transcribed using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara,

Japan) and used for cDNA synthesis.

2.2 Cloning of HSP20, HSP70 and HSP90B

The HSP20, HSP70 and HSP90B genes of *B. orientalis* (BoHSP20; BoHSP70; BoHSP90B) were amplified from cDNA using primers listed in Table 1. The thermal cycling parameters included the activation of *FastPfu* DNA polymerase (TransGen Biotech, China) at 95 ℃ for 2 min, 40 cycles of (denaturation at 95 ℃ for 20 s, annealing at 55 ℃ for 20 s, extension at 72 ℃ for 3 min), and a final extension of 10 min at 72 ℃. The amplified fragments were electrophoresed using 0.8% ethidium bromide-stained agarose gel and purified by EasyPure@ Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). The purified DNA fragments were ligated into pEGFP-N1 vector by NovRec enzyme (TransGen Biotech, China).

2.3 Cell transfection and heat-shock treatment

293T cells and HeLa cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Sigma) with 5% CO_2 at 37 ℃. Cells were transfected separately with pBoHSP20, pBoHSP70, and pBoHSP90B plasmids in equal amounts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfecting condition was optimized by the complex of 100 ng DNA and 0.2 ul transfector reagent per well for 96-well plate, 500 ng DNA and 1 ul transfector reagent per well for 24-well plate, 2500 ng DNA and 5 ul transfector reagent per well for 6-well plate. Cell transfection was conducted when the

cell density reached 60%-80% confluence. After the complex of DNA and transfector reagent actuation duration for 6 h, culture supernatants were removed, along with adding fresh culture medium and further culturing cells for 24 h. pEGFP-N1 and pEGFP-N1-CAM were used as a negative control and a blank control, respectively. After transfection and maintenance culture of 24 h, the cells were transferred to an incubator. The recovery experiment was conducted by maintaining cells at 37 ℃ temperature for further culture after 40-minute 45 ℃ heat-shock treatment.

2. 4 Protein thermal-stability assays

The effect of heat shock on protein solubility was analyzed by ArrayScan VTI 600 series High Content Screening (HCS) (Thermo Scientific, American). Briefly, pBoHSP20, pBoHSP70, and pBoHSP90B proteins were expressed as EGFP-fusion proteins in 293T transfection cells after transfection and 24 h maintenance culture, and the intensities of the green fluorescent proteins in these cells were recorded directly using HCS machine every 2 h after heat-shock treatment (55 ℃). In order to improve the credibility and facticity of this method, one unrelated plasmid pEGFP-N1-CAM was prepared as a blank control, and pEGFP-N1 was prepared as a negative control.

2.5 MTT assay

Cell viability was examined with MTT (Thiazolyl blue tetrazolium bromide) assay kit (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI)

according to the supplier's instructions. Briefly, Hela cells were seeded into 96-well plates for 24 h of culture, and subjected to 45 ℃ heat-shock treatment at 0-4 intervals. Next, 10 μL MTT solution was added per well to achieve a final concentration of 0.45mg/mL, followed by incubation at 37 °C for 1-4 h and addition of 10 μl solution into each well to dissolve formazan crystals. Culture plate was vigorously vibrated to ensure complete mixture and solubilization. Absorbance at 570 nm was recorded on a micro-plate reader (BioTek, Winooski, VT). In order to eliminate the deviation of the empirical method, two control groups were prepared, with Lipofectamine2000 as a blank control and pEGFP-N1 as a negative control.

2.6 Western blot

The collected cells were treated with RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min on ice. Briefly, 30 ug protein quantified by BCA protein assay kit (Beyotime Institute of Biotechnology) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane. After blocking with 1% BSA for 1 hour, the membrane was exposed to anti-GFP (1:1,000) at 4 ℃ overnight. After washing with PBS, the membrane was incubated with the secondary antibody (1:2000, HRP labeled goated anti-mouse IgG, Beyotime, Beijing) for 2 h at room temperature. The protein was detected with Enhanced chemiluminescence (ECL, Applygen Technologies Inc., Beijing, China). The signals were captured and the intensity of the bands was quantified using Image Lab software (Bio-Rad).

176

177 **2.7 Statistical analysis**

178 The experiments were performed in three independent replicates. The results were
179 presented as the mean values ± standard errors (SE) of three experiments. The pooled
180 data were subjected to one-way analysis of variance (ANOVA) followed by least
181 significance difference (LSD) test at $p< 0.05$ and/or 0.01 using the GraphPad Prism
182 5.0 Software.

183

184 **3. Results**

185 **3.1 Construction of BoHSP20, BoHSP70, and BoHSP90B eukaryotic expression**
186 **plasmids**

187 The HSP20, HSP70 and HSP90B genes of *B. orientalis* were amplified from cDNA
188 by the homologous recombination method using primers in Table 1, which were
189 designed based on published sequences (Accession number: BoHSP20: JX993941.1;
190 BoHSP70: EF512547.1; BoHSP90-B: KF379585.1). The PCR amplified products
191 with sizes of 534 bp, 1944 bp and 2349 bp were ligated into the pEGFP-N1 vector
192 and sequenced. Their fusion proteins with EGFP tag were expressed in 293T cells,
193 and identified by Western blot with predicted sizes of 47 kDa, 97 kDa, and 118 kDa
194 (Figure 1).

195

196 **3.2 Expression of BoHSPs**

197 The expression of these fusion proteins were observed by fluorescence microscopy.

198 BoHSP20 was expressed mainly in cytoplasm, BoHSP70 was well-distributed in the
199 intracellular, and BoHSP90 was present in cytoplasm with a relatively low expression;
200 meanwhile, the control group with pEGFP-N1 blank plasmid was expressed in both
201 cytoplasm and nuclei (Figure 2 A-D). Expression of the three fluorescent proteins
202 remained unchanged under 45 ℃ heat shock treatment but not under 55 ℃
203 heat-shocking. Figure 2 E-H showed the difference in the three fluorescent proteins in
204 293T cells under 55 ℃ heat-stress for 2 h. The fluorescence intensity faded faster in
205 the pEGFP-N1 blank group than in the experimental groups, and the bright
206 aggregation in the pBoHSP20 group was transformed into a homogenization state.
207
208 **3.3 Protein stability analysis**
209 The thermal-stability characteristics of the eukaryotic expression of *B. orientalis*
210 HSP20, HSP70 and HSP90B were investigated by treating 293T cells expressing
211 BoHSPs under heat stress 55 ℃ for different time intervals. The fluorescent intensity,
212 which represents the thermotolerance of heterologous proteins, was determined. As
213 shown in Figure 3, after 55 ℃ heat shock for different intervals, the fluorescence
214 intensities of pBoHSP20, pBoHSP70 and pBoHSP90B were relatively strong and
215 even enhanced over a long time, while those of the two controls, pEGFP-N1 and
216 pEGFP-N1-CAM, gradually faded and even disappeared after 6 h. Collectively, the
217 fluorescent intensities showed a significant difference between experimental groups
218 and control groups ($p<0.05$). Notably, pBoHSP20 exhibited better thermotolerance
219 than pBoHSP70 and pBoHSP90B. Throughout the heat-shock treatment, pBoHSP20

maintained significant differences ($p=0.04$, $p<0.05$, after 2 h; $P=0.005$, $p<0.01$; after 4 h; $P=0.0005$, $p<0.001$, after 6 h), while both BoHSP70 and BoHSP90B only showed the difference $P=0.01$ at 4^{th} and 6^{th} hour.

3.4 Cell survival assay

The chaperone activity of HSPs was known to protect cell viability under stress conditions. Cell survival rate was measured via MTT assay to evaluate the role of BoHSPs in safeguarding heat-shocked cells. Hela cell rather than 293 T cell was applied in this experiment because of its weak adherence. A significant difference was observed in the survival ratio between pBoSHPs and controls (pEGFP-N1 and lipidosome) at the 2nd hour after 45 ℃ heat-shock treatment ($p=0.0037$, $p<0.01$), but not difference among the pBoSHP groups including pBoHSP20, pBoHSP70 and pBoHSP90B (Figure 4).

4. Discussion

Heat shock proteins are abundant, ubiquitous and evolutionary conserved proteins in most of cells and have a similar role in organisms from bacteria to humans. *B. orientalis* is an intracellular protozoan parasite and responsible for debilitating or lethal diseases in animals. In common with most other organisms, *B. orientalis* possesses HSPs to protect cells against intracellular condition and variable environment. Good stability and perfect protection characteristics of BoHSPs guaranteed *B. orientalis* against variable thermal stress when living in different niches,

including mammalian hosts, insect vectors and outside environment. The HSPs of *B. orientalis* (BoHSP20, BoHSP70 and BoHSP90B) have been verified to have good sequence similarity with the HSPs of other *Babesia*[7-9]. The ACD-sHSP-like domain, one of the major domains in sHSP family[27], was predicted in 74aa -168aa of BoHSP20 by NCBI and Expasy Software. Crystal structures of the ACD domain possessed dimeric arrangement and oligomeric assembly, which determined the thermostability of sHSPs [23,28]. BoHSP70 possesses a NBD-sugar-kinase-HSP70-actin super family structure, which is involved in the allosteric control mechanism of HSP70 chaperone function[29]. The ribosomal protein S5 domain 2-like domain of BoHSP90B was predicted in 313 aa-797aa of the whole amino acid sequence, which participated in binding clients[9].

The EGFP fluorescent protein is generally expressed in cytoplasm and nucleoplasm, and the fusion expression of pBoHSP plasmid can represent the locations and shapes of BoHSPs in cells. pBoHSP20 was almost expressed in the form of aggregation in cytoplasm, and the aggregation was transformed into homogenization after 55 ℃ heat-shock treatment. It should be noted that HSP20 exists in the form of stacked oligomers, which are mainly shaped in the dimerization domain of the ACD domain. Therefore, over-expression of pBoHSP20 in cells can be a good method to demonstrate the nature of BoHSP20, and the disappearance of dimerization in the cells after 2 h of 55 ℃ heat shock is probably attributed to a charge region contained in the C terminus of sHSP, a region essential for the heat stability of sHSP[30]. pBoHSP70 and pBoHSP90B were mainly expressed in cytoplasm and their locations

were not shifted under heat-shock conditions. We also treated the cells at other temperatures, and found no change in the shape or location. This result can be attributed to the sequence difference between human host HSP genes and *B. orientalis* exogenous genes.

The fluorescence intensities of the recombinant proteins with EGFP tag were monitored by HTC to reveal the difference in the half-time of pBoHSPs during heat-shock treatment. As shown Figure 3, after 55 ℃ heat shock for different intervals, the drop differences of fluorescence intensities were obvious between pBoHSPs groups and the two controls. Comparing to the controls, pBoHSP20 group show significant repression of descend with $p<0.05$ at 2^{th} h, $p<0.01$ at 4^{th} h and $p<0.001$ after 6^{th} h, while pBoHSP70 and pBoHSP90B groups both remarkably with $p<0.05$ after 4^{th} h, but no difference at 2^{th} h. There are two possible explanations for the elevated fluorescence value in the pBoHSP groups. Firstly, the chaperone activity of pBoHSPs enhanced with an increased temperature, leading to a slight ascent of fluorescence intensity in the fluorescent proteins. Secondly, the structures of the proteins such as pBoHSP20 were transformed from aggregation to homogenization. In the process of heat-shock, the cell morphology was destroyed, leading to the complete exposure of proteins and a slight deviation in the fluorescence value, which was understandable under the unchanged photoing conditions, such as the exposure of photograph and focal distance. These observations demonstrate that pBoHSPs do possess good thermostability, and monitoring the fluorescence intensities in cells is an effective method to detect the stability of pBoHSPs. Many mathematical models were

proposed to elaborate the dynamic HSPs synthesis induced by heat shock, and the heat shock response was generally based on the mechanism of HSPs:HSF:HSE molecular response[17,21,31]. According to the temperatures proposed in the computational models and our previous research, we designed the 45 ℃ heat-shock treatment in cell viability assay and confirmed the good capacity of pBoHSPs in protecting cell from apoptosis. As shown by the cell survival curve in Figure 4, the pBoHSPs showed significant difference form the two control groups in protection characteristics at the second hour of treatment, which disappeared after 4 hours of heat-shock treatment. Additionally, a qPCR experiment was conducted in this study, and both pBoHSP70 and pBoHSP90B showed unstable expressions except for pBoHSP20 (data not shown). This result implies that pBoHSPs, as heterologous proteins, played a role in cell survival through the protein chaperone activity rather than totally by regulation on the mRNA level. The effect of over-expressing exogenous pBoHSPs on protecting Hela cell might be due to the early formed contributions of chaperoning and degradation to protein homeostasis[32-35].

In conclusion, the strategy in our study exhibited good performance of pBoHSPs in the chaperone activity. Compare to other traditional methods, such as bacterial survival experiment[8], the eukaryotic expression of BoHSPs in mammalian cells was an effective method to study their biological characteristics, and the fluorescent intensity of the pBoHSPs recombinant proteins was a formidable and effective index to show the HSPs protein stability.

5. Conclusions

As a protozoan parasite, the pathogenic mechanism of *B. orie*ntalis was complex, among which, HSPs have been one of its common strategies to cope with environmental stress. Herein we developed a novel strategy to verify three BoHSPs (BoHSP20, BoHSP70 and BoHSP90B) thermal-stability activities and cell protection characteristics in eukaryotic expression, which may facilitate further research on the pathogenic mechanism of *B. orientalis* HSPs.

Abbreviations

HSPs: heat shock proteins; BoHSPs: *Babesia orientalis* heat shock proteins; BoHSP20: *Babesia orientalis* heat shock protein 20; BoHSP70: *Babesia orientalis* heat shock protein 70; BoHSP90B: *Babesia orientalis* heat shock protein 90 B; pBoHSPs: *Babesia orientalis* heat shock proteins eukaryotically expressing on pEGFP-N1 vector; pBoHSP20: *Babesia orientalis* heat shock protein 20 eukaryotically expressing on pEGFP-N1 vector; pBoHSP70: *Babesia orientalis* heat shock protein 70 eukaryotically expressing on pEGFP-N1 vector; pBoHSP90B: *Babesia orientalis* heat shock protein 90B eukaryotically expressing on pEGFP-N1 vector; ACD: alpha-crystallin domain; NBD: nucleotide-binding domain; MTT: Thiazolyl blue tetrazolium bromide; s: Seconds; m: Minutes; h: Hours.

Availability of data and material

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Authors' Contributions

JH wrote the draft of the manuscript. JZ and LH revised the manuscript. LF, YH, and PH had workn in Parasitology lab, Huazhong Agricultural University, China. They contribute the basic need for *B. orientalis* heat shock protein research. All authors read and approved the final manuscript.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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440

441 **Table1** Oligonucleotide primers used for cloning the plasmids of pBoHSP20,
442 pBoHSP70, pBoHSP90B.

443 **Figure legends**

444 **Figure 1** Identification of the recombinant proteins by Western blot. Lane M:
445 pre-stained molecular weight marker; Lane 1: lysate of 293T cells transfected with
446 plasmid pBoHSP90B; Lane 2: lysate of 293T cells transfected with plasmid
447 pBoHSP70; Lane 3: lysate of 293T cells transfected with plasmid pBoHSP20.

448 **Figure 2** Fluorescent proteins of pBoHSP20, pBoHSP70, and pBoHSP90B expressed
449 in 293T. A-D: Fusion expression of pEGFP-N1, pBoHSP20, pBoHSP70 and
450 pBoHSP90B in 293T after 24-h transfection at 37 ℃, respectively. E-H: Fusion
451 expression of pEGFP-N1, pBoHSP20, pBoHSP70 and pBoHSP90B in 293T under
452 55 ℃ heat-stress for 2 h, respectively.

453 **Figure 3** Relative fluorescent intensities of 293T cells expressing pBoHSP20,
454 pBoHSP70, and pBoHSP90B under 55 ℃ heat-shock. Data are mean±SD, *$p<0.05$,
455 **$p<0.01$, ***$p<0.001$, the results were repeated in three independent experiments. N:
456 Relative fluorescent intensities of pEGFP-N1 group, as negative control; E20:
457 Relative fluorescent intensities of pBoHSP20 group; E70: Relative fluorescent
458 intensities of pBoHSP70 group; E90: Relative fluorescent intensities of pBoHSP90B
459 group; E-CAM: Relative fluorescent identity of pEGFP-N1-CAM group, as unrelated
460 control.

Figure 4 Cell survival rates of heat-shocked cells as identified by MTT Assay. Data are mean ± SD, **p<0.01, the results were repeated in three independent experiments. N: Cell survival rate of pEGFP-N1group, as negative control; E20: Cell survival rate of pBoHSP20 group; E70: Cell survival rate of pBoHSP70 group; E90: Cell survival rate of pBoHSP90B group; L: Cell survival rate of lipidosome group, as blank control.

Supplementary Files

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- Table1.doc