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Heat shock proteins as a defense mechanism in midgut and haemocytes of *Bombyx mori* larvae subjected to various stressors

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Abstract
A common physiological response of organisms to environmental stress is the increase in expression of heat shock proteins. In this study, an attempt was made to evaluate the role of HSPs in maintaining the homeostasis of ROS in silkworm, *Bombyx mori*, and larvae under various stressors. A twenty four hours exposure to cold, hypoxia and nuclear polyhedral virus resulted a significant up regulation of HSP70 and HSP90 in midgut tissue and haemocytes. However, the differential expression of proteins were observed in the tissues. Our results clearly indicate HSPs as a possible transitory defense mechanism afforded by silkworm to lessen the oxidative cellular damage induced by short term stressors. The study also brings an insight into the stressor specific and tissue specific expression of HSPs as an immediate defence mechanism to overcome the oxidative insult.

Keywords: heat shock proteins, oxidative stress, cold, hypoxia, virus, stress

1. Introduction

Induction of heat shock proteins (HSPs) is a hallmark of cell under stress and induced HSP may act as chaperones. The flux of non-native proteins induces the stress signal that activates the heat shock response. HSPs are induced and accumulated in the cells during different stress such as hypoxia, serum deprivation and cold shock [1. 2]. The expression of these proteins during stress response has been shown to correlate with increased survival of cells exposed to cytotoxic stimuli and is found in different type of cells [3]. Stress signals are well known targets of HSP70 and include many consecutive as well as stress inducible proteins with overlapping or unique functions in different cell compartments and in different cellular context [4]. Some have mainly a cytosolic localization like the major inducible HSP70 (HSP72) whereas, the consecutively expressed HSC70 (mtHSP70) is localized in mitochondria and the other in endoplasmic reticulum (GRP 78 / Bip) [5]. The overexpression of HSP70 inhibits the apoptotic cascade stimulated by nitric oxide and heat stress triggered translocation of Bax from cytoplasm to the mitochondria [6]. It interacts with the intrinsic and extrinsic pathways of apoptosis at a number of steps and inhibits cell death through chaperone dependent as well as independent activities [7, 8]. HSP70 prevents cell from forced expression of caspase 3 and prevent late caspase dependent events such as activation of cytosolic phospholipase A2 and changes in nuclear morphology [5].

Prominent members of the HSP90 family of proteins such as HSP90α and HSP90β [9] are essential for the viability of eukaryotic cells. They are constitutively abundant, make up to 1-2% of cytosolic protein and can be further induced by stress [10]. HSP90, mostly promote cell survival through its involvement at different steps in the formation of active NFkB which interferes with apoptosis. It is essential for the stability of RIP, which is recruited by activated TNFR-1 following binding with its ligand TNF, for sustained NFkB activity [11, 12]. HSP90 also directly interacts and maintains the activity of Akt by inhibiting its dephosphorylation [13] and when this interaction is prevented by HSP90 inhibitors, Akt is dephosphorylated, destabilized and the likelihood of apoptosis increases. HSP90 Akt complex indirectly promotes cell survival by the inhibition of JNK-mediated cell death through phosphorylation and consequent inactivation of ASK-1 [14]. A common physiological response of organisms to environmental stress is the increase in expression of heat shock proteins. In insects, this process has been widely examined for heat stress, but the response to cold stress has been less understood. Increased HSP70 concentration may be the most rapid response to thermal stress in *Drosophila melanogaster* [15]. Heat shock at 37ºC induced synthesis of HSP64 immediately whereas, the level of HSP70 increased after its synthesis began during recovery in malpighian tubule of *D. melanogaster* [16]. In onion maggot *Delia antiqua* pupae HSP90 expression was up-regulated following heat (35ºC) and cold (-10ºC) [17]. During cold stress and recovery from chill coma inflected a rise in HSP68, HSP70 and HSP83 in adult *D. melanogaster* [18]. Accumulation of HSP70 is also considered as a complex cold tolerance adaptation in the insect *Pyrrhocoris apterus* [19].

Anoxia exposure to 24h elevated levels of HSP70 in gall fly larvae, *Eurosta solidaginis* [20]. In adult flesh fly *Sarcophaga crassipalpis*, HSP70 was the most responsive HSP with several hundred fold increase in expression on hypoxia of 3% oxygen [21]. Overexpression of HSP70 in haemocytes of *D. melanogaster* provides a remarkable survival benefit to flies exposed to severe hypoxia for days [22]. Baculovirus induce HSP70 in *Spodoptera frugiperda* (Sf-9) cells transfected with *Autographa californica* multiple nucleopolyhedrovirus (ACMNPV) [23]. Heat shock cognate 70B (HSC 70B) plays important role in suppression of O'nyong nyong virus (ONNV) replication in the vector *Anopheles gambiae* [24].

The midgut and hemocytes of insects are considered to be highly metabolic, and the midgut is usually susceptible to oxidative injury during food digestion with strong redox potential; the oxidising condition often causes the production

of ROS [25]. High ROS concentration impairs the absorption of ingested nutrients and can cause oxidative damage to the midgut cells [26]. On the other hand, insect hemocytes play an important role in immunity and the respiratory burst of hemocytes is often associated with immune [27, 28].

The main aim of the work was to evaluate the role of HSPs in maintaining the homeostasis of ROS in silkworm, *Bombyx mori*, larvae under various stressors. In the present study, we analyzed the altered HSP expression in the midgut and hemocytes of two instars. We report differential HSP expression in the tissues of silkworm to overcome OS when larvae were stressed for a short time, with low temperature, hypoxia, and virus. Our results clearly indicated a possible transitory defense mechanism afforded by HSPs to lessen the OS-induced cellular damage.

2. Materials and Methods

2.1 Insects and experimental design

The second instar larvae were procured from Kunigal seed area, Karnataka, India and were maintained in laboratory throughout the larval stages and were fed ad libitum on M5 variety mulberry leaves [29, 30]. The uniformly grown healthy larvae of IV and V instars were used in all experiments and were maintained at 24 – 25ºC with relative humidity of 70-75 %. They were made into six groups and each group consisted of hundred silkworms. Experimental animals of group I were not subjected to any stress and was considered as control. Group II larvae were subjected to cold treatment at 5ºC for 24h, whereas group III was also subjected to cold treatment and maintained at room temperature for an additional period of 12h as recovery period. Group IV was subjected to hypoxia for 24h and group V larvae were subjected to hypoxia for the same period and were allowed to recover for an additional period of 12h. Hypoxia was induced by closure of 4 pairs of posterior spiracles with dental wax and during recovery period all the spiracles were in open state. Group VI larvae were inoculated with 10 µl of 1 x 10^6 *Bombyx mori* nuclear polyhedral virus (*Bm* NPV) suspension / g body weight. Larvae that were injected with 10µl of insect ringer served as sham.

Midgut epithelial cells were isolated by 1% collagenase treatment for 10 min after micro dissection at ice cold. Haemolymph was collected in a pre-cooled 2 ml vial containing 5mg thiourea by gentle incision on caudal horn of the larvae. Haemocytes were separated by centrifuging the two times diluted haemolymph at 3000 rpm for 10 min in cold. Cold phosphate buffer of pH 7.4 was used for the 5% tissue homogenate preparation by giving 10 strokes and for the separation of haemocytes or for the isolation of midgut epithelial cells.

2.2 Protein extraction and SDS PAGE

The isolated tissue were homogenized in Tris HCl (pH-6.8) containing dithiothreitol (DTT) and phenyl methane sulfonyl fluoride (PMSF). Supernatant obtained after centrifugation at 6700g was mixed with SDS along with mercaptoethanol, a reducing agent and was subjected to high temperature to dissociate the proteins. Protein samples were electrophoresed on SDS PAGE (12%) along with 100 KDa marker and stained with Coomassie Brilliant Blue R-250 [31]. After destaining the quantitative estimation of HSPs expressed was performed using molecular imaging system (Vilber Lourmat, France) and with the Photo-Capture software.

2.3 RNA extraction and semi quantitative RT-PCR

Total RNA from midgut epithelial cells and haemocytes were isolated using Trizol reagent, a mono phase solution of phenol and guanidine isothiocyanate and treated with DNase I (Chromous Biotech, Karnataka, India). 2 µg of total RNA was reverse transcribed (RT) using 500 ng of oligo dT primer to obtain complementary DNA (cDNA). 50 µl of final volume reaction mixture contains 100 mM magnesium chloride, 750 mM potassium chloride and 500 mM Tris (hydroxymethyl) aminomethane hydrochloride (pH-8.3). 2.5 mM each dNTPs, 40 U of ribonuclease inhibitor (RNasin) and 10 U of Avian myeloblastosis virus (AMV) reverse transcriptase. The mixture was incubated at 42ºC for 1h and reaction was terminated by incubating at 94ºC for 5 min. PCR reaction was performed using Taq DNA polymerase (Chromous Biotech, Karnataka, India) according to the manufacturer's instruction. PCR was performed on an automated thermal cycler (Quanta Biotech, UK). Actin was used as control gene and the sequence of primers along with the cycle conditions are shown in Table-1. The PCR products were resolved on 1.5% agarose gel in Tris-Acetic acid/EDTA buffer with a constant voltage of 80V in parallel with standard markers. Gel was stained with ethidium bromide (0.5 µg/ml) and bands were digitized by molecular imaging system (Vilber Lourmart, France). Densitometric analysis was performed on a negative image using Photo-Capt software. Data were calculated and presented as fold induction of mRNA for HSP 70 and HSP 90 normalised to Bm actin.

Table 1: Primers and the PCR cycle conditions used for the expression of HSP70 and HSP90 in midgut and haemocytes of silkworm *B. mori*.

Genes	Primer pairs	Cycle conditions				
		Initial Denat.	Denat.	Anneal.	Extn.	Final Extn.
		94ºC	94ºC	55ºC	72ºC	72ºC
HSP70	F 5' - TTCAGCAGGACGTGAAGCAC -					
	R 5'- ATGCCGGAACTGTGACTACC -	5 m	30s	30s	30s	5 m
HSP90	F 5' - CAAGTCCATGCTTCCCGTAT -					
	R 5'- ACACCGATGCACAAAAACAA -					
BmA3	F 5' - GAAGCTGTGCTACGTCGCTC -	5 m	30s	30s	1m 30s	5 m
	R 5' - CCGATGGTGATGACCTGACC -					

Statistical Analysis

Data are shown as the mean ± SD of six observations. Changes between the groups were analysed by MANOVA and further tested by the Bonferroni post-hoc test using Statistical Package for Social Sciences (SPSS) software [32] and $p<0.05$ was considered significant. Statistically significant data are presented in the text.

3. Results

3.1 SDS page analysis for stress proteins

Induction of stress protein in silkworm *B. mori* in response to stressors like cold, hypoxia and virus was studied. Cytoprotective protein such as HSPs play a central role in the cellular mechanisms of stress tolerance. PAGE analysis indicated that exposure to low temperature of 5ºC and hypoxia for 24h have induced a 90 kDa protein in midgut tissue of IV instar silkworm. The protein expression of HSP90 was reduced in midgut tissue of silkworm in 12h recovery from low temperature and hypoxia. The induction

of HSP90 protein was not evident in control tissue as well as on exposure to viral infection in midgut tissue of IV instar silkworm. However, the expression of HSP70 protein was evident in midgut tissue of IV instar silkworm on infection with NPV virus (Fig 1). In midgut tissue of V instar silkworm the newly expressed stress protein of 90 kDa was more evident on exposure to low temperature and hypoxia for 24h. Midgut tissue of control silkworm and 12h recovery from these stressors did not show any evidence of HSP90 protein. HSP90 protein expression was not evident in midgut tissue of V instar silkworm on exposure to viral infection, whereas HSP70 protein was induced in midgut tissue of silkworm on viral infection (Fig 2).

In haemocytes of IV instar silkworm, exposure to low temperature, hypoxia and viral infection for 24h resulted in HSP90 expression. Recovery from the stressors for 12h and in control HSP90 expression was not observed (Fig 3). Similarly, in haemocytes of V instar silkworm exposed to various stressors like low temperature, hypoxia and viral infection showed a marked increase in HSP90 protein. However, HSP90 protein expression was not evident in control group as well as on recovery for 12h from low temperature and hypoxia (Fig 4). Expression of HSP70 was not observed in haemocytes of IV and V instar silkworm on exposure to any of the stressors studied.

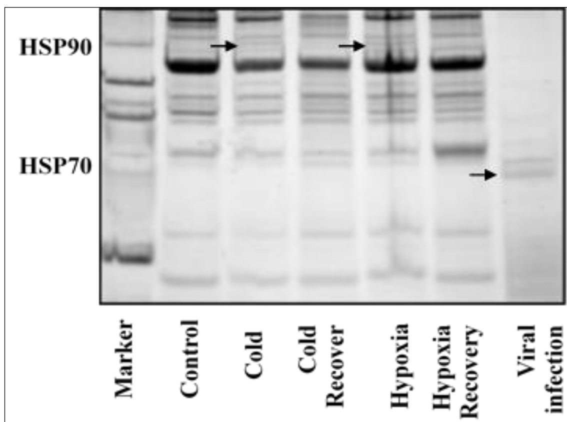

Fig 1: Heat shock protein profile derived from midgut tissue of IV instar silkworm *Bombyx mori* on exposure to cold, cold recovery, hypoxia, hypoxia recovery and viral infection. Stressor specific expression of 90 kDa and 70 kDa heat shock protein is indicated by arrow

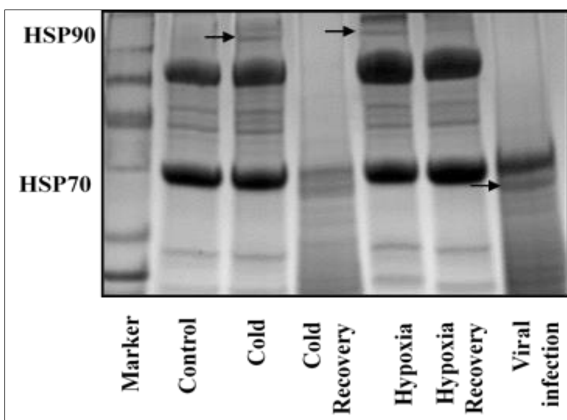

Fig 2: Heat shock protein profile derived from midgut tissue of V instar silkworm *Bombyx mori* on exposure to cold, cold recovery, hypoxia, hypoxia recovery and viral infection. Stressor specific expression of 90 kDa and 70 kDa heat shock protein is indicated by arrow

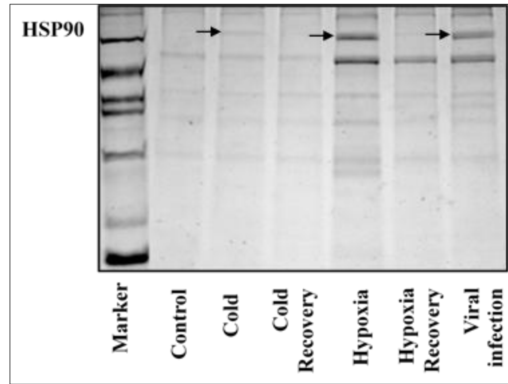

Fig 3: Heat shock protein profile derived from haemolymph of IV instar silkworm *Bombyx mori* on exposure to cold, cold recovery, hypoxia, hypoxia recovery and viral infection. Tissue specific expression of 90 kDa heat shock protein is indicated by arrow

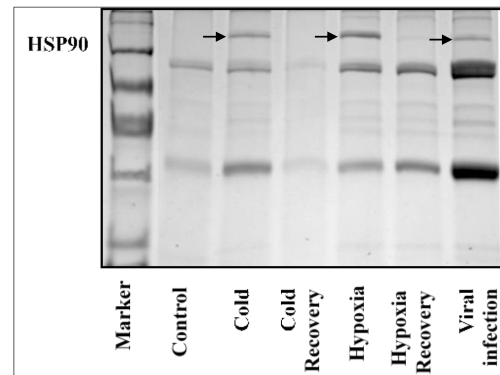

Fig 4: Heat shock protein profile derived from haemolymph of V instar silkworm *Bombyx mori* on exposure to cold, cold recovery, hypoxia, hypoxia recovery and viral infection. Tissue specific expression of 90 kDa heat shock protein is indicated by arrow

3.2 mRNA expression studies for HSP70

HSP expression promotes the functional recovery of oxidative damaged proteins and protects cells from stress related cell damage. In the present study, the variation in expression of HSP70 in midgut and haemocytes of silkworm on exposure to low temperature, hypoxia and virus is clearly evident. mRNA expression of HSP70 was up-regulated during cold, hypoxia and viral infection and the same was quantified by densitometry in midgut tissue of silkworm (Fig 5 and 6). The mRNA expression of HSP70 has shown similar pattern in haemocytes as that of midgut epithelial cells of silkworm larvae (Fig 7 and 8).

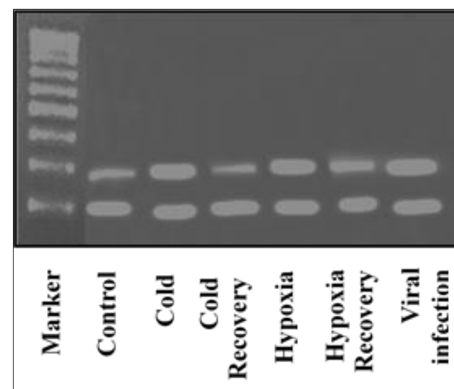

Fig 5: Representative mRNA expression resolved on agarose gel: HSP70 in midgut of silkworm *B. mori* on exposure to various stressors.

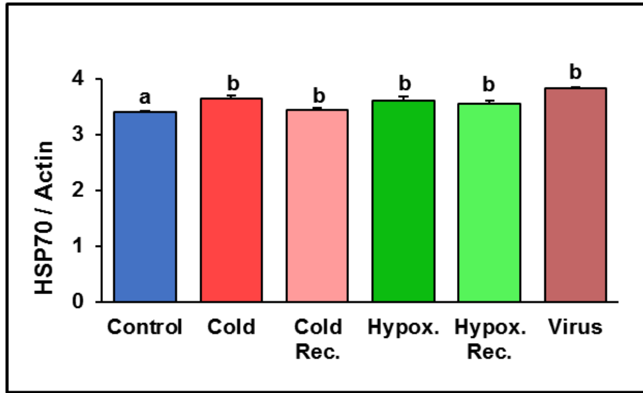

Fig 6: HSP70 mRNA in midgut tissue of silkworm *B. mori* is represented by the ratio of HSP70/ βactin RT-PCR products. Data are means ± SE (n = 4). P <0.05 was considered significant. Values between the stressors are represented in lower cases (a, b). Those not sharing the same letters are significant.

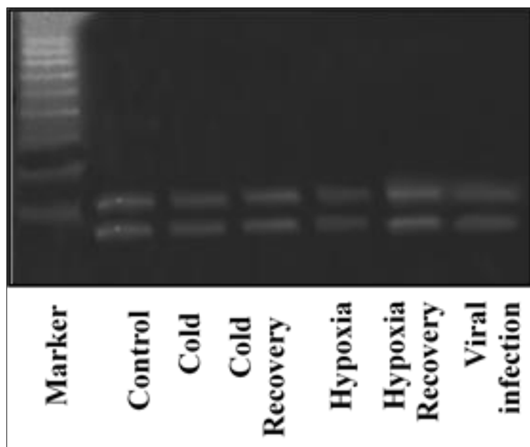

Fig 7: Representative mRNA expression resolved on agarose gel: HSP70 in haemocytes of silkworm *B. mori* on exposure to various stressors.

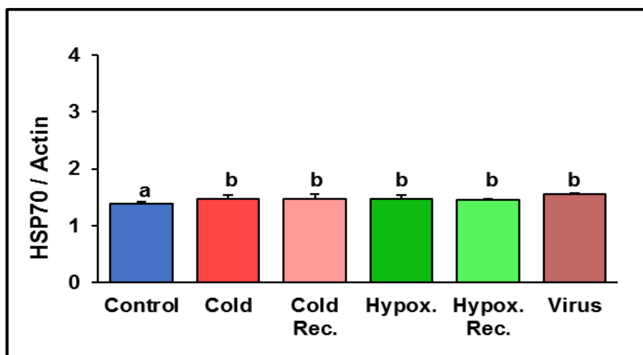

Fig 8: HSP70 mRNA in haemocytes of silkworm of silkworm *B. mori* is represented by the ratio of HSP70/ βactin RT-PCR products. Data are means ± SE (n = 4). P <0.05 was considered significant. Values between the stressors are represented in lower cases (a, b). Those not sharing the same letters are significant.

3.3 mRNA expression studies for HSP90

HSP90 genes were up-regulated during low temperature, hypoxia and viral stress in midgut tissue of silkworm (Fig 9) and the same was quantified by densitometry (Fig 10). A significant up-regulation of HSP90 was also observed during 12h recovery period from low temperature and hypoxia (Fig 10). A similar trend was also observed in the mRNA expression of HSP90 in haemocytes of silkworm of silkworm as that of midgut epithelial cells (Fig 11 and 12).

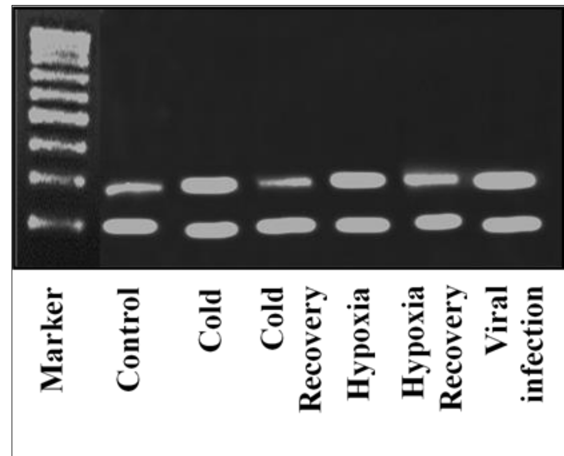

Fig 9: Representative mRNA expression resolved on agarose gel: HSP90 in midgut of silkworm *B. mori* on exposure to various stressors.

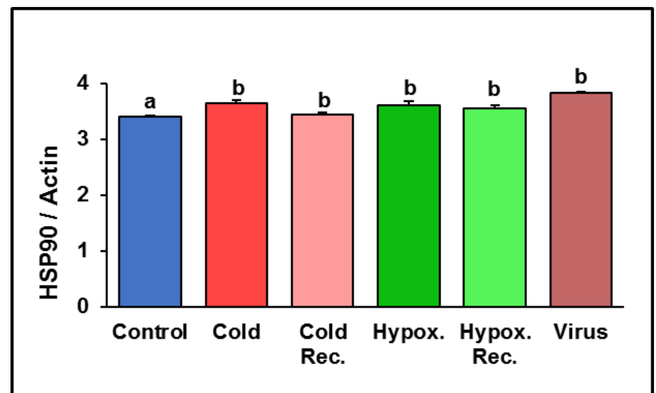

Fig 10: HSP90 mRNA in midgut tissue of silkworm *B. mori* is represented by the ratio of HSP90/ βactin RT-PCR products. Data are means ± SE (n = 4). P <0.05 was considered significant. Values between the stressors are represented in lower cases (a, b). Those not sharing the same letters are significant.

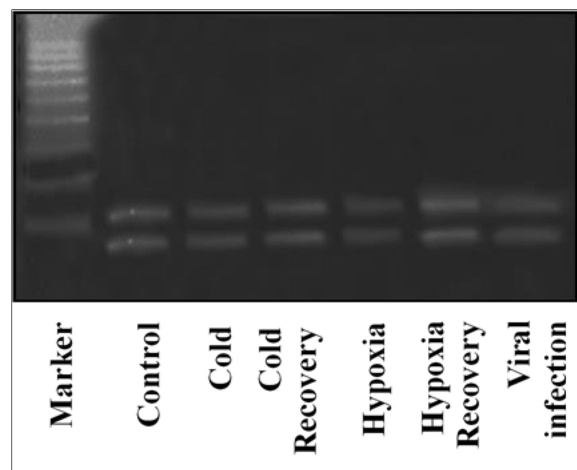

Fig 11: Representative mRNA expression resolved on agarose gel: HSP90 in midgut of silkworm *B. mori* on exposure to various stressors.

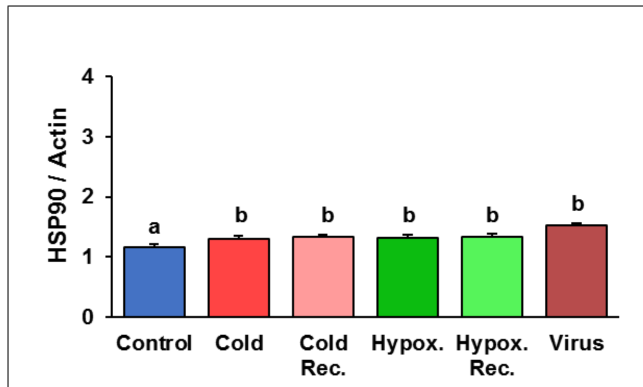

Fig 12: HSP90 mRNA in haemocytes of silkworm of silkworm *B. mori* is represented by the ratio of HSP90/ βactin RT-PCR products. Data are means ± SE (n = 4). P <0.05 was considered significant. Values between the stressors are represented in lower cases (a, b). Those not sharing the same letters are significant.

4. Discussion

In general, organisms are equipped with an interdependent cascade of antioxidant defense system to relieve oxidative stress and to remedy the damaged macromolecules produced during exposure to stressor. In this cascade, antioxidant enzymes are the most important components in the scavenging system of ROS [33, 34]. Antioxidant cascade consist of antioxidant enzymes and other being heat shock proteins. HSPs, named according to their molecular weight such as HSP100, 90, 70, 60 and small HSPs are a class of functionally related protein involved in chaperoning of other protein [35]. Of these, HSP90 is constitutively expressed and acts intra cellularly as molecular chaperones, whereas others, particularly HSP70 is usually expressed at low basal levels and increases in response to environmental and physiological stressors. HSPs are best known for their responses to multiple forms of stress, including low temperature [36, 37]. In leaf miner *Liriomyza huidobrensis* on exposure to cold temperature (10°C) have shown significant accumulation of HSP 70 [38] and similar expression of HSP70 was also observed in Antarctic midge *Belgica Antarctic* [39]. However, recovery from cold elicited HSP70 and HSP90 expression in corn ear worm *Helicoverpa zea* [37]. Entry into diapause a cold induced physiological state elicits a developmental up-regulation of HSP70 in *Ostrinia nubulalis, Sarcophaga crassipalpis, Manduca sexta,* walnut husk maggot *Rhagoletis suavis* [2], apple maggot *Rhagoletis pomonella* [40] and *Pyrrhocoris apterus* [19]. HSP90 response to diapause is variable and the gene encoding this HSP can be up/down regulated or involved during diapause in different species. During larval diapause, HSP90 is up-regulated in *Delia antiqua* [17] and in rice stem borer *Chilo suppressalis* [41]. Whereas it is down regulated in green bottle fly *Lucilia sericata* [42] and in bamboo borer *Omphisa fuscidentalis* [43]. In our study expression of HSP90 was evident in both midgut tissue and haemocytes of silkworm of IV and V instar silkworm on exposure to low temperature. Increased expression of HSPs have been reported during hypoxia [44] and anoxia exposure elevated levels of HSP70 in gall fly *Eurosta solidaginis* [20]. Overexpression of HSP70 in haemocytes of silkworm provided a remarkable survival benefits to *D. melanogaster* exposed to severe hypoxia [22]. In *Sarcophaga crassipalpis*, hypoxia induced several hundred folds of HSP70, whereas the response of HSP90 was negligible [21]. However, in the present study exposure to hypoxia induced increased expression of HSP90 in midgut tissue and haemocytes of silkworm of *B. mori*. Infection with *Autographa californica* multiple nucleopolyhedrovirus induced HSP70 that acted as cryoprotectant in *Spodoptera frugiperda* cell (Sf-9) and thereby suppressed the replication of O'nyong-nyong, virus in *Anopheles gambiae* [24]. In silkworm *B. mori* the switch of cellular translation machinery to polyhedrin synthesis at late stages of infection is accompanied by the inability to synthesis HSP [45]. Our results, illustrated the expression of HSP70 in midgut tissue and the expression of HSP90 in haemocytes of silkworm of *B. mori* infected with NPV. Similar, differential expression of HSP70 and HSP90 in response to other stressors was reported in different races of *B. mori* [46]. In contrary to our studies, baculovirus infection did not elicit HSP70 mRNA level in silkworm larvae [47]. Heat shock response operates through the transcriptional induction of heat shock genes by the activated heat shock factor [48, 49, 50]. Variation in the heat shock induced transcription of the HSP and the turnover of their transcript is cell and developmental stage specific in *D. melanogaster* [16, 51].

Heat shock quickly induced the transcription of HSP70 in malpighian tubule of *D. melanogaster* but the protein is not detectable, except in the small stellate cell and opined that a post transcriptional regulation prevents HSP70 synthesis in the cells [52]. In our results, both HSP70 and 90 mRNA expression was observed in the tissues studied on exposure to cold, hypoxia and viral infection. However, the differential expression of proteins were observed in the tissues. The present study brings an insight into the stressor specific and tissue specific expression of HSPs as an immediate defence mechanism to overcome the oxidative insult.

5. Acknowledgments

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