



Time and Tissue Dependent Expression of Heat Shock Protein 27, 70 and 90 in Mice Following Hyperthermia

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Abstract

Heat shock response is rapidly induced to protect cells from irreversible injury by stabilizing cellular synthetic and metabolic activities. Particularly, the response differentially protects against stresses, infections and inflammations. While it could be time- and tissue-dependent, it is not established as to how it exhibits. This study explored the expression of heat shock protein (Hsp) 27, 70 and 90 in intestines, kidneys, livers and lungs of mice undergoing hyperthermia at 42°C for 1 hr and recovering for 1-72 hr. The expression of these Hsps was determined by immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections. There was an increase in the expression of Hsp27, 70 and 90 that peaked at 6-12 hr and differentially declined at 48-72 hr. The peak expression was reached earlier in the lung and dropped sharply in the intestine while sustained for a longer time in the liver and kidney. At 72 hr only Hsp90 exhibited moderate expression in the lung and kidney. It is concluded that optimal expression of Hsps is time- and tissue- dependent and has narrow margin of peak expression in some tissues. This knowledge might contribute to designing therapeutic agents for curbing stresses, infections and inflammations that can be suppressed by Hsps.

Key words: Hsp27; Hsp70; Hsp90; hyperthermia; mice.

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1. Introduction

Heat shock or stress response is a complex phenomenon that is rapidly induced to protect cells from irreversible injury by stabilizing the synthetic and metabolic activities in the cell.

The most obvious characteristics of this response are enhanced synthesis of heat shock proteins (Hsps) commonly known as molecular chaperones and concomitant inhibition of overall protein synthesis [1-3]. This heat shock response is induced by various stimuli including thermal stress, heavy metals such as sodium arsenite and zinc ions, bacterial and bacterial exo- and endotoxins, viral infections, ischemia, nutritional deficiency, ionizing radiation, oxidants, some interferon (IFN) inducers and cytokines [4].

The Hsps are groups of evolutionary conserved proteins ranging in size from 8 kDa to 150 kDa synthesized by most cells and organisms when they react to heat and other stressors [5, 6]. They are classified into several families according to their molecular weights. The major Hsps families include HSP150, HSP110, HSP90, HSP70, HSP60, HSP40, HSP20, and HSP8.5 [4]. In mammalian cells, induction of Hsps is considered to be regulated mainly at the transcriptional level by the activity of a specific heat shock transcriptional factor (HSF). They are produced after transactivation of genes by a family of DNA-binding proteins called the HSF 1-4, of which the best known is HSF 1 [4, 7].

Heat shock proteins have been demonstrated in normal, unstressed cells in low constitutive levels. These levels are further enhanced upon stress [8]. Whether constitutive or enhanced, Hsps act as chaperones through protein-protein interaction to protect the already synthesized proteins against further injury and down regulation of the inflammatory response [9]. Specific protective roles of Hsps to various organs and systems have been reported. Under physiological conditions, Hsp27 and Hsp70 protect kidneys against their hyper osmotic environment [10] and kidney cells in diabetic rats [11]. Increased production of Hsp25 and Hsp72 in the kidney, liver and heart and skeletal muscles is effective in cytoprotection against various stresses in rat [12]. Further, over expression of Hsp25 and Hsp27 by intestinal epithelial cells protects the cells against oxidant injury [13] and acute inflammation [14]. In the nervous tissue, oral induction of Hsp70 by geranylgeranylacetone confers neuroprotection against cerebral ischemia [15] whereas over expression of Hsp25, Hsp27 and Hsp70 in cerebral arteries and vascular smooth muscles protects rats against cerebral ischemia and other types of injury [16].

Although Hsps are known to confer protection some studies have indicated failure of these protections despite of their over expression [4, 17]. For instance, the expression of Hsp70 and Hsp90 induced by *Salmonella* during its invasion and infection does not protect cells against the bacterial invasion. Instead, induction of these Hsps before infection may protect the intestinal cells against the bacteria [4]. It has further been shown that although expression of Hsp60, Hsp72 and Hsp90 occurs in the colonic mucosa, only Hsp72 and Hsp90 have protective role against acetic acid-induced intestinal lesions [17]. The failure to confer protection could, at least in part, be accounted for by the timing of Hsps expression which seems to be critical in Hsp-mediated protection. The current study was therefore designed to explore the time course production of Hsp27, 70 and 90 in the kidney, intestines, liver and lungs in mice undergoing hyperthermia so as to get an insight in the optimal time for Hsp expression in order to efficiently exploit their protective potency.

2. Materials and Methods

2.1. Experimental animals

This study used a total of 52 adult male mice. They were grouped randomly in separate cages of 13 mice each and kept at 12 hr day/night cycles at room temperature throughout the experiment. The animals were fed standard diet and provided with clean water *ad libitum*.

2.2. Induction of heat shock response

The heat shock response was induced by exposing mice to 42°C for 1 hr in an aerated oven. Mice were then allowed to recover for 1, 2, 4, 8, 12, 18, 24, 36, 48 and 72 hrs at room temperature. Control mice were kept in the same oven set at 37°C for 1 hr and recovered for 8 hrs. Four mice were humanely sacrificed using chloroform at the end of each recovery time. Sacrificed mice were opened by midline laparotomy. The intestines, kidneys, livers and lungs were sampled, fixed with 10% neutral buffered formalin and embedded in paraffin. Tissue sections (4 µm thick) were obtained and underwent immunohistochemical staining.

2.3. Immunohistochemistry techniques

2.3.1. Subbing

Subbing was done to avoid detachment of tissue sections from microscopic slides when exposed to various chemicals during immunohistochemistry. Subbing solution was made by dissolving 10 g of gelatin and 1 g of $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 1000 ml of warm non boiling distilled water. After stirring until dissolved, a thymol crystal was added. The solution was allowed to cool before further dissolving 0.188 g of $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in the solution.

Microscopic slides were placed in racks and soaked in soap solution for 1 hr then rinsed in deionized water that was changed several times to remove all the soap. The slides were then dipped into the subbing solution, drained onto a paper towel and allowed to dry for 1 hr. A second dipping was done followed by draining and loosely covering the slides with plastic wrap or bench paper. When thoroughly dry, the slides were stored in slide boxes until use.

2.3.2. Antigen retrieval

To unmask antigens, the tissue sections were first deparaffinized in three changes of xylene for 5 min each, hydrated in two changes of 100% then 95% ethanol for 10 min each change, and in distilled water for 1 min. They were then placed in a container and covered with 10 mM sodium citrate buffer (pH 6.0) with 0.01% (w/v) EDTA and heated at 95°C for 5 min.

The container was topped off with fresh buffer and heated again. The slides were allowed to cool at room temperature for 20 min while in buffer solution and subsequently washed in three changes of distilled water for

2 min each.

2.3.3. Quenching

Quenching was done to block endogenous peroxidase activity by incubating the tissue sections in 3% H₂O₂ in distilled water for 2 min. They were then washed in three changes of phosphate buffered saline (PBS) (0.01M Na₂HPO₄, 0.01M NaH₂PO₄, 0.9% (w/v) NaCl; pH 7.3) for 5 min each.

2.3.4. Immunoperoxidase staining by Horseradish peroxidase-Streptavidin (HRP) system

The tissue sections were incubated for 10 min in ready to use pre-blocking solution. This was followed by incubation in a humid chamber with primary monoclonal anti-Hsp27, anti-Hsp70 and anti-Hsp90 (Novus biological, Littleton, CO, USA) diluted in 2% normal blocking goat serum in PBS for 60 minutes at room temperature. The sections were then rinsed in 3 changes of PBS for 2 min each and incubated for 10 min with broad spectrum biotinylated secondary anti-goat antibody and rinsed in 3 changes of PBS for 2 min each. Afterwards, tissue sections were incubated with HRP-streptavidin (SPlink HRP detection bulk kit, GBI Labs, Mukilteo, WA, USA, Cat No D01-60) for 10 minutes at room temperature and rinsed in 3 changes of PBS for 5 min each. Pre mixed 3,3-diaminobenzidine plus (DAB) chromogen (adding 1 drop of DAB chromogen concentrate in 1 ml of DAB substrate buffer and mixed well) (GBI Labs, Mukilteo, WA, USA, Cat No C09-12) was applied to cover the tissue sections and incubated for 5 min or until desired colour was attained. The tissue sections were then counter stained in haematoxylin (10-20 sec), rinsed thoroughly under tap water (1-2 min), put in PBS (30-60 sec, until showed blue color) and rinsed well in distilled water. Subsequently, the tissue sections were dehydrated in ascending grades of alcohol (95% then 100% ethanol, 2 changes in each solution for 10 sec each change) and then dipped in xylene for 10 min before mounted with DPX. All stained tissue sections were observed under light microscope (Olympus BX 41, Japan) and pictures taken by digital camera (Olympus DP 21, Japan).

2.4. Scoring of Hsp27, 70 and 90

In order to assess the expression of Hsp27, 70 and 90, microscopic slides were examined for staining of the Hsps by considering the size of staining area, intensity of staining and the pattern of staining (Table 1). The final degree of expression was deduced from the average scores of the area, intensity and pattern of staining and termed “no expression”, “mild expression”, “moderate expression” and “intense expression” (Table 2).

Table 1: Scoring of Hsp27, 70 and 90 expression

Score	Size of staining area	Staining intensity	Staining pattern
1	0	none	none
2	<0.25	faint brownish	focal
3	0.25-0.5	brown	patchy
4	>0.5	deep brown	diffuse

Table 2: Interpretation of Hsp expression based on average scores shown in Table 1

Average score	Hsp expression
1-1.4	No expression
1.5-2.4	Mild expression
2.5-3.4	Moderate expression
3.5-4	Intense expression

The Hsp average score was obtained by combining the scores of size of staining area, intensity and pattern of staining (Table 1) divided by 3.

2.5. Statistical analysis

Statistical significant of Hsp27, 70 and 90 expression between the mean values of control mice and those undergoing hyperthermia was assessed by one way ANOVA with comparison of means. The differences were considered significant at $p < 0.05$ level using the Student's t-Test. All numerical values are presented as mean \pm SEM.

3. Results

3.1. Expression of Hsp27, 70 and 90

The extent of expression of Hsp27, 70 and 90 by mice at different recovery time after hyperthermia is exhibited in Figure 1-12. As seen in Figure 1, control animals did not show expression of any of the Hsps tested. On the contrary, intense expression was observed in the liver at 8 hr (Figure 2), the kidney at 18 hr (Figure 3) and the lung at 36 hr (Figure 4). As seen in these figures, the whole tissue section stained well for the respective Hsps. Moderate expression was observed in intestinal sections stained for Hsp27 at 18 hr (Figure 5) and Hsp70 at 24 hr (Figure 6) as well as the kidney stained for Hsp90 after 72 hr of recovery (Figure 7). It is obvious from these figures that the expression was not distributed throughout the section and was faint when compared to intense expression in Figures 2-4. Mild expression was observed in lung sections stained for Hsp90 at 1 hr of recovery (Figure 8), intestine stained for Hsp27 at 36 hr (Figure 9), kidney stained for Hsp27 at 48 hr (Figure 10), and the liver staining for Hsp70 at 72 hr of recovery (Figure 11). In all mild expressions, sections were unevenly stained, less than 50% of cells in a given section stained for the respective Hsp, and the staining was faint. At 72 hr of recovery, neither expression of Hsp27 nor 90 could be observed in the intestine (Figure 12).

3.2. Score of Hsp27, 70 and 90 expression

The four levels of Hsp27, 70 and 90 expression scoring, i.e. no, mild, moderate, and intense expression in the intestine, kidney, liver, and lung are depicted in Figure 13-15. A general trend of sharp increase in expression Hsp27, 70 and 90 within 8 hr of recovery was observed. The expressions of Hsp27 (Figure 13), Hsp70 (Figure 14) and Hsp90 (Figure 15) were already intense at 4 hr in the lungs. No other organ exhibited intense expression of the Hsps at 4 hr of recovery but mild to moderate expressions (Figure 13, 14 and 15).

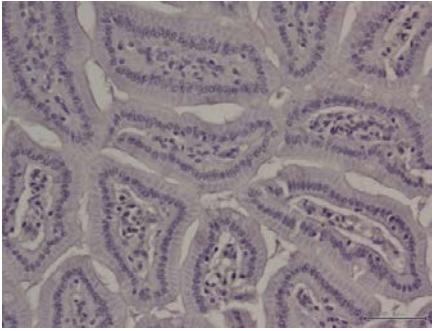


Figure 1: No expression of Hsp27, 70 nor 90 in the intestine, control,

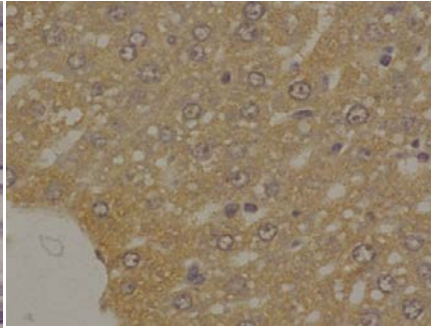


Figure 2: Intense expression of Hsp27 in the liver at 8 hr. 400x

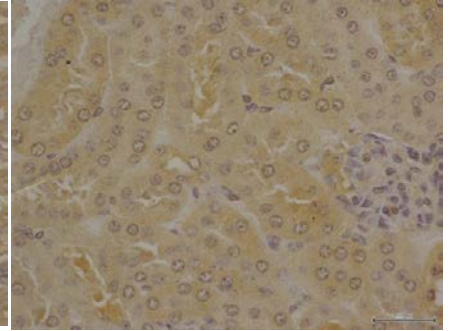


Figure 3: Intense expression of Hsp70 in the kidney at 18 hr. 400x

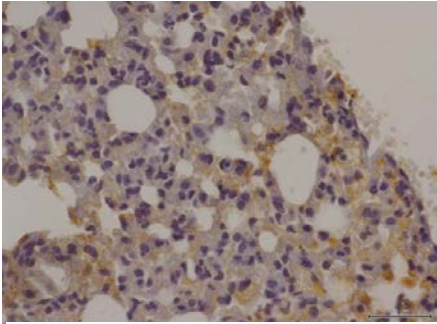


Figure 4: Intense expression of Hsp90 in the lung at 36 hr. 400x

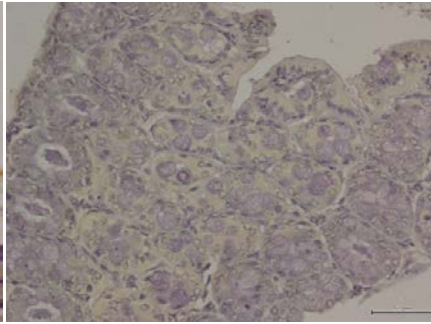


Figure 5: Moderate expression of Hsp27 in the intestine, 18 hr. 400x

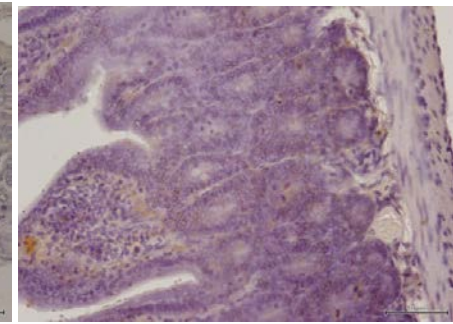


Figure 6: Moderate expression of Hsp70 in the intestine at 24 hr. 350x

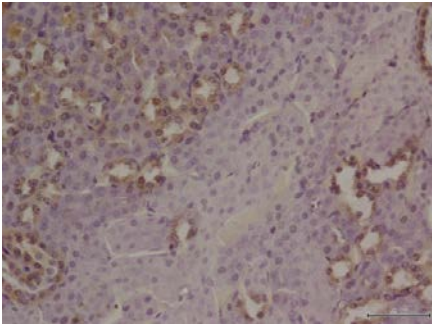


Figure 7: Moderate expression of Hsp90 in the kidney at 72 hr. 350x

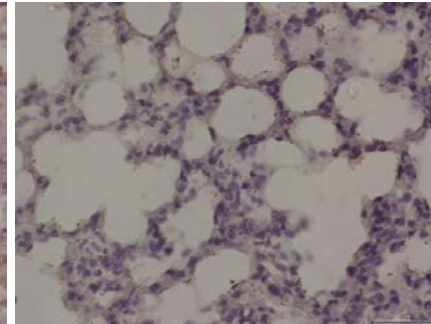


Figure 8: Mild expression of Hsp90 in the lung, 1 hr. 400x

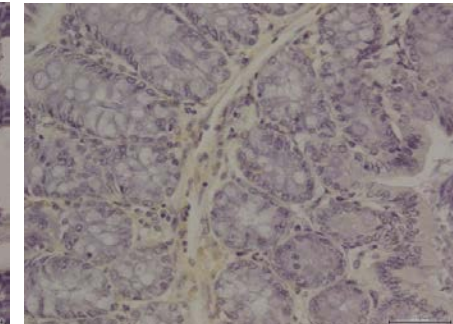


Figure 9: Mild expression of Hsp27 in the intestine, 36 hr. 400x

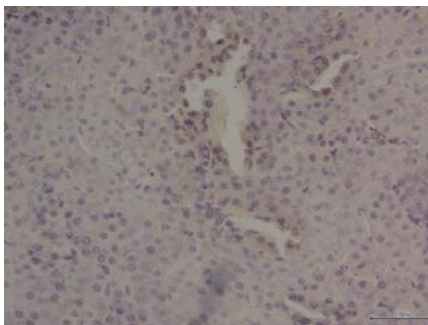


Figure 10: Mild expression of Hsp27 in the kidney, 48 hr. 400x

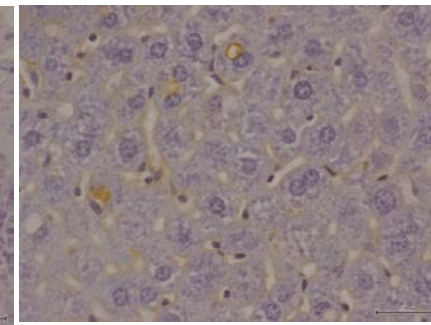


Figure 11: Mild expression of Hsp70 in the liver, 72 hr. 400x

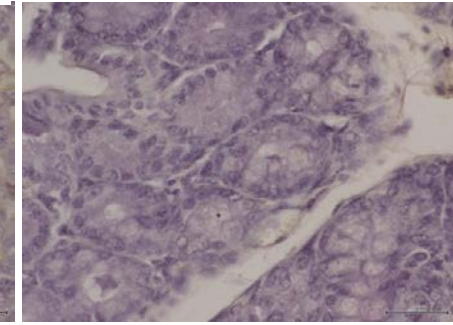


Figure 12: No expression of Hsp27 in the intestine, 72 hr. 400x

Acquisition of intense expression of Hsp27, 70 and 90 in other organs was delayed and achieved at 8 hr except for Hsp70 in the liver that was delayed to 12 hrs (Figure 13, 14 and 15). The intense expression of these Hsps persisted until 18 hr in the intestine where Hsp27 was moderate. Beyond 18 hr the expression of Hsp27, Hsp70 and Hsp90 in the intestines dropped sharply until it disappeared at 72 hr (Figure 13, 14 and 15). The intense expression of Hsp27 and 70 in the kidney dropped steadily after 18 hr to mild expression at 72 hr (Figure 13, 14, and 15).

In the liver and lung, beyond 24 hr the expression of Hsp27 dropped to moderate (lung) or mild (liver) at 72 hr (Figure 13). That of Hsp70 remained intense until 36 hr (liver) or 48 hr (lung) before dropping to moderate (lung) or mild (liver) at 72 hr (Figure 14).

Expression of Hsp90 in the liver was still intense at 48 hr while that of the lung had dropped to moderate until 72 hr where that of the liver dropped further to mild (Figure 15). There was no expression of Hsp27, 70 or 90 in control animals (Figure 13, 14, and 15).

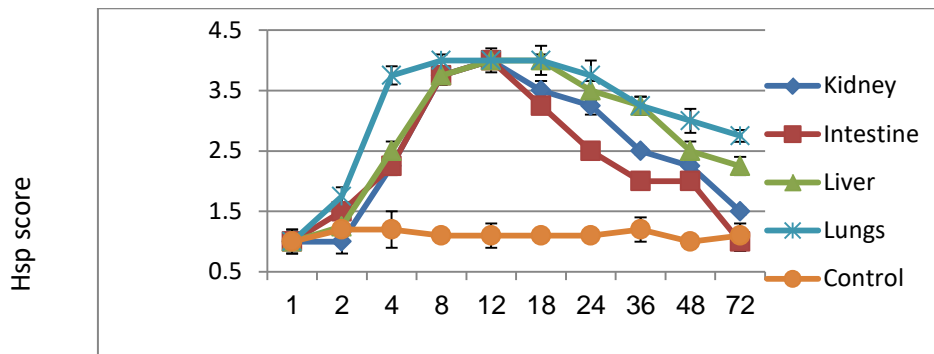


Figure 13: Time dependent expression of Hsp27 in mice kidney, intestine, liver and lung. Mice were heat shocked at 42°C for 1 hr and allowed to recover for 1, 2, 4, 8, 12, 18, 24, 36, 48 and 72 at room temperature prior to sacrifice at the end of each recovery time.

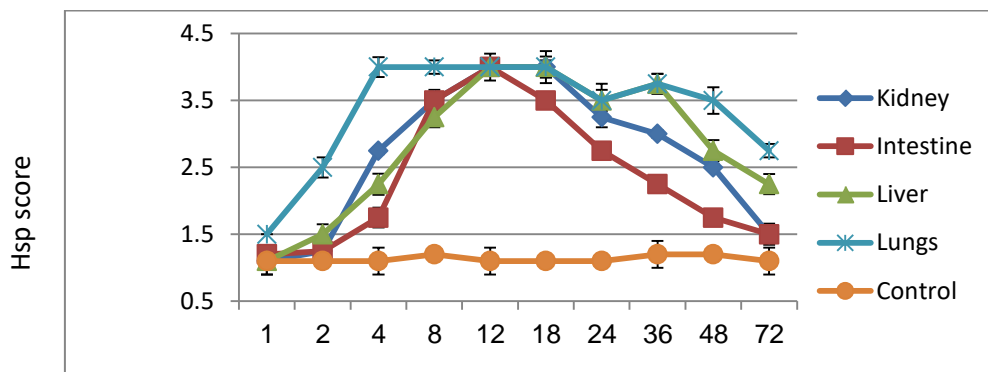


Figure 14: Time dependent expression of Hsp70 in mice kidney, intestine, liver and lung. Mice were heat shocked at 42°C for 1 hr and allowed to recover for 1, 2, 4, 8, 12, 18, 24, 36, 48 and 72 at room temperature. After recovery they were sacrifice and the obtained tissues processed routinely and stained for anti-Hsp 70.

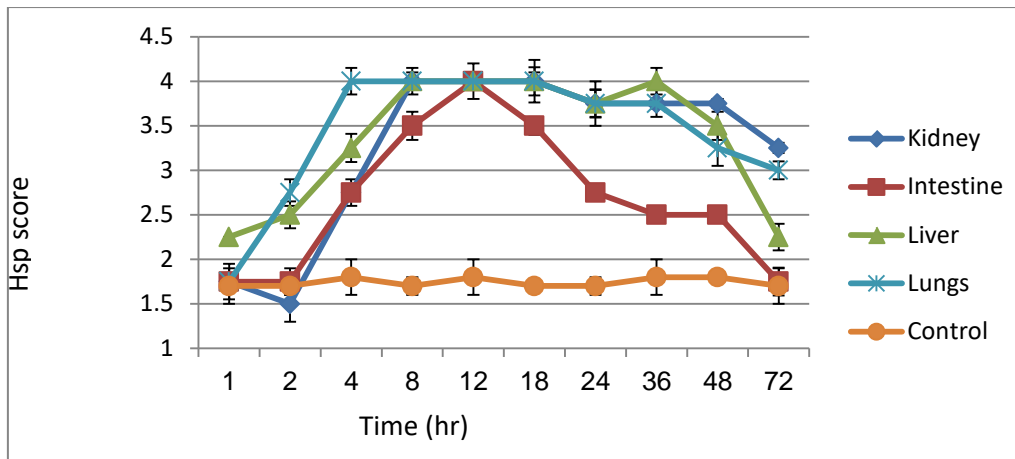


Figure 15: Time dependent expression of Hsp90 in mice kidney, intestine, liver and lung. Mice were heat shocked at 42°C for 1 hr and allowed to recover for 1, 2, 4, 8, 12, 18, 24, 36, 48 and 72 at room temperature. After recovery they were sacrifice and the obtained tissues processed routinely and stained for anti-Hsp 90.

4. Discussion

The expression of Hsps with respect to different recovery time in the kidney, intestine, liver and lung of mice exposed to hyperthermia was investigated in the present study. It is known that the adaptive heat shock response induces expression of the molecular chaperone proteins, the Hsps, such as Hsp27, Hsp70 and Hsp90 that prevent stress induced cell death by inhibiting key signaling pathways designated for cell death [18]. The levels of these Hsps are further enhanced upon stress. They are optimal in various organs after 6-8 hr of hyperthermia [19]. The present study shows that the expression of Hsps exhibit a pattern featured by no, mild, moderate and intense expression followed by a decline. This pattern is time and tissue dependent (Figure 13, 14, and 15).

Time and tissue dependent expression of Hsp70 has been partly described by some researchers both in vitro and in vivo. Studies using intestinal epithelial cell lines have shown that expression of Hsp70 and 90 increases gradually and peaks in intensity and amount of proteins produced around 6-8 hrs. Thereafter it persists as far as 72 hr but with fading staining intensity and decreased protein production [4, 20]. Ostberg and colleagues [21] also observed increased expression of Hsp70 in heart, kidney, lung, lymph nodes and thymus and Hsp110 in lung, lymph nodes and thymus in mice at various times following fever range (39.5-40°C for 6 hr) hyperthermia. In another study, mice exposed to 42°C after five days of acclimation to 35°C, increased the expression of Hsp72 and Hsp90 in lung, heart, spleen, liver, and brain [22]. Collectively, these earlier observations suggest a certain pattern or trend of Hsp expression even though no single study followed up the exact pattern. Nonetheless, the observations seem to harmonize with the findings in this very study.

The observations that the lung achieved intense staining earlier than liver, kidney and intestine and that the expression dropped sharply in intestine but sustained longer in liver and kidney tissues could be attributable to the nature of these tissues to adapt to stressful conditions. Tissue variations in Hsp expression have been observed previously. Manzerra and others [23] reported higher levels of constitutive Hsp70 in neural tissues than non-neural tissues. On the contrary, following hyperthermia, induction of Hsp70 was greatest in non-neural

tissues such as liver, heart, muscle, spleen, and kidney compared to nervous system. These researchers suggested that the amount of preexisting constitutive Hsp70 protein may modulate the level of induction of Hsp70 under stressful conditions. It has further been shown that Hsp genes are expressed constitutively in tissues exposed directly to the environment such as the epidermis and cornea as well as in certain internal organs like the epithelium of the tongue, esophagus, and forestomach, and the kidney, bladder, and hippocampus [24]. Exposure of mice to hyperthermia leads to rapid expression of Hsps in organs not constitutively expressing Hsps, namely the liver, pancreas, heart, lung, adrenal cortex, and intestine [24]. The rapid expressions observed in this very study, particularly for lungs, liver, and intestine are in consistency with earlier studies by Huang and colleagues. The observation herein that the expressions in kidneys were delayed for 2 hours further harmonizes with earlier observations [21].

The over expression Hsp27, Hsp70 and Hsp90 observed in this study could be beneficial in protecting the animal against various noxious stimuli. For instance, it has been shown that heat shock response in whole body (hyperthermia) increases Hsp70 expression in ventricular muscles and protects against ischemia reperfusion injury [25]. Further, over expression of Hsp27 in rats improves cardiac function due to stabilization of cytoskeleton structure [26]. In the kidney, Hsps confer protection as demonstrated by Neuhofer and others [10] that, Hsp27 and Hsp70 protect renal medullary cells, papillary collecting ducts and papillary interstitial cells against their hyper osmotic environment. Similarly, it is evident that over expression of Hsp70, Hsp71 and Hsp85 in liver cells protects the liver against ischemia and have physiological functions in the normal growth and development in rats [27, 28). Other studies have shown that Hsps protect the intestine against colonocytes preservation in epithelial cells under stress, inflammatory bowel disease, infections, and various other conditions [29, 30]. Furthermore, hyperthermia-enhanced Hsp expression protects against various systemic stressful conditions like sepsis [31] and tumours [32]. Interestingly, epidemiologic studies in military personnel operating in hot environments and elite athletes suggest that repeated exposure to hyperthermia may exert long-term health effects [22]. This may relate to the enhanced immune responses that are observed during febrile temperatures since immunostimulation by hyperthermia involves both direct effects of heat on the behavior of immune cells as well as indirect effects mediated through Hsp release [33].

5. Conclusions

In conclusion, this study has shown a time and tissue dependent expression of Hsps. The data generated allude to the importance of establishing optimal time for Hsp expression in a particular tissue so as to exploit the protective potency of Hsps. Hsp27, Hsp70 and Hsp90 are optimally produced around 6-12 hr in the lungs, kidney, intestine, and the liver. However, in some organs such as the intestines, the expression does not persist long. In tissues where it falls abruptly, maneuvers to exploit Hsp27, Hsp70 and Hsp90 protective potency should timely be instituted.

6. Recommendations

This study explored the expression of Hsp 27, 70 and 90 in intestines, kidneys, livers and lungs of mice undergoing hyperthermia at 42°C for 1 hr and recovering for 1-72 hr.

This time was long enough to explore the trend of Hsp production that peaked at 6-12 hr and differentially declined at 48-72 hr. This is a clear indication of tissue and organ variations regarding induction and recovery from stress response that may subsequently impact on protection.

Thus when designing and applying therapeutic agents and maneuvers that employ Hsps, one should take into account the variations in tissue response to stress stimuli. Missing the optimal time for Hsp expression that may fail to curb stresses, infections and inflammations would lead to wrong conclusions on the potential of Hsps to protect the organs and tissues.

7. Compliance with ethics guidelines

The author, Joshua Joseph Malago declares that he has no conflict of interest in the present work.

All institutional and national guidelines for the care and use of laboratory animals were followed.

8. Constraints of the study

This study has some constraints and limitations that future studies in this field are needed to supplement it. For instance, the stress response was induced at one temperature value for 1 hr. A wide range of temperature and time should be exploited. Further, besides hyperthermia, agents known to induce heat shock proteins especially that are edible and routinely included in the diet should be assessed on their trend to induce Hsp expression to different tissues and organs. Since this was an experimental designed study, extrapolation of results should cautiously and carefully be done.

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