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Role of Heat Shock Protein -70 and *Helicobacter pylori* in Atherosclerosis.

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Abstract

Background: Coronary Artery Disease due to atherosclerosis is one of the major causes of mortality and morbidity. It occurs in a significantly large number of patients without any risk factors. Even in patients with known risk factors, the exact mechanism is not known. Hence, there has been a great interest in the infectious origin of the disease. Many studies have demonstrated *Helicobacter pylori* association with atherosclerotic plaques. However, exact mechanism is still not established. In our present study, we have tried to find association between HSP-70 and Cag-A gene for causing atherosclerosis in coronary arteries.

Methods: Serum, LIMA and endarterectomy specimen of 45 patients undergoing CABG were collected. The serum was subjected to ELISA for HSP-70 Antibodies, LIMA for HSP-70-IHC and endarterectomy specimen for IHC and PCR for 16SrRNA and Cag-A gene.

Results 45 samples were positive for HSP-70 Ab by ELISA. Of 45 endarterectomy plaques, 41 were positive by IHC for HSP-70 and 4 were negative whereas, 2 of 45 LIMA samples were IHC positive and 43 were negative. 43 out of 45 samples were positive for 16SrRNA genes and 2 were negative. Out of 45 endarterectomy specimens, 38 were positive and 7 were negative for Cag-A gene as detected by PCR. Of 41 IHC positive samples, 38 were positive and 3 were negative for Cag-A gene. Of 4 negative samples, all 4 were negative for Cag-A. LIMA specimens were not subjected to PCR.

Conclusion: In our present study, we have clearly demonstrated circulating antibodies in serum against HSP-70(45), presence of HSP-70 in plaques (41) along with Cag-A gene of *H. pylori* (38). Presence of Cag-A gene indicates infection by *H. pylori*. This chronic *H. pylori* infection induces overexpression of HSP-70 in vascular wall of coronary arteries. Interaction between HSP-70 and HSP-70 antibodies initiates immune-mediated injury to vascular wall, leading to inflammation and atherogenesis.

Introduction

Multifactorial nature of atherosclerosis is a well-established fact for causing atherosclerotic coronary artery disease leading to myocardial infarction and death[1]. Clinico-epidemiologically, many risk factors like hypertension (HT), diabetes (DM), smoking, stress and hyperlipidaemia are identified[2]. However, there is a large sub-set of population which develops atherosclerotic coronary artery disease (CAD) without any risk factors and even the presence of risk factors does not suggest the underlying mechanisms[3-7]. Hence, for long, infective nature involving molecular and immunological mechanisms have been extensively studied.

The presence of inflammatory cells in the atherosclerotic plaque is well-established[8], however, the exact mechanism of activation of inflammation is not known but the autoimmunity triggered by infections is the most favoured hypothesis[9-11]. In this regard, *Helicobacter pylori* is extensively investigated as a causative organism[12] based on sero-epidemiological studies[13] by detecting anti *H. pylori* antibodies. Chronic infection by *H. pylori* can trigger expression of Heat Shock Proteins[14-16] in vessels. This could be mediated through Cytotoxicity associated gene A (Cag-A) which is pathogen marker of *H. pylori.* Antibodies against HSP-70 can react with HSP-70 leading to immune activated inflammation and plaque formation. In our study, we sought to demonstrate presence of Cag A gene, 16SrRNA and HSP-70 in atherosclerotic plaques and anti HSP-70 antibodies in serum.

Methods

45 patients undergoing coronary artery bypass graft (CABG) surgery along with endarterectomy were enrolled for the research purpose. Serum, Left Internal Mammary Artery (LIMA) and endarterectomy specimens were collected during the surgery. In all the patients, severe coronary artery disease (CAD) was established angiographically and surgery was advised.

Enzyme Linked Immuno-Sorbent Assay (ELISA):ELISA was performed on all serum samples to detect anti HSP-70 antibodies. Immunohistochemistry (IHC):IHC was performed on LIMA and endarterectomy specimen to detect presence of HSP-70.Polymerase Chain Reaction (PCR):PCR was performed on endarterectomy specimen to detect presence of Cag A and 16SrRNA genes.

Results:

All 45 serum samples were positive for HSP-70 Anti-bodies by ELISA. (Table 1) ,Of 45 endarterectomy plaques, 41 were positive by IHC for HSP 70 and 4 were negative whereas, only 2 of 45 left internal mammary artery (LIMA) samples were IHC positive. (Table 2). Of 45 endarterectomy plaques, 43 were positive for 16SrRNA gene and 2 were negative. (Table 3) Out of 45 endarterectomy specimen, 38 were positive and 7 were negative for Cag-A gene as detected by PCR. (Table 3)

Of 41 IHC positive samples, 38 were positive and 3 were negative for Cag-A gene. Of 4 negative samples all 4 were negative for Cag-A. (Table 4).LIMA specimen were not subjected to PCR.

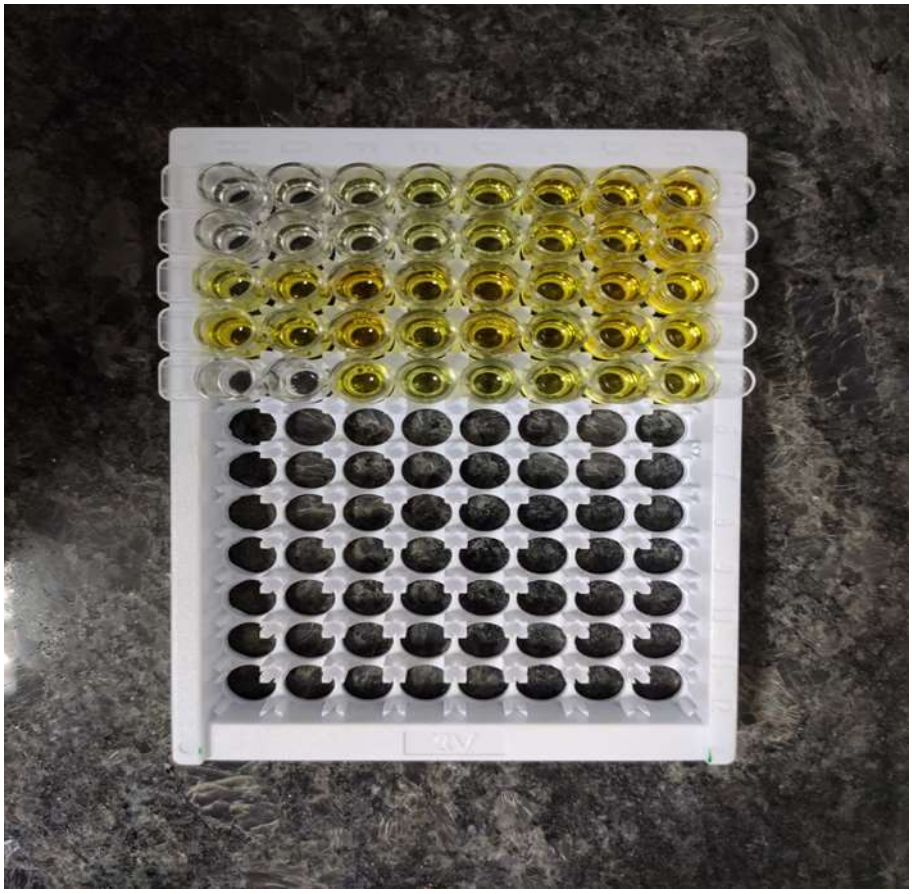


Fig 1:ELISA test for HSP-70 Antibodies

Fig 2: gel electrophoresis of the amplified product of ----PCR 16SrRNA gene

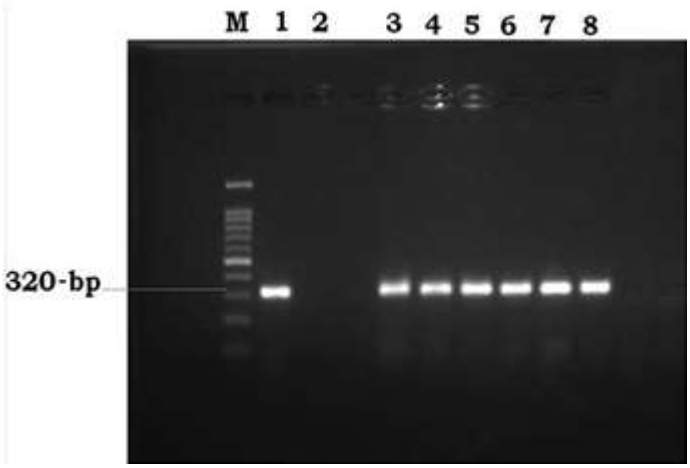


Fig3 :PCRAmplification product of --- Cag-A gene

Endarterectomy specimens for IHC

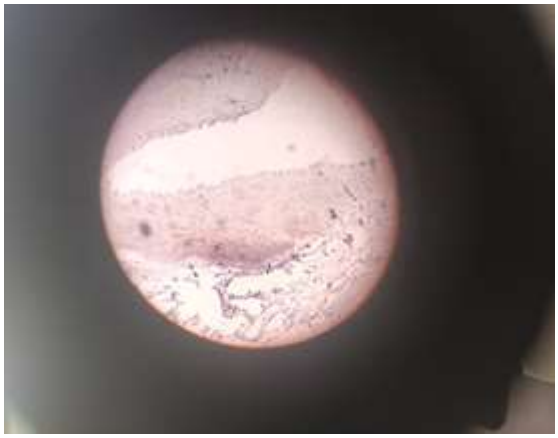
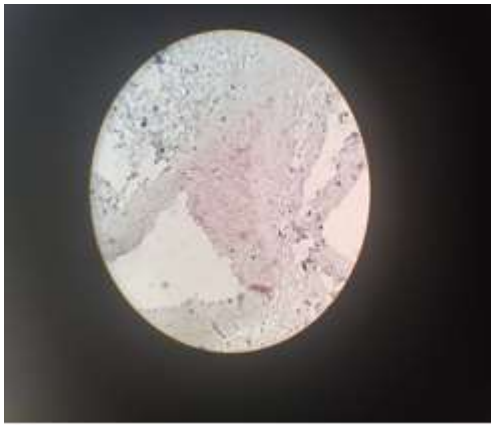


Fig 4:IHC for HSP-70



IHC for HSP-70

Table 1: Age Distribution

Age groups	No	%
30-40	3	6.66%
41-50	3	6.66%
51-60	18	40%
61-70	19	42.2%
71-80	2	4.44%
Total	45	100.00

Table2: Gender Distribution

Gender	No	%
Male	33	73.33%
Female	12	26.66%
Total	45	100.00

Table 3: Distribution according to the risk factors

Risk factor	No	%
Diabetes	18	40%
Hypertension	4	8.88%
Diabetes and Hypertension	12	26.66%
No Diabetes and Hypertension	11	24.44%
Total	45	100.00

Table 4: Comparison of Male and Female with ELISA test

	ELISA		
	Positive	Negative	Total
Male	33	0	33
Female	12	0	12
Total	45	0	45

Table 5: Comparison of Endarterectomy and LIMA with IHC

	IHC for HSP 70		
	Positive	Negative	Total
Endarterectomy	41	4	45
LIMA	2	43	45

Table 6:comparison of PCR for 16SrRNA and CagA with endarterectomy

	PCR-Endarterectomy		
	Positive	Negative	Total
16SrRNA	43	2	45
Cag-A gene	38	7	45

Table 7:Comparison of IHC and CagA PCR with endarterectomy

Endarterectomy				
	Positive		Negative	
IHC	41		4	
Cag-A	Positive	Negative	Positive	Negative
	38	3	0	4

Discussion

Ritoss, in 1962, first detected HSP in *Drosophila Melanogaster*[17]. Subsequent works have demonstrated the highly phylogenetically conserved and ubiquitous nature of HSP family. Apart from many other reasons, their expression is induced by infections also[18]. HSPs act as molecular chaperones[19]. Their extracellular presence can induce pro-inflammatory processes.

In many infections, HSP offers protection via humoral and cellular immune response[20-21]. However, altered immune response is implicated in development of IHD and neurodegeneration. CD-14 TLR-2 and 4 and CD-19 receptors interact with HSP-70 for initiation of immune response[22].

Based on above finding, we investigated role of HSP-70, HSP-70 antibodies and Cag-A and 16SrRNA in development of atherosclerosis in human coronary arteries.

We hypothesised that chronic infection by *H. pylori* as indicated by presence of its nuclear material [Cag A and 16SrRNA genes] in atherosclerotic plaques[23] induce HSP-70 expression. Already circulating HSP-70 antibodies against *H. pylori* derived HSP-70 antigens cross react with host HSP-70 antigens[24]. This enhances release of pro-inflammatory cytokines[25].

We demonstrated *H. pylori* infection in our study subjects by detecting Cag A gene and 16SrRNA genes in the plaque. We also demonstrated expression of HSP-70 antigens in plaques by IHC. We also demonstrated anti HSP-70 antibodies against *H. pylori* in almost all patients' serum.

Many studies have pointed towards *H. pylori* infection as a possible cause for atherosclerosis[26]. However they have not proposed exact mechanism. Some studies have partially explained the role of HSPs in atherogenesis[27].

Another study has demonstrated interaction between *H. pylori* antibodies and antigens in the walls of atherosclerotic carotid arteries[28]. This finding suggested towards the phenomenon of molecular mimicry in which *H. pylori* antigens mimicked vascular antigen. However, this study did not elicit the exact mechanism to its full extent.

H. pylori and its genome have been demonstrated in atherosclerotic plaques[28] by IHC using antibodies against *H. pylori*. This made the authors believe that presence of *H. pylori* itself is cause for atherosclerosis. However, it also failed to provide complete explanation for atherogenesis.

In our present study we have clearly demonstrated circulating antibodies in serum against HSP-70, presence of HSP-70 in athero-slerotic plaques along with Cag-A gene of *H. pylori in many samples*. Presence of Cag-A gene indicates infection by *H. pylori*. This chronic *H. pylori* infection induces over expression of HSP-70 in vascular wall of coronary arteries. Interaction between HSP-70 and HSP-70 antibodies initiates immune-mediated injury to vascular wall leading to inflammation and atherosclerosis. The control arm of study in which LIMA was studied did not demonstrate presence of Cag-A gene in any specimen along with no expression of HSP-70 proteins. This indicates that LIMA doesn't harbour *H. pylori* infection. We don't have clear explanation to why. *H. pylori* affects coronary arteries only, not LIMA. One possible explanation could be mechanical stress injury to which coronary arteries are more prone.

Limitations of our study have been, not all the specimens of endarterectomy have demonstrated Cag-A gene and HSP-70 proteins. This points towards other possible mechanisms for atherosclerosis. We have also not studied exact molecular mediators. We have not explored the role of anti-Cag-A antibodies as has been suggested by some in a study.

To conclude, our study provides experimental evidence for antibody mediated injury involving HSP-70 in majority of the specimen studied in established cases of coronary artery disease.

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