Clinical Microbiology & Case Reports

ISSN: 2369-2111 Research Article

Induction of Sterile Inflammation

This article was published in the following Scient Open Access Journal:
Clinical Microbiology & Case Reports
Received July 17, 2015; **Accepted** August 13, 2015; **Published** August 20, 2015

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Abstract

Higher temperature ranges have been found to be associated with the death of cells and the increase of intracellular HSP 72 in jurkat cells. HSP 72 relates with membrane lipids and are released from cells when heat shocked. Depending on the localization of HSPs on the cell surface, either membrane bound or embedded, HSPs will possibly induce apoptotic cell death or protect cells from dying and other damages related to cellular stress. They may bring out the physiological response of the innate immunity such as the secretion of cytokine and cell activation, thereby promoting roles in stimulating cell protection, death and immune activation under normal physiological conditions and on exposure to stress stimuli. HSP72 is a significant marker for various environmental stresses and diseases; therefore this research seeks to describe the intracellular expression of endogenous HSP 72 by flow cytometry, under control conditions and in response to stress using various heat treatment with the control temperature set at 37ºC.

Keywords: HSP 72, Apoptosis

Background

Sterile inflammation from cell death occurs as a result of release of cell contents normally inactive and sequestered within the cell [1]. The induction of sterile inflammation is led by liberation of a plethora of damaged associated molecular patterns following tissue injury and necrotic cell death. While inflammation can be activated by either viral or bacterial infection, sterile inflammation is the cause of most chronic and acute diseases [2]. More often than not, inflammatory responses are stimulated by dying cells which occur internally. Coupled with this is the fast flow of neutrophils and shortly monocytes into injured tissues. This response is demonstrated by most injured cells and is originally use to know the particular time an injury occurred, such as, in myocardial infarction. In the absence of infection, these responses from sterile inflammation can cause tissue damage and give rise to the pathogenesis of a number of diseases, example in rheumatoid arthritis, atherosclerosis and other skin diseases such as psoriasis. This research seeks to bring to the fore, how cell activity is influenced by different temperature ranges. It is therefore important to understand the fundamentals of sterile inflammation to tissue injury and how cells react after a heat shock [3].

A family of greatly preserved proteins known as HSPs play significant roles in defending cells from stress and maintaining internal homeostasis during inflammation. Though the roles of HSPs intracellularly, are undoubted, there is an indication that, cells have indispensable machinery to actively produce specific HSP in responding to cellular stress. Additionally the discovery of HSP 72 in cellular stress environment particularly during physical exercise, fever or physiological stress has led to the escalating interest in the study of the role of intracellular and extracellular HSPs, where they have been discovered as ancestral 'danger signal' that alerts the immune system during dangers and assist in the generation of immunity. During immunization, HSP generally have shown to hinder autoimmune disorders such as arthritis and diabetes in both animal models and in clinical trials in patients with chronic inflammatory diseases.

Moreover, HSP 72 is a potential DAMP, it is consistent with the danger model and are elevated in serum following a number of stress conditions for example elevated temperatures, other cardiovascular diseases and infections. They stimulate proinflammatory responses, therefore making it important to understand under what conditions it is released from cells. The aim of this research is to find out how heat shock affects cell survival and the concentration of HSP 72 when heat treatment was applied to jurkat cells [4,5].

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Materials and Methods

The samples used in this research were jurkat cells, collected from leukaemia patients from the countess of Chester hospital. Fresh non sterile media (KPml + Ab), fixative and permeabilization (BD Cytofix/Cytoperm™), Dulbecco`s Phosphate Buffered Saline (DPBS), anti HSP 72 solution were used.

Before the experiment, total cell activity and viability was carried out on all samples used, by means of a haemocytometer. Cell viability was carried out using a dilution of 1:1 for the sample and trypan blue solution. It is also essential to monitor cell status before and during the experiment with the help of a good electronic microscope. Although a number of assays can be use to determine cell status the MTS assay was use in this experiment because of its high solubilisation. The data obtained was subjected to analysis by a one way ANOVA with Dunnett's post hoc test. Differences were considered to be significant when $P<0.05$.

Measuring cell activity with an MTS assay

Jurkat cells were pippetted into test tubes and placed into the temperature gradient bar and left for an hour. A 100 µl pipette was use to get cells transferred into 1.5 ml centrifuge tubes with three replicates for each of the temperature ranges. Cells in eppendorf tubes were spun in a centrifuge for 5 minutes at room temperature, after which the supernatant was removed leaving the cell pellets at the bottom. The pellets of cells were diluted with a 100 µl of fresh media and place in a 96 well microtitre plate. Furthermore, an addition of 20 µl of MTS was added to all wells containing the solution, left for 2 hours in the incubator after which the spectrophotometer records the absorbance at 490 nm. The assay was repeated for two hours and three hours at the same temperature ranges with controls at 37ºC [6].

Measuring HSP 72 with flow cytometer

Jurkat cells were pippetted into test tubes and placed into the temperature gradient bar for 3hrs (500 µl), 110 µl of cells were pippetted into 96 well plate for all 3 replicates at the different temperature ranges with the control set at 37ºC.The cells were then placed in a plate centrifuge at 500 g for 5minutes, a 70 µl fixative and permeability solution was added to the cells to stop any biochemical reactions from taking place, so HSP protein remains. The reaction was placed in the fridge and left overnight after which it was centrifuge and the supernatant taken of, an addition of 100 µl of DPBS was added to wash off the fixative. After which the plate was centrifuged and solution thrown off. A 100 µl of blocking solution was added to wells. An addition of 100 µl of blocking solution prepared from a 5% concentrated FBS-non sterile solution and DPBS was added to the wells to prevent any further reaction from taken place. The plate was spun again after leaving the set up for 15 minutes at room temperature. Supernatant was then discarded and an addition of 50µl anti HSP 72(FITC) mixed with blocking solution was put in each of the wells, with the exception of the no staining (control) wells followed by a careful mixing of the solution in the wells (only blocking solution was added to the controls). The assay was then put in the fridge for close to 50 minutes after which a 50 µl of blocking solution was added and centrifuged. The solution was discarded and an addition of 100 µl DPBS added to prevent cells from bursting. Cells were assessed by flow cytometry using Cell Quest software on a FACS Calibur flow cytometer (BD) to determine the activity of HSP 72. The experiment was repeated for an hour of heat shock and 3 hours recovery period.

Results and Discussion

Cell activity

Over several weeks of data collection, eleven different temperature ranges was set up with the control set at 37ºC. An MTS assay was used to measure cell activity and a flow cytometer to determine the concentration of intracellular HSP 72 during heat shock. The data were subjected to an ANOVA with Dunnett's post hoc test to determine the significant levels. During the exposure of jurkat cells to heat shock for an hour, there was no significant effect of temperature on the cells below a temperature of 41ºC. At a temperature of 41ºC there was a significant effect of heat on the cell activity ($P<0.05$) compared to the control temperature. At 44ºC there was a very significant effect of heat on cell activity ($P<0.001$) whiles apoptosis increased up to a peak of 53ºC ($P<0.001$) (Figure 1). The lowest temperature ranges showed little to no cell death with the percentage of survival at 100% for the control at a temperature of 37ºC. When cells were exposed to heat treatment for two hours, there was a significant effect of heat on cell activity ($P<0.05$) at a temperature of 48ºC and a further decrease in cell activity between temperatures 49ºC and 53ºC ($P<0.001$). Furthermore, the exposure of jurkat cells to three hours of heat shock showed a very significant decrease in cell activity from temperatures 41ºC to 53ºC ($P<0.001$). Moreover, there was a significant ($P<0.05$) decrease in the survival of cells at a temperature of 40ºC when cells were treated with an hour of heat and left for three hours to recover (Figures 2-4).

Intracellular HSP concentration

HSP72 intracellular levels are modified with heat shock: A confirmation of various temperature treatments were giving to jurkat cells under heat shock response, subsequently, intracellular HSP72 concentration was measured. After treating cells to different temperatures for a period of 3hours, there was a significant increase in the concentration of HSP72 at a temperature of 42ºC ($P<0.05$) as compared to the control

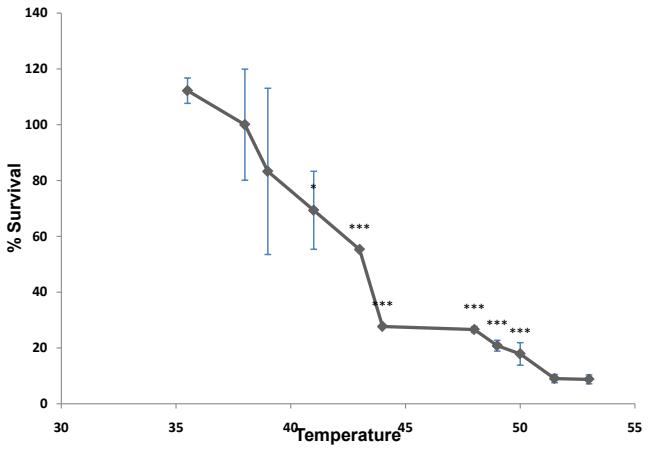

Figure 1. MTS on jurkat after an hour of heat shock at varying temperatures. Jurkat cells showed a decreased in activity as temperature increased. Percentage survival also decreased as temperature increased with a 100% survival for the control at 37ºC.

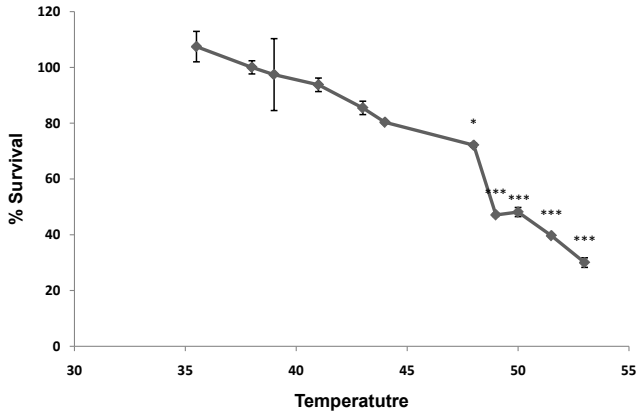

Figure 2. MTS on jurkat after 2 hours of heat shock at varying temperatures. Jurkat cells showed a decrease in cell activity as the temperature increased. The percentage survival of cells was significantly less as the temperature increased, with a 100% survival rate at the control.

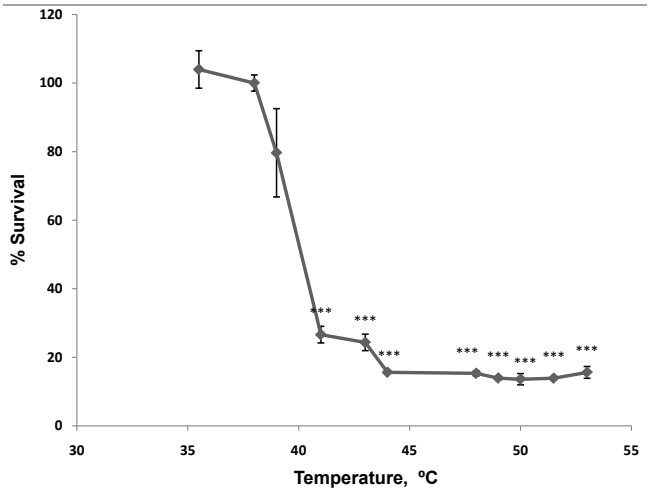

Figure 3. MTS on jurkat cells after 3 hours of heat shock at varying temperatures. Jurkat cells showed a sharp decrease in cell activity. The percentage survival decreased significantly as cells were subjected to heat shock for a period of 3hours.

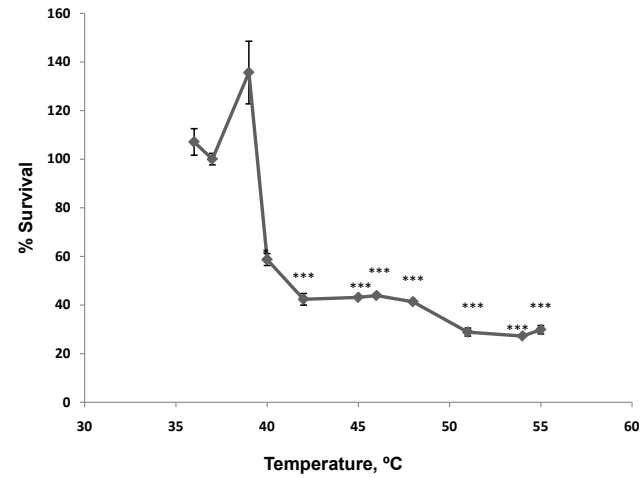

Figure 4. MTS on jurkat after an hour of heat shock and 3 hours of recovery period.

temperature at 37ºC (Figure 5). In addition intracellular HSP 72 concentration increased from a temperature of 36ºC to 39ºC but decreased between 40ºC and 46ºC with a very significant increase at a temperature of 54ºC when jurkat cells were exposed to heat shock after an hour and a period of 3 hours recovery (Figure 6).

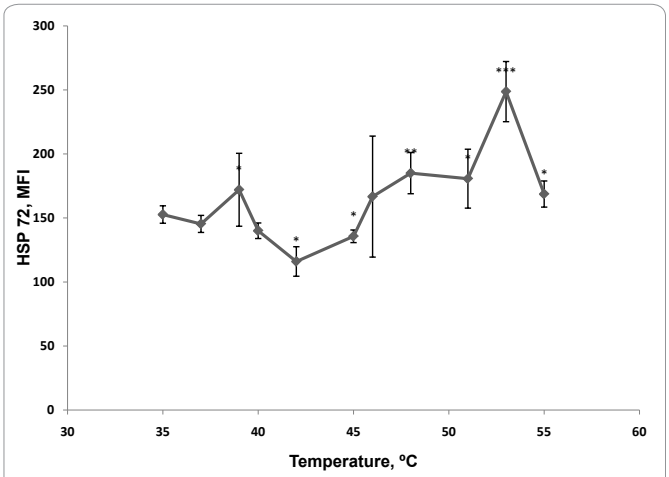

Figure 5. Intracellular HSP 72 concentration after 3 hours heat shock at varying temperatures. HSP 72 release increases at higher temperatures when jurkat cells were exposed to heat shock.

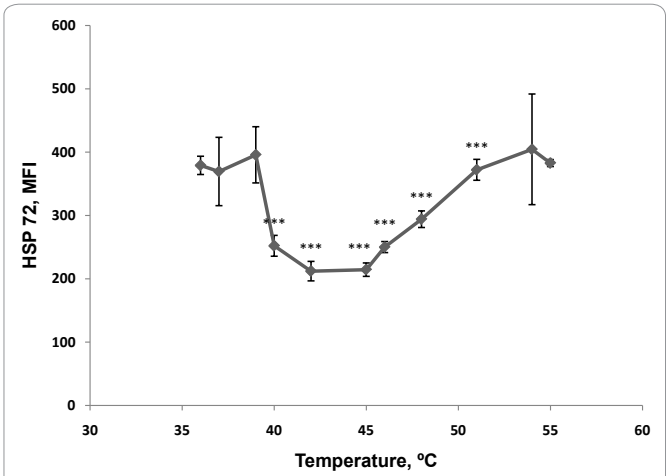

Figure 6. Intracellular HSP 72 concentration after an hour of heat shock and a 3 hours recovery period. A higher rise in the release of HSP 72 at increasing temperatures.

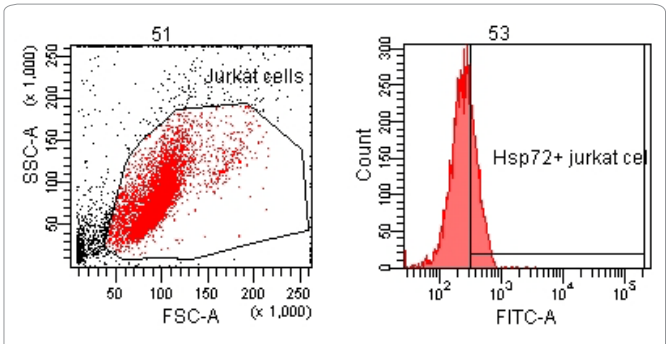

Figure 7. Identification of HSP 72 in jurkat cells using FSC/SSC on the flow cytometer at 42ºC.

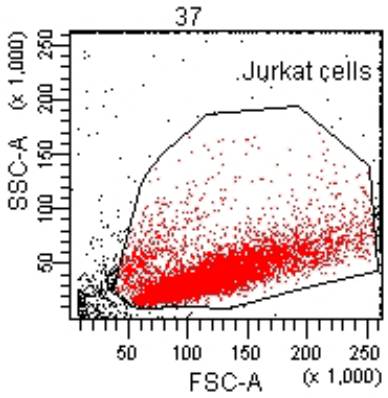

Figure 8. Identification of HSP 72 in jurkat cells at a temperature of 37ºC.

Dot plot and histogram of HSP 72 release at different temperatures and control temperature on a side scatter flow cytometer (Figures 7 and 8).

Discussion

The basic function of HSPs, an intracellular molecular chaperone is to control the folding, transport and degradation of various types of naïve and denatured polypeptides and proteins. This occurs under both physiological conditions and following exposure of cells to potentially harmful stress stimuli such as heat shock. More importantly HSPs promote cell survival during stress by facilitating the right folding of denatured proteins [7]. The significant death of cells at higher temperatures shows that, apoptosis occurs at a temperature of 42ºC and or above, leading to cell and tissue damage as indicated by previous studies. After heat shock inducing apoptosis up to a certain temperature, necrosis is the next dominant cell death pathway. Induction of apoptosis by heat shock activates mitochondrial pathway and also induce cytochrome-c release. The activities of a cell, including its transportation system, DNA, RNA and protein synthesis slows down or stops when a cell experiences environmental stress such as heat shock and nutrient deprivation. Cells undergo apoptosis or necrosis on exposure to heat, depending on the temperature applied. An MTS assay and flow cytometry protocol on jurkat cells revealed a decrease in the percentage of survival of cells at higher temperatures and an increase in the intracellular concentration of HSP 72 respectively. The release of small HSPs or inducible HSP 72 has been proven to enhance the survival of mammalian cells when exposed to numerous types of stimuli especially heat and other forms of stress stimuli [8]. The release of HSPs are essential in protecting cells from various environmental stress such as high temperatures and normal cell function [9,10]. Most of these stress impedes cellular functions, damage cell structures and leads eventually to necrosis or apoptosis [11]. Jurkat cells are apoptotic after heat shock at 42°C and leads to the apparent movement of HSP 72 and HSP 60 to the cell surface. Further studies suggest that, the release of HSP 72 indicates how HSPs remain at the cell surface and stabilises cell membrane before the production of newly synthesized HSPs [12]. Most of the HSP70 family are expressed strongly when cells are stressed by factors such as heat or deprivation of nutrients, including inflammation and other diseases. Many proteins begin to unfold, expose hydrophobic domains, increases their likelihood of aggregation, lose their cellular functions and finally cell death when exposed under these conditions. In this study, it was observed that, the concentration of HSP72 increased at increasing temperature and eventually, cell survival decreased significantly with high temperatures. Intracellular HSP72, when expressed after heat shock treatment, protects cells against apoptosis and thermo tolerance but then moderate heat shock situations have been used as potential therapy against various cancer types, by effectively activating the immune system's response specifically to cancer cells [13].

The surface levels of HSP 72 are low in leukemic cells at normal growing temperatures, conversely, their release increases when cells are heat shocked at 42°C and undergo apoptosis, evident by the activation of the pro caspase 2 and the effector caspase 3. Hence a cellular model with a percentage of apoptotic cells, expressing HSP 72 on cell surface and secreting them into extracellular space is the first indication that apoptotic cells can secrete HSP 72. HSPs are therefore, important in physiological processes in that, they can help cells to recover from their initial shock and stabilize other membranes in the human system through covering the hydrophobic domains of the unfolded proteins, until cellular conditions become normal again and help cells to recover from the heat shock [14].

Conclusion

It is evident that when cells die, a number of important processes are set in motion. One of them is the immediate recruitment of innate immune components from the blood, as part of a process we recognize as inflammation. One other similar process is the recruitment of highly specific T and B cell defenses from more distal sites, all these processes induced by cell injury ensure adequate host defense [15]. It is evident that HSP and peptides have an immune modulatory role. Although immune modulation is vital in any organism in controlling excessive inflammation and subsequent damage, inflammatory reactions are requirements needed to destroy harmful pathogens. Therefore the immune system has developed variety of mechanisms which it uses to regulate the immune response. HSPs play crucial roles in such regulatory mechanisms and may be a target for emerging therapies in different inflammatory disorders including rheumatoid arthritis. Additionally, the central role HSPs play in immune regulation and in the human immune system could be strategically used in our pursuit to develop better therapies for sterile induced inflammations and other autoimmune disorders [16,17]. In conclusion, sterile inflammatory signals and adaptive immune responses are often concealed in the interior of cells and released when cells lose the integrity of their plasma membrane, thereby initiating host responses. Although much progress has been made in identifying some of these signals and their modes of action, there is still much to be studied about the mechanisms of action of these factors and their roles in sterile inflammation [18].

Acknowledgment

First and foremost, I would like to thank the almighty God for seeing me through this course successfully; secondly, I would like to thank Prof. John Williams and Dr Nina Dempsey for their guidance and immense contribution towards this research and to my parents Mr. and Mrs. Ampadu and my sister Janet Ampadu G. for their support.

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Appendix

Tables

Table 1. One hour heat shock data for MTS assay

Temp	% survival where control is 100%			average	SD	SIG
35.5	117.3593	108.8022	110.4067	112.1894	4.548582	
38	116.7242	105.2925	77.98329	100	19.90535	
39	75.07521	58.42897	116.2897	83.26462	29.78701	
41	61.27019	61.23677	85.47075	69.32591	13.98186	*
43	55.25348	55.32033	55.28691	55.28691	0.033426	***
44	27.37604	27.61003	28.04457	27.67688	0.339239	***
48	26.50696	25.57772	27.67688	26.58719	1.051879	***
49	22.93036	19.11978	20.38997	20.81337	1.940255	***
50	21.69359	13.63788	18.21727	17.84958	4.040422	***
51.5	8.924791	7.587744	10.52925	9.013928	1.472777	***
53	8.490251	7.286908	10.4624	8.746518	1.60318	***

Where * = P<0.05, ** = P<0.01 and *** = P<0.001

Table 2. Two hours heat shock data for MTS assay

Temp	% survival where control is 100%			average	SD	SIG
35.5	104.5501	96.74991	121.0058	107.4353	12.38266	
38	103.9343	85.76805	110.2976	100	12.72927	
39	97.63941	76.70202	117.8584	97.39993	20.57922	
41	81.1495	113.8898	86.17858	93.73931	17.6311	
43	87.10229	100.1711	69.10708	85.46014	15.59696	
44	81.79952	69.14129	90.04447	80.32843	10.52895	
48	82.31269	75.81252	58.19364	72.10628	12.47936	*
49	45.56962	43.38009	52.34348	47.09773	4.673002	***
50	46.80123	44.88539	52.6856	48.12407	4.064878	***
51.5	37.46151	51.18029	30.34554	39.66245	10.59032	***
53	37.35888	19.94526	32.70612	30.00342	9.015928	***

Where * = P < 0.05, ** = P<0.01 and *** = P<0.001

Table 3. Three hours heat shock data for MTS assay

Temp	% survival where control is 100%			Average	SD	SIG
35.5	98.61953	103.7673	109.5343	103.9737	5.460288	
38	98.46471	98.81306	102.7222	100	2.363945	
39	93.20088	67.53967	78.22216	79.65424	12.8904	
41	24.69359	25.77732	29.33815	26.60302	2.429882	***
43	21.98426	24.22913	26.78364	24.33234	2.401355	***
44	15.13353	15.6754	16.02374	15.61089	0.448596	***
48	15.09483	14.70778	16.13985	15.31415	0.740801	***
49	13.66275	13.54664	14.55296	13.92078	0.550551	***
50	14.94001	14.0885	11.76622	13.59825	1.642709	***
51.5	13.81757	13.54664	14.35944	13.90788	0.413857	***
53	14.51426	14.66907	17.57193	15.58509	1.722395	***

Where * = P < 0.05, ** = P<0.01 and *** = P<0.001

Table 4. One hour heat shock data for MTS assay with a three hour recovery period.

Temp	% survival where control is 100%			average	SD	SIG
36	133.9522	86.81829	100.4641	107.0782	24.25305	
37	103.1098	107.3567	89.53353	100	9.309633	
39	136.0408	136.5282	134.3003	135.6231	1.171219	
40	70.5268	60.01392	45.53261	58.69111	12.5495	*
42	42.39963	49.15294	35.50708	42.35321	6.823047	***
45	33.90578	53.95683	41.56417	43.14226	10.11825	***
46	37.87422	41.00719	52.84289	43.9081	7.894727	***
48	41.00719	42.46925	40.51984	41.3321	1.014504	***
51	29.24112	27.50058	29.86772	28.86981	1.226476	***
54	23.81063	24.92458	33.0007	27.2453	5.015342	***
55	25.76004	37.59573	26.17777	29.84451	6.716001	***

Where * = P < 0.05, ** = P<0.01 and *** = P<0.001

Table 5. Three hours heat shock data for HSP 72

Temp	Absorbance			Aver	SD	SIG
35	145	158	155	152.667	6.806	
37	151	147	138	145.333	6.658	
39	197	141	178	172	28.478	*
40	144	133	143	140	6.082	
42	120	103	125	116	11.532	*
45	138	139	130	135.6667	4.932	*
46	130	220	150	166.6667	47.258	
48	203	180	172	185	16.093	**
51	180	204	158	180.6667	23.007	*
53	225	249	272	248.6667	23.501	***
55	157	173	176	168.6667	10.214	*

Where * = P < 0.05, ** = P<0.01 and *** = P<0.001

Table 6. An hour heat shock data for HSP 72 with a three hour recovery period

Temp	Absorbance			Aver	SD	SIG
35	363	391	383	379	14.422	
37	324	429	355	369.3333	53.947	
39	355	389	443	395.6667	44.377	
40	243	242	271	252	16.462	***
42	195	225	216	212	15.394	***
45	224	216	203	214.3333	10.598	***
46	254	256	240	250	8.717	***
48	282	292	308	294	13.114	***
51	373	388	355	372	16.522	***
53	395	496	322	404.333	87.374	
55	377	383	388	382.6667	5.507	

Where * = P < 0.05, ** = P<0.01 and *** = P<0.001