TO ASSESS HEAT SHOCK PROTEIN 70 IN MAXILLOFACIAL INJURIES: A PILOT STUDY

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ABSTRACT

PURPOSE: The purpose of the study was to assess the levels heat shock protein 70 preoperatively, intraoperatively and postoperatively in patients with maxillofacial fracture. **METHODS:** This is a pilot study conducted on the patients admitted in Accident and Emergency Medicine of Sri Ramachandra institute of higher education and research from 2021 to 2023 with clinically and radiologically diagnosed facial fractures. In this study, specific serum sample were collected. The collection time included initial assessment – two hours after admission, second assessment – intraoperatively, final assessment – second day postoperatively. **QUANTIFICATION:** Venipuncture with withdrawal of 10 ml of blood for HSP70 quantification and kept for an interval of 15 -20 minutes for thorough coagulation. Centrifugation done at 3000 rpm for 15 minutes at room temperature. Samples will be aliquoted and stored at -80C until measurements. Quantification of hsp70 will be done through ELISA and values are assessed. **RESULT:** In this study we compared the HSP levels in control group with the study group. The result shows higher mean values in study group (intraoperative 41.0650 ng/ml > preoperative 35.9809ng/ml > postoperative 30.2492 ng/ml) compared to control group (27.7200 ng/ml). The comparison of mean difference of OD absorbance among the control and study group (preoperative) found p value of 0.028 implying statistical difference was seen among the control and preoperative group. **CONCLUSION:** Compared to healthy individuals and patients with isolated injuries, increased serum concentrations of HSP 70 were demonstrated. The present study provides the first in vitro evidence that the early release of HSP 70 protein into the bloodstream after injury was associated with a signi ficant increase in prognosis of traumatized patients.

KEYWORDS: Heat shock protein, Facial injury severity score

Introduction:

Heat shock proteins (HSP's) are heterogeneous gene products of a highly conserved family of stress proteins. Their molecular weights vary roughly from 16 killodalton to 110 killodalton (KDa), and they are rapidly expressed in cells exposed to a variety of stress. HSP's after their release in the blood, also participate in signal transduction.[1]

HSP's canmodulate cellular adaptive response by modulating CD8+ cytotoxic cell receptors on one hand and can directly stimulate an innate immune response by toll-like receptor (TLR) mediated signaling on the other .[2]

HSP70 lowers oxidative damage of fibroblasts and supports cell proliferation in the wound area by inhibiting stress - induced apoptosis and Toll-like receptor activation [3]. The functioning of HSP70 is aided by the expression of HSP47, which is predominantly involved in pro- collagen synthesis and binding with collagen types II and III. Small HSP' s like HSP27 also support wound healing by stabilizing actin microfilaments, supporting endothelial cell migration in the wound bed, protecting sensory neuron degeneration, and inhibiting stress-induced apoptosis [4].

HSP (HSP 70 in particular) may also exert protective effects on the immune system by contributing to th e processing and presentation of bacterial and tumoral antigens [5]. HSP70 increases alkaline phosphatase activity and promotes human mesenchymal stem cell (hMSC) mineralization. Under osteogenic induction conditions, HSP70 significantly upregulates the expression of osteo-specific genes [6]. It prevents protein aggregation and renatures unfolded proteins in the cytoplasm andnucleus [7].

So far there has been no study conducted to evaluate the effect of heat shock protein 70 in the maxillofacial region. In this study we have tried to correlate the levels of heat shock protein 70 expression with the severity of maxillofacial injury by using facial injury severity score. The levels of heat shock protein 70 are analyzed at three-time intervals preoperatively, intraoperatively and postoperatively.

Aim:

To assess the levels of Heat Shock Protein 70 in maxillofacial injuries.

Methodology:

This is an observational prospective study to evaluate levels of heat shock protein 70 in patients having maxillofacial injury coming to the Emergency Room and at the Department of Oral and Maxillofacial Surgery in Sri Ramachandra Dental College and Hospital between 2021-2022.

Inclusion criteria:

- Patients who presented to the Emergency Service Department of Sri Ramachandra Medical Center and the Department of Oral and Maxillofacial Surgery of Sri Ramachandra Institute of Higher Education and Research.
- Patients with maxillofacial injuries.
- Patients who were willing to investigations and treatment.

Exclusion criteria:

- Patients without maxillofacial injuries.
- Patients who were not willing to further investigations andtreatment.

- Patients who left the hospital against medical advice.

Venipuncture with the withdrawal of 10 ml of blood for HSP70 quantification and kept for interval of 15 -20 minutes for thorough coagulation. Centrifugation was done at 3000 rpm for 15 minutes at room temperature. Samples will be aliquoted and stored at - 80C until measurements. Quantification of HSP70 was done through ELISA.

Procedure:

Using commercially procured ELISA kit (RayBio). Procedurefor assay was mentioned below as follows:

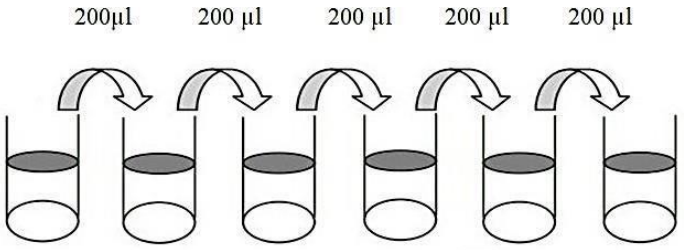
Reagent Preparation:

- All reagents and samples were brought to room temperature (18 - 25ºC) before use.
- Assay Diluent B was diluted 5 - fold with deionized or distilled water before use.

Figure 1: Collected human serum stored samples

Preparation of standard:

- Standard vial was centrifuged at 5000 rpm for 2 mins. 400 µl of Assay Diluent C was added to standard vial to prepare a 600 ng/ml standard solution. The powder was dissolved thoroughly by a gentle mix. 400µl of Assay Diluent was pipetted into each tube. Standard vial (600 ng/ml) was used to produce a dilution series (S1 - S6). Each tube was mixed thoroughly before the next dilution. Assay Diluent C served as the blank.
- 20 ml of wash buffer concentrate was diluted to yield 400ml of 1X Wash buffer.
- Detection Antibody vial was centrifuged at 5000 rpm for 2mins. 100µl of 1X Assay Diluent B was added into vial to prepare a detection antibody concentrate. Tube was mixed thoroughly. The detection Antibody was diluted 80 –fold with 1X Assay Diluent B.
- HRP – Streptavidin concentrate vial was centrifuged at 5000 rpm for 2 mins and gently mixed. This was diluted 500 – fold with 1 X Assay Diluent B.



	Std1	Std2	Std3	Std4	Std5	Std 6
Diluent volume	Standard+ 400 µl	400 µl	400 µl	400 µl	400 µl	400 µl
Conc.	600 ng/ml	200 ng/ml	66.67 ng/ml	22.22 ng/ml	7.407 ng/ml	2.469 ng/ml

Figure 2: Preparation of dilutant for standard series

Assay procedure:

- Plate map for the assay was prepared for standards.
- 100µl of each standard/ sample were added to appropriate wells as per plate map. Wells were covered and incubated for 2.5 hours at room temperature with gentle shaking.
- The solution was discarded and washed 4times with 1X Wash Solution. Each well was washed by filling with w ash buffer(300 µl) using multichannel pipette. After wash, the plate was aspirated or decanted. The plate was inverted & blotted against clean paper towels.
- 100µl of 1 X prepared biotinylated antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking.
- The solution was discarded, washed for 4 times with 1X wash buffer.
- 100µl of prepared Streptavidin solution was added to each well. Plate was incubated for 45 minutes at room temperature with gentle shaking.
- The solution was discarded, washed for 4 times with 1X wash buffer.
- 100µl of TMB One Step Substrate Reagent was added to each well. Plate was incubated for 30 minutes at room temperature in the dark with gentle shaking.
- 50µl of Stop Solution was added to each well to stop the reaction. Absorbance was read at 450 nm immediately.
- 10. Standard graph was derived with that samples, HSP concentration of samples was calculated.

RESULTS:

Demographic data

In our study a total of 45 patients were included, among which 41 patients were males and 4 patients were females between the age group 16-65 years. The mean age of patients in the study was 35.8 years. The control group included of 5

samples.

Samples were collected preoperatively, intraoperatively, postoperatively for study group and levels were compared with the control group and pairwise comparison was done among each group.

TABLE 1: Mean difference of OD absorbance at three-time intervals

ANOVA					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.060	2	.030	2.550	.042*
Within Groups	1.490	126	.012		
Total	1.551	128			

Table 1: Shows the mean difference of od absorbance at three - time intervals. Statistical difference was seen among the different time intervals.

Table 2: Mean difference of HSP 70 at three time intervals

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2518.119	2	1259.059	.306	.037*
Within Groups	518614.271	126	4115.986		
Total	521132.390	128			

Table 2: Shows the mean difference of HSP 70 at three - time intervals. P- value <0.05 was considered to be statistically significant. While assessing the mean difference, p- value was found to be <0.05 which implies statistical difference is seen among the different time intervals.

Table 3: Comparison of mean difference of HSP 70 among the control and study group (pre-operative values)

					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95 % Confidence Interval of the Difference			

				Lower	Upper			
CONTROL AND STUDY GROUP (PRE-OPERATIVE VALUES)	-8. 26537	1.250	17. 94474	-44. 34569	27. 81495	-. 461	48	0.001*

Table 3 shows the comparison of mean difference of HSP 70 among the control and study group (pre-operative values). While assessing the mean difference, p-value was found to be <0.05 which implies statistical difference was seen among the control and study group (pre- operative values).

Discussion:

Heat shock proteins belong to a highly conserved family of proteins normally localized in the endoplasmic reticulum, mitochondria, cytoplasm, and nucleus.[8]

The primary objective of this study was to determine whether HSP 70 protein could be detected in the serum of patients immediately after trauma when assessed preoperatively, intraoperatively, postoperatively and to check the serum levels of HSP 70 correlate with the facial injury severity score, postoperative prognosis and survival of patient.

In our study, the total sample size was 45 out of which 41 were males and 4 were females. The mean age was found to be in between 30-40 years with the lowest 16 years of age and highest 65 years of age. The control group consisted of 5 samples. In this study we compared the HSP levels in control group with the study group. The result shows higher mean values in study group (intraoperative 41.0650 ng/ml > preoperative 35.9809ng/ml > postoperative 30.2492 ng/ml) compared to control group (27.7200 ng/ml). The comparison of mean difference of OD absorbance among the control and study group (preoperative) found p value of 0.028 implying statistical difference was seen among the control and preoperative group. This increased serum concentrations of HSP might be involved in high NF- κB activity after severe trauma as reported by Li et al. [9] A G Pockley et al[2] in his study found that, the mean serum Hsp70 concentrations in females mean value of (2543+/3808) were approximately twice the levels detected in male (1131+/ - 254), which corresponds to similar results as in our study.

Guisasola et al[10] did a pilot study with 18 patients showed an increase of HSP 70 serum concentrations following severe injuries with peak levels immediately after trauma and gradual reduction. He evaluated the early inflammatory and stress response of 18 polytraumatized patients at 12 hours, 24hours, and 48 hours post trauma. HSPA1A levels were significantly higher immediately after the accident followed by gradual lowering. Anti -Hsp70 antibodies showed a significant reduction in all the studied time - points. The significant reduction in the levels of anti - Hsp70 antibodies could reflect a part of post traumatic immunosuppression. These findings are in line with the observation of our study. The mean difference of HSP 70 at three time intervals in study group was statistically significant with p value of 0.037 and post hoc analysis of pairwise comparison of study group showed mean difference of 5.08414

between preoperative and intraoperative with standard error of 13.83623 and which shows statistically insignificant p value of 0.928. Mean difference between preoperative and postoperative was 5.73168 with standard error of 13.83623 and showing it's not statistically significant with p value of 0. 910. Comparison between intraoperative and postoperative group had mean difference of 10.81582 with standard error of 13.83623 and p value of 0.035 which shows significant statistical difference. Hashiguchi et al[11] in his study found that trauma elevates expression of HSPs in leukocytes. Based on this report, leukocytes might be one of the major cellular origins of elevated HSPs serum concentrations following severe trauma.

Basu et al[12] in his study found that in heat-mediated induction of the stress response, immunocompetent cells such as neutrophils or macrophages expresses Hsp 72 at their plasma membrane, leaving open the possibility that this protein could be released into the blood stream secondary to a cleavage by proteases. It has been suggested that necrotic but not apoptotic cells release intracellular heat shock proteins into the extracellular space. This observation could provide an explanation for the fact that most of the patients in our study had higher initial serum levels of Hsp 70 protein in the preoperative period and Intraoperative period.

Liu et al[12] reported a progressive decline in the serum levels of Hsp 72 protein as a function of aging. In addition, a number of in vitro findings have shown that the expression of Hsp 72 is decreased in senescent cells, indicating that the process of aging may be associated with reduced Hsp 72 production. Another possible contributing factor could be the production of antibodies against the Hsp 72 protein that in turn could bind to the antigen and thereby prevent the detection of Hsp 72 in the serum of older pat ients. Rea et al[13] reported an apparent increase in the serum levels of anti - Hsp 72 antibody in the older but otherwise normal subjects.

Our study has some limitations. We did not account for preexisting conditions in our patient cohort. Furthermore, the preoperative sample collection time varied depending on the admission of patients post injury at our hospital.

Conclusion:

In conclusion, our study provides further insight in heat - shock response following severe trauma. Compared to healthy individuals and patients with isolated injuries, increased serum concentrations of HSP 70 were demonstrated. The present study provides the first in vitro evidence that the early release of HSP 70 protein into the bloodstream after injury was associated with a significant increase in prognosis of traumatized patients. Although the mechanisms of protection associated with elevated levels of serum Hsp 70 protein early after trauma remain yet to be defined, experimental evidence has suggested that circulating heat protein peptide complexes could be involved in the activation of innate immunity, leading to a down - regulation of the inflammatory response without the need for induction of thermo-tolerance. Future clinical studies will therefore be needed to determine how circulating heat proteins including Hsp70 could modulate the severity of the inflammatory response to trauma in a standardized fracture condition.

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