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Short communication

Non-lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *Vibrio campbellii*

Parisa Norouzitallab [a], Kartik Baruah [a], Dechamma M. Muthappa [b], Peter Bossier [a, *]

[a] Lab of Aquaculture & Artemia Reference Center, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Rozier 44, Gent 9000, Belgium
[b] Department of Fisheries Microbiology, College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Mangalore, Karnataka, India

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1. Introduction

Unlike vertebrates that depend on both arms (innate and adaptive) of the immune system, the invertebrates rely only on the innate arsenals to fight against invading pathogens [1]. Several lines of evidence have suggested that microbial components (such as lipopolysaccharide, proteoglycans, bacterial DNA) and/or signal molecules released from dying cells can readily activate the innate immune system and initiate multiple inflammatory cascades [2,3]. High-mobility group box 1 (HMGB1) and heat shock proteins (HSPs) are well-known mediators of these inflammatory responses [4]. HMGB1 is a highly conserved, ubiquitous, non-histone chromatin-associated protein, which function is to stabilize nucleosome (histone/DNA complex) formation and to act as transcription-factor like protein that regulates gene expressions by bending DNA and promoting access to transcriptional proteins on specific DNA targets [3,5–9]. Additionally, HMGB1 has recently been reported to induce cytokines and inflammatory responses once secreted to the extracellular environment [2]. In presence of stressing agent, it acts as a danger signal and inflammatory mediator by passive secretion from necrotic cells [10] and/or by an active leakage from immune cells e.g. macrophages, monocytes and dendritic cells [6,11]. Like HMGB1 protein, the highly conserved HSPs also play very comparable role under similar physiological conditions in the organisms [12,13]. Under normal biological conditions, HSPs are constitutively produced (intracellularly) and are involved in upholding protein biogenesis and protein homeostasis in the cells [14,15]. However, under stressful conditions, these proteins are induced and are released either actively or passively into the extracellular environment in order to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins and inhibit protein aggregation, thus protecting cells from harmful environmental stresses [16,17]. Besides these, extracellular HSPs are also implicated in eliciting immune responses against many diseases as demonstrated in a wide variety of experimental models [18–20]. These proteins range in size from 27 to 110 kDa and are categorized into five main families according to their molecular mass: HSP100, HSP90, HSP70, HSP60 and small HSPs [21]. Among the different HSP families, the HSP70 family molecules, such as the constitutive HSP70 (HSC70 or HSP73) and the stress-inducible HSP70 (HSP70 or HSP72), are the most well-characterized HSPs [19].

The proteins HMGB1 and HSPs share many common characteristics. For instance, they function as molecular chaperone for DNA and protein, respectively, both function as an extracellular signaling molecule and damage associated molecular protein (DAMP) during inflammation and various cellular processes [see review [7,22]]. In presence of pathogenic biotic stressors, extracellular HMGB1 and HSPs participate in the activation of cell surface innate immune receptors, typically Toll-like receptors (TLRs), thereby affecting many aspects of host's immune responses [6,23]. In addition to these, abiotic stressors such as heat stress can also readily alter the levels of both these proteins [7,24].

In our previous study, we have shown that exposure to a non-lethal heat stress (NLHS) at 37 °C for 30 min followed by 6 h recovery period induced HSP70 production within the host *Artemia fransciscana* nauplii and this induced HSP70 contributed to improve resistance of the host against subsequent *Vibrio* challenges [25].

* Corresponding author. Tel.: +32 926 43754; fax: +32 926 44193.
E-mail address: Peter.Bossier@UGent.be (P. Bossier).

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Considering the fact that HMBG1 protein is an intracellular molecular chaperone, and it is released into the extracellular medium upon cellular stress or activation, similar to HSP70 [4], it is possible that the observed protective effects of NLHS is mediated by the induction of HMGB1 in combination with HSP70. In this first study, using the gnotobiotic *Artemia* model organism (GART) system, we investigated whether these two molecular chaperones are the effector molecules in mediating downstream protection to *Artemia* against pathogenic *Vibrio campbellii* in *Artemia*. We used the GART system to conduct this study since in this system *Artemia* can be cultured under germ-free environment, and a controlled species and population of micro-organism can be added. This system is a crucial tool for such studies because it allows eliminating the interference of the microbiota that are naturally present in any type of aquatic environment and furthermore facilitates the interpretation of the results in terms of a cause effect relationship [26,27].

2. Materials and methods

The gnotobiotic system was developed by hatching of *Artemia* cysts axenically following decapsulation and hatching procedures as described previously [28]. Briefly, 12 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 ml of sterile distilled water for 1 h. Sterile cysts and nauplii were obtained after decapsulation via using 3.3 ml NaOH (32%) and 50 ml NaOCl (50%). The reaction was stopped after 2 min by adding 50 ml $Na_2S_2O_3$ (10 g l^{-}). Thereafter the decapsulated cysts were washed with sterile artificial seawater (35 g l^{-1}, Aquarium Systems, Sarrebourg, France) and then suspended in 1-l glass bottles containing sterile artificial seawater, and then incubated at 28 °C for 28 h with constant illumination of approximately 27 μE m^{-2}·sec for hatching. All the manipulations were performed under a laminar flow hood and all tools were autoclaved at 121 °C for 20 min in order to maintain sterility of the cysts and nauplii. After 28 h of incubation, the axenicity of the hatched *Artemia* nauplii was verified both by spread plating (100 ml) and by adding (500 μl) hatching water on Marine Agar and in Marine Broth (Difco, Detroit, USA), respectively followed by incubation at 28 °C for 5 days [28]. Experiments starting with non-sterile nauplii were discarded.

Swimming nauplii at instar II stage were collected, counted volumetrically and then distributed in 4 groups, each with 3 replicates. Each group was maintained in 1-l glass bottle containing sterile seawater and placed in rectangular tank containing water maintained at 28 °C using a thermostatic heater with constant illumination (approximately 27 μE m^{-2} sec.) and aeration. Two groups were given non-lethal heat shock treatment by exposing the nauplii to a temperature of 37 °C for 30 min following the procedure described previously [25]. The other two groups were maintained isothermally at 28 °C. One group, each from the heat shocked and non-heat shock, were challenged with *V. campbellii* at 10^7 cells ml^{-1} for 12 h [29]. Samples containing a group of live nauplii weighing in total 0.1 g were harvested from each group at 6 and 12 h post challenge, rinsed in sterile distilled water, immediately frozen in liquid nitrogen and preserved at −80 °C for further analysis.

Total RNA was extracted from each *Artemia* sample using the RNA extraction kit (Promega, Belgium). First strand cDNA was synthesized from 1 μg total RNA using the RevertAid™ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) following the manufacturer's guidelines. The expression of *hsp70* and *hmgb1* genes in the nauplii was analyzed by qRT-PCR using a following pair of primers (*hsp70*: forward – cgataaaggccgtctctcca, reverse-cagcttcaggtaacttgtccttg; *hmgb1*: forward-ggatgaaagcaaaccccgtg, reverse – gtgctcttctctgcaagtctg). The primers for the *hmgb1* gene were designed based on the region of the *Artemia hmgb1* gene (information available through an internally available *Artemia* draft genome) that is highly conserved with human *hmgb1* gene (sequence submitted to EMBL). The qRT-PCR amplifications were carried out in a total volume of 25 μl, containing 9.8 μl of nuclease free water, 0.4 μl of each primer, 12.5 μl of Maxima SYBR Green qPCR Master mix (Fermentas, Cambridgeshire) and 2 μl of cDNA template. The qRT-PCR was performed in a One Step qRT-PCR instrument (Applied Biosystems) using a four-step amplification protocol: initial denaturation (10 min at 95 °C); 40 cycles of amplification and quantification (15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C); melting curve (55–95 °C with a heating rate of 0.10 °C s^{-1} and a continuous fluorescence measurement) and cooling (4 °C). The *β-actin* gene was used as a reference gene for standardizing the expression of target genes. Master mixes were prepared in duplicate for each sample and qRT-PCR for target and reference genes was performed. Relative quantification of target gene transcripts with a chosen reference gene transcript was done following the Pfaffl method [30,31].

Artemia samples were homogenized in cold buffer K, centrifuged at 2200 × *g* for 1 min at 4 °C and the supernatant was collected. Protein was quantified by the Bradford method [32] using bovine serum albumin as standard. Loading buffer was added to the supernatant samples, vortexed, heated at 95 °C for 5 min and electrophoresed in 10% and 4–20% SDS-PAGE gel for HSP70 and HMGB1, respectively, with each lane receiving 10 μg of protein. HeLa (heat shocked) cells (Enzo Life Sciences, USA) (6 μg) were loaded on to one well to serve as a positive control and for calculating the amount of target proteins in the sample. Proteins were then electrotransferred to polyvinylidene fluoride membranes (BioRad Immun-Blot™ PVDF) for antibody probing. The membranes were incubated with blocking buffer [50 ml of 1*x* phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature followed by with primary antibodies for HSP70, which recognizes both constitutive and inducible Hsp70 (1:5000; Affinity BioReagents Inc., Golden, CO) [32] and HMGB1 (1:2500; Affinity Abcam, ab18256, United Kingdom). The membranes were then incubated with horseradish peroxidase conjugated donkey anti-mouse IgG (1:2500; Affinity BioReagents Inc., Golden, CO) and horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibodies (1:10,000; Gentaur BVBA, Belgium) for HSP70 and HMGB1, respectively. The membranes were then treated with enhanced chemiluminescence reagent (GE healthcare, UK) and the signals were detected by a ChemiDoc MP Imaging System (Biorad, Belgium). The relative signal intensity was quantified by densitometry with Biorad Image Lab™ Software version 4.1.

The HSP70 and HMGB1 protein data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences version 20.0. *P* values ≤ 0.05 were considered significant. Results for *hsp70* and *hmgb1* mRNA quantification are presented as fold expression relative to *Artemia* actin. The expression level in the control group was regarded as 1.000 and thereby the expression ratio of the treatments was expressed in relation to the control. Significant differences in expression between control and treatments were analyzed by Pfaffl method [31].

3. Results and discussion

In our previous studies, HSP70 was investigated for its role in controlling bacterial disease in (shrimp) aquaculture [25,33]. For that HSP70 protein was induced within the aquaculture model organism *Artemia* by exposing the animal to a classical stress inducer i.e. a non-lethal heat shock at 37 °C for 30 min followed by 6 h recovery period. In those studies, HSP70 protein was

analyzedanalyzed immediately following the end of the recovery period, and this phenomenon of increased HSP70 production level was shown to be associated with conferring protection to *Artemia* against subsequent *Vibrio* attacks [25]. The current study is an extension of our earlier studies [25,33]. However, here we analysed HSP70 and HMGB1, both at the transcriptional and translational levels, in *Artemia* exposed to a non-lethal heat shock at 37 °C for 30 min (but without a recovery period) and immediately challenged with *V. campbellii* for an indicated period, with the aim to determine if besides HSP70, HMGB1 protein is also induced in response to non-lethal heat shock, and whether these two proteins are involved in conferring protection to *Vibrio*-challenged *Artemia*. Our results showed that at 6 h post challenge, non-lethal heat shock treatment caused a significant effect on the expression of *hsp70* gene in *Artemia* (Fig. 1). In fact, due to non-lethal heat shock treatment, the *hsp70* expression level in the unchallenged (NLHS group) and *Vibrio*-challenged (NLHS + VC group) *Artemia* increased by respectively 3.1-fold and 2.7-fold relative to the corresponding control. Exposure of *Artemia* to *V. campbellii* (VC group) for 6 h also caused a significant increase (6.3-fold to the control, $P < 0.05$) in the expression level of *hsp70* mRNA. However, at 12 h post challenge, the *hsp70* mRNA level did not differ significantly among the different groups ($P > 0.05$, Fig. 1). Having observed these, we next verified if the transcribed *hsp70* in *Artemia* due to different treatments was translated to functional protein to exert its functions. To this end, we carried out Western blot analysis and the results revealed that non-lethal heat shock treatment significantly increased HSP70 production in the *Artemia* as compared to the untreated control group at 6 h post challenge (Fig. 2A and C). From these results, it can be suggested that induction of HSP70 at the protein level was associated with the induction of Hsp70 at the gene level in *Artemia* exposed to non-lethal heat shock. Accordingly, an increased induction of HSP70, at the transcription or protein level or both, in different experimental aquaculture animals in response to sub-lethal heat stress has already been reported by other investigators [25,33–35]. However, at 6 h post challenge, the HSP70 protein level in the untreated *Artemia* challenged with *V. campbellii* (VC group) was significantly reduced (Fig. 2A and C), and this reduction in the HSP70 production level occurred in spite of the increase in the expression level of *hsp70* gene in this group

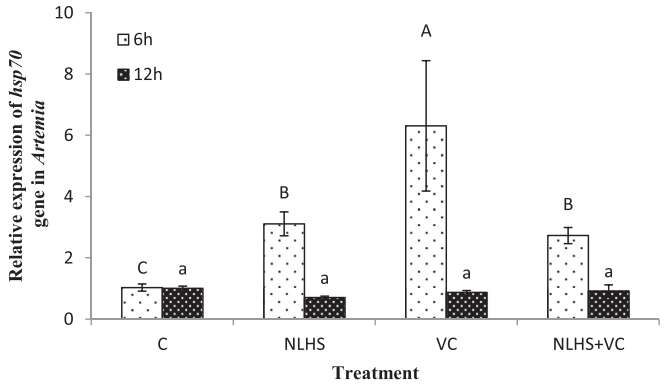

Fig. 1. Expression of *hsp70* gene in *Artemia* nauplii. The nauplii were exposed to non-lethal heat shock (NLHS) treatment at indicated conditions. The nauplii were then either challenged for 12 h with *V. campbellii* (NLHS + VC) or not. Untreated *Artemia* challenged with *V. campbellii* (VC) or not (C) served as controls. Samples were collected for *hsp70* gene expression at 6 and 12 h post challenge. The expression of *hsp70* mRNA in the control (C) group was regarded as 1. Results, which are the mean of 3 replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. [31]. Bars indicate standard error. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

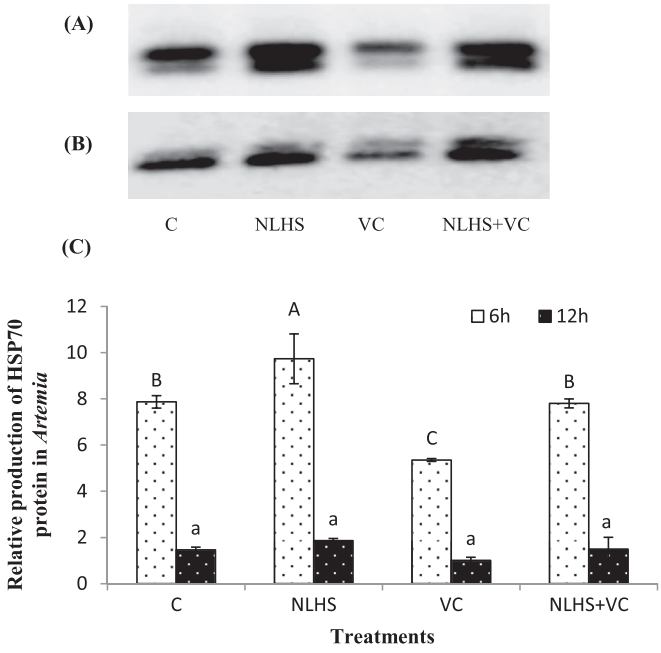

Fig. 2. Production of HSP70 protein in *Artemia* nauplii. For the control and treatment groups, refer to Fig. 1 for explanation. Extracted protein from *Artemia* samples collected at (A) 6 h and (B) 12 h post challenge was resolved in SDS-PAGE gel, transferred to polyvinylidene fluoride membranes and then probed with anti-HSP70 antibody. *Artemia* protein (10 μg) was loaded in each lane. HeLa (heat shocked) cells (6 μg) were loaded on to one well to serve as a positive technical control and for calculating the relative amount of HSP70 in the samples. (C) Quantitative analysis of HSP70 in *Artemia* (expressed relative to the amount in HeLa cells). Bars indicate standard error of 3 replicates. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$). Dual bands appeared in Fig. 2A and B. The upper and lower bands are predicted to represent constitutive HSP70 (HSC70 or HSP73) and the stress-inducible HSP70 (HSP70 or HSP72).

(Fig. 1). By inducing HSP70, organisms temporally and spatially respond swiftly to an ever-changing array of environmental conditions (for review, see 13). For instance, it has been unequivocally demonstrated that synthesized HSP70 protein in animals contributes to the generation of protective (immune) responses against subsequent bacterial attacks [12,17,33,36]. However, there are also evidences suggesting that to survive the host immune response, bacteria respond to the elevated signaling molecules (such as HSP70 mRNA) by targeting the host mRNA translation machineries and thereby inhibiting the synthesis of functional proteins involved in cellular homeostasis and cellular survival [37,38]. From this compendium of evidences, we can hypothesize that the reduced HSP70 production in the challenged *Artemia* (VC group) could be due to the interference of the pathogenic *V. campbellii* with the HSP70 translation machineries of the host. Our results also showed that the HSP70 production level in heat-shocked group challenged with *V. campbellii* (NLHS + VC group) was significantly reduced as compared to the NLHS group (Fig. 2A and C; $P < 0.05$), however, not to a level that it differed significantly from that in the control. This indicates that NLHS exposure might have minimized the functional activity of *V. campbellii* in interfering with the host HSP70 translation machineries, a hypothesis that needs further verification.

At 12 h post challenge, similar to what was observed for *hsp70* gene expression, the HSP70 protein level did not differ significantly among the different groups (Fig. 2B and C). Despite the absence of increased (or limited) production of HSP70 in NLHS-*Artemia* challenged with *V. campbellii* at the indicated time points, we observed a significant increase in the survival of NLHS-*Artemia* challenged with *V. campbellii* as compared to the untreated *Artemia* challenged

with *V. campbellii* [data not shown, see Refs. [25,33]]. In this study, HSP70 was analyzed at about 6 h and 12 h post exposures to NLHS/*V. campbellii*. It is possible that early production (prior to 6 h) of HSP70 and/or other members of HSP family like HSP60, HSP90 protein in response to NLHS might have contributed to the increased survival of *Vibrio*-challenged *Artemia*. Also the observed survival could be due to the elevated levels of HMGB1 molecule instigated by increased levels of HSP70.

HMGB1 is a nuclear protein, which functions as a nucleosome stabilizer and a regulator of transcription [6]. But the activity of HMGB1 is not solely mediated by its ability to bind to DNA. Indeed, recent evidences suggested that in response to stimuli such as infection and injury (oxidative stress) this DNA chaperone can get released into the extracellular environment and can instigate the host immune system to mount a nonspecific biological responses at the site of infection or injury [39,40]. Since a link exists between HMGB1 induction and oxidative stress and downstream survival of an organism [7], we further analyzed the induction of HMGB1 both at the gene and protein levels in *Artemia* exposed to different factors. As shown in Fig. 3, there was no significant difference in the expression level of *hmgb1* gene among the different groups at any of the time points tested. However, a significant change in the level of HMGB1 protein in response to the NLHS and/or *V. campbellii* was observed (Fig. 4). At 6 h post challenge, the HMGB1 protein level in *Artemia* exposed to NLHS and *V. campbellii* decreased by 1.4- and 1.6-fold, respectively compared to the control ($P < 0.05$, Fig. 4A and C). However, in the NLHS-*Artemia* immediately challenged with *V. campbellii* (NLHS + VC group), the HMGB1 protein level was comparable to that of the control group. At 12 h post challenge, the HMGB1 protein level in untreated *Artemia* challenged with *V. campbellii* (VC group) remained altered compared to the control group ($P > 0.05$, Fig. 4B and C). However, in response to NLHS exposure, there was a significant increase in the production of HMGB1 protein in both the unchallenged (NLHS group, 1.3-fold) and challenged (NLHS + VC group, 1.4-fold) *Artemia*. These phenomena, interestingly, corresponded well with increased survival of *Artemia* in these groups [data not shown; see Ref. [25]]. Taken together, these results indicate that in parallel to HSP70, HMGB1 protein is also induced in *Artemia* in response to exposure to NLHS and these two molecular chaperones (possibly) through an unexplored cascade of biochemical and immunological reactions might have contributed to the protection of *Artemia* against *V. campbellii*. This finding of our study corroborates another report that is

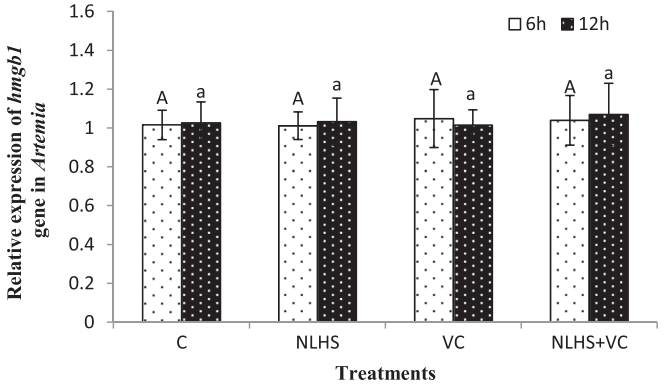

Fig. 3. Expression of *hmgb1* gene in *Artemia* nauplii. For the control and treatment groups, refer to Fig. 1 for explanation. Samples were collected for *hsp70* gene expression at 6 and 12 h post challenge. The expression of *hmgb1* mRNA in the control (C) group was regarded as 1. Results, which are the mean of 3 replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. [31]. Bars indicate standard error. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

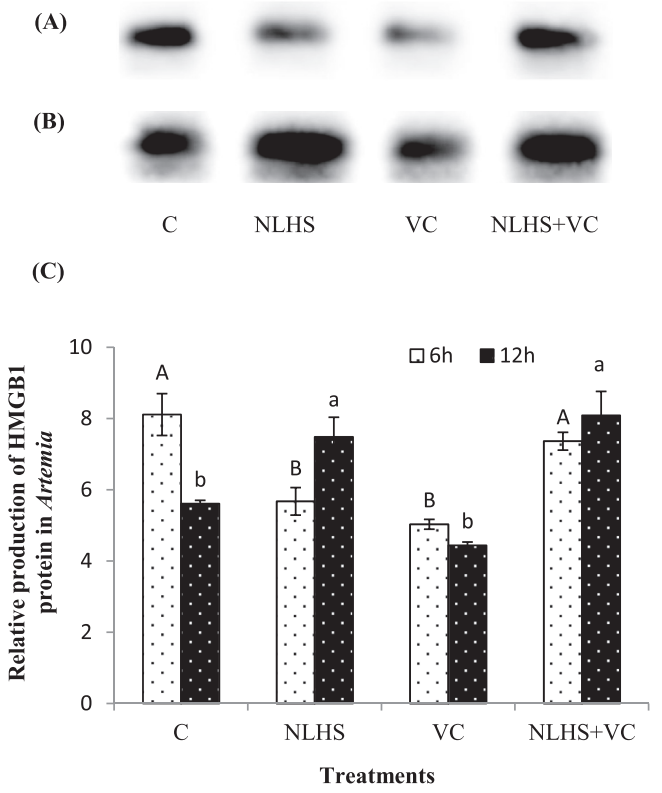

Fig. 4. Production of HMGB1 protein in *Artemia* nauplii. For the control and treatment groups, refer to Fig. 1 for explanation. Extracted protein from *Artemia* samples collected at (A) 6 h and (B) 12 h post challenge was resolved in SDS-PAGE gel, transferred to polyvinylidene fluoride membranes and then probed with anti-HMGB1 antibody. *Artemia* protein (10 μg) was loaded in each lane. HeLa (heat shocked) cells (6 μg) were loaded on to one well to serve as a positive technical control and for calculating the relative amount of HMGB1 in the samples. (C) Quantitative analysis of HMGB1 in *Artemia* (expressed relative to the amount in HeLa cells). Bars indicate standard error of 3 replicates. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

pointing towards a critical role of HMGB1 protein in conferring protection to mice against LPS–induced endotoxemia and bacterial infection by *Listeria monocytogenes* [40].

A striking observation that was made in this study was that the induction pattern of HSP70 and HMGB1 proteins in response to NLHS was inversely related, suggesting that these two molecular proteins are induced sequentially to execute their cytoprotective and immunoregulatory functions. The mechanisms underlying the regulation of active HMGB1 release, association with HSP70 are complex and remain elusive, and therefore need further verification.

In conclusion, this study provides strong *in vivo* evidences that the levels of HSP70 and HMGB1 in a gnotobiotically-grown *Artemia* are elevated sequentially, rather than simultaneously, in response to mild heat stress. These significant alterations in the level of these two proteins were associated with increased protection of *Artemia* against *V. campbellii*. In view of the fact that HSP70 and HMGB1 proteins showed anti-bacterial functions, further studies are warranted to unravel the molecular mechanisms that underlie the protective effect of these two proteins against bacterial infection.

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