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Role of αB-Crystallin on β-Amyloid with Systemic Amyloidotic Mice Brain

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Abstract: **αB-Crystallin is one of the heat shock proteins and its Chaperonic activity is well established. AlphaB-Crystallin forms complexes with denaturing proteins thereby preventing their uncontrolled aggregation. Chaperonine process has been shown to follow a saturation type of complexing, when the ability of the available αB-Crystallin to bind the target protein is exceeded, the excess target protein aggregates and eventually precipitates out of solution. The role of αB-Crystallin on Aβ fibril formation under systemic amyloidodsis in the brain, we have determined the level of abeta's in the brain. In the present study, in-vivo studies on the role of the chaperones were investigated by Congo red stain, Immuno fluorescence, Radio labeled alphaB-Crystallin and RP-HPLC methods. The results indicated that there was amyloid deposits in brain, increases the abeta's level in the brain and the alphaB-Crystallin crosses blood brain barrier and abeta's level comes near to control level. In conclusion the implication of abeta's in the brain. The significance in-vivo study has shown αB-Crystallin as a therapeutic use for amyloid disease.**

Keywords: **Aβ, Alzheimer's disease, Systemic amyloidosis, Crystallin, Chaperonine, Aggregate, Fibril.**

I. INTRODUCTION

Small heat-shock proteins (sHSPs) are one of four families of heat-shock proteins (HSPs) expressed in response to heat shock and other forms of stress (Parsell and Lindquist 1993). They are a diverse family of proteins that appear to be ubiquitous in nature, being found as surface antigens in eukaryotic parasites, as inclusion body-binding protein in E. Coli, and as structural proteins in the vertebrate lens (Caspers et al 1995). Despite their low molecular mass (12-40 kDa), sHSPs are isolated as large oligomeric complexes of 2-40 subunits depending on the physiological state of the cell. The role of these proteins in thermotolerance appears to be a consequence of their ability to function as molecular chaperones and to modulate actin filament dynamics (Lee et al 1995). sHSPs from many species inhibit the unfolding-induced aggregation of proteins in an ATP-independent manner and form a stable complex with their protein substrate. Thus, during periods of stress, sHSPs act as energy-independent traps preventing the irreversible aggregation of proteins. Recently, it has been shown that upon establishment of refolding conditions, proteins bound to sHSPs are efficiently refolded in cooperation with other chaperones (Lee et al 1997)

The research subunit is investigating the in-vivo functions of the lens alpha-Crystallin proteins (αA and αB). These proteins, abundant in vertebrate lenses, were originally thought to be solely structural proteins, but in recent years have been shown to possess a myriad of activity in-vitro including molecular chaperone activity, autokinase activity, DNA binding activity and binding to and regulating the Polymerization State of several cytoskeletal proteins. Mice lacking the alphaB-Crystallins have been generated and are being analyzed. Mice lacking αB-Crystallin appear to be completely normal. Mice lacking αA however develop cataracts beginning early in life and progressing in severity with age. In the absence of αA, the αB forms large insoluble masses, 1 to 3 um in diameter, in the cytoplasm in lens fiber cells. Lenses of mice lacking αB-Crystallin appear to be completely normal.

In AD, the plaque around the brain cell surface contains mainly aggregated Aβ and is responsible for neurotoxicity Selkoe 2000). The $A\beta_{40 \text{ and } 42}$ sequences are the proteolytic degraded products of the amyloid precursor protein. Some reports indicate that the small heat shock proteins Hsp27 and αB-Crystallin colocalize in the plaques (Renkawek et al 1995).

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Further, αB-Crystallin is widely expressed in other tissues and its expression increases with neurodegenerative disease (Sax and Piatigorsky 1994). The role of αB-Crystallin in the development of these diseases is largely unknown but may be related to its functional role as a small heat shock protein (Caspers et al 1995). Recently, alphaB-Crystallin was reported to act as a chaperone molecule capable of protecting other proteins from denaturation (Horwitz 1993). Whether this chaperone function is active *in-vivo* either in the lens, or, in other tissues, is unknown. To understand the effect of αB-Crystallin on Aβ fibril formation, we need to determine whether they interact with each other. In the present study, *in-vitro* studies on the role of the interaction of SAA/SAA fibrils with chaperones were investigated. The results indicated that there was interaction between SAA and alphaB-Crystallin when they were incubated together. A possible mechanism for this interaction and its implicated significance *in-vivo* are discussed.

II. MATERIALS AND METHODS

Production of Systemic Amyloidosis:

Colony-bred adult male Swiss White mice (30-35 g) were used. Animals were selected randomly and caged in groups of four at room temperature (25-35°C) and supplied with food (Commercial pelleted animal feed marketed by M/s. Hindustan Lever, Bombay, India under the name "Gold Mohur rat feed") and tap water ad libitum (control n=5, test n=5). All animal procedures were carried out as approved by the Animal Care and Use Committee of Central Leather Research Institute, Chennai. Mice were given 0.5 ml of 10% Vitamin free Casein (1CN Pharmaceuticals, Cleveland, OH, USA) as subcutaneous injection for 66 days to induce systemic amyloidosis (Botto et al 1997). Control animals were treated with saline. After 66 days alphaB-Crystallin was treated 100 mg/kg-body weight/day for three weeks. At the end of treatment, animals were decapitated. Immediately after decapitation, brain cerebral cortex, liver and spleen were removed in the ice-chilled condition for further analysis. Systemic amyloidosis was confirmed by "ladder formation" in the liver. The presence of amyloid fibrils in the liver and Spleen is further confirmed by Congo red staining.

Congo red staining of Brain Amyloid Deposits:

Frozen tissue sections were analyzed for amyloid deposition with Congo red staining by following the reported procedure (Westermark et al 1999). In brief, the deparaffinized sections were stained with Mayer's solution for 1 min and then rinsed in saline solution for few minutes. Sections were treated in solution containing 10 gm NaCl to 100 mL in 80% (v/v) ethanol for 20 min. These sections are transferred directly to a solution of 2gm Congo red and 10 gm NaCl in 1000 mL 80% ethanol and stained for 20 min. The sections were then washed twice with absolute ethanol. The sections were then washed with xylene thrice and mounted under cover glass in a synthetic-mounting medium, DPX. Stained sections were observed with crossed high intensity polarized light in polarization microscopy (EUROMAX, Holland).

Immunostaining of Brain sections:

The antisera against abeta$_{25-35}$ were prepared as per the following procedure. New Zealand White rabbits were given intradermal injection of 0.1 mg/ mL of mouse abeta$_{25-35}$ weekly once. Prior to the injection, the abeta$_{25-35}$ peptide was dissolved in PBS and emulsified in complete and incomplete adjuvant. Three weeks later a fourth intradermal injection was given and the animals were bled in the following week. The antisera were stored for further processing at -20°C. The immunostaining of abeta$_{25-35}$ in the brain was carried out as follows; after blocking nonspecific binding with 2% BSA solution, the brain tissue sections were incubated with anti abeta$_{25-35}$ (1:1000) for 30 min at 4°C. After washing in PBS, the sections were incubated with FITC conjugated rabbit anti mouse antibody for 30 min at 4°C. After washing in PBS, incubated with FITC conjugated rabbit anti mouse antibody for 30 min at 4°C, the FITC fluorescence was observed by fluorescence microscopy.

Purification, Extraction and Estimation of Aβs:

Brain tissue was extracted as described by Naslund et al (1996). The tissue was extracted repeatedly in a buffer containing 1% sodium dodecyl sulfate (SDS), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, yielded an SDS-insoluble pellet, which was extracted with 1, 1, 1, 3, 3, 3-hexafluoro 2-propanol (HFIP) (Sigma, USA). After removal of debris, the HFIP extract was dried under the stream of nitrogen.

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RP-HPLC:

The HFIP brain extract (Naslund et al 1996) containing Aβ peptides was dissolved in 70% formic acid and separated using acidic RP-HPLC buffer systems on a Waters system, equipped with a Shimadzu SPD 10A UV-Visible detector (Shimadzu, Kyoto, Japan). Separation were performed using Spherisorb-ODS2 25 cm × 4.6 mm, 5 µm, C_{18} (Waters, Milford, Massachusetts, USA) silica column. The buffers used were 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.1% TFA in acetonitrile (ACN) (buffer B). Solvents were filtered through 0.45 µ nylon filter (Sartorius, AG, Gottingen, Germany). Samples were eluted with a linear gradient of buffer B at a flow rate at 1 mL/min while monitoring UV absorbance at 220 nm.

Isolation of αB-Crystallin:

Bovine lenses (8-10 lenses) were decapsulated and homogenized (Luthra and Balasubramanian 1993) in 0.1 M Tris buffer, pH 7.4 containing 0.5 M NaCl, 1 mM EDTA, and 0.1% NaN_3. The insoluble protein fraction and membrane debris was removed by centrifugation at 30,000g for 30 min. The supernatant was chromatographed on a Sephacryl-S200 (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (0.8 × 90 cm) to separate the alphaB-Crystallin using Gradifrac™ FPLC system (Fig. 1a) (Pharmacia Biotech, Uppsala, Sweden). Each of the alphaB-Crystallin fractions was dialyzed repeatedly against water using FILTRON concentrating 10000 D. Molecular weight cut off membrane (Gelman Sciences, India) and lyophilized and stored at -20°C. Protein was determined by Lowry et al (Lowry et al 1951). Homogeneous 12% SDS-polyacrylamide gels were run on the mini gel electrophoresis system (Fig. 1b) (Laemmli, 1970).

Fluorolabelling of Abeta$_{25-35}$ Fibrils:

Purified abeta$_{25-35}$ proptofibrils (400 µg/400 µl 0.001% NH_4OH, pH 9.0) were labeled with fluorecein isothiocyanate (FITC) (Fluka, USA) by dialysis through a 1000 Da cutoff membrane. FITC (0.4 mg) was dissolved in 40 µl DMSO and added to 40 ml of PBS adjusted to pH 9.0 with NH_4OH. After 12 hrs at 4°C the abeta$_{25-35}$-FITC and abeta$_{25-35}$ protofibrils-FITC were dialyzed against milli-Q water, sterile filtered (0.45 µm) and held at 4°C (Linke et al 1991). 1.25-6.25 µM of SAA-FITC and SAA protofibrils-FITC were assayed with 0.15-15 nM of αB-Crystallin.

Radio labeling:

The alphaB-Crystallin was labelled with ^{125}I (Baba Atomic Research Center, New Mumbai, India) using the method by Hamazaki (Hamazaki 1995) and stored in phosphate buffered saline containing 0.02% NaN_3. Labelled proteins to a final specific activity of approximately 2mci/mM were given to amyliodotic and control mice. ^{125}I BSA used as a control. After 24 hrs, animals were sacrificed and the brain and plasma were measured in a 1270 Rack Gamma II γ counter (Pharmacia Biotech (LKB), Uppsala, Sweden). All the groups compared statistically using the Chi-spuare test, U-Test (Mann-Whitney-Wilcoxon) and analysis of variance (ANOVA) with 2-tailed significance thresholds.

III. RESULTS AND DISCUSSION

The systemic Amyloidosis results in amyloid deposits in liver, spleen and brain as shown (Asokan 2015). The brain sections of the alphaB-Crystallin treated mice with systemic amyloidosis were also examined by this procedure. The Congo red staining of the brain cerebral cortex (Fig. 2a) revealed that in control mice there was no fibril deposition. This was already observed by several groups (Baltz et al 1980). The diffused amyloid deposits were dispersed throughout the cerebral cortex, with occasional accumulation in few places in the casein discontinued mice brain (Fig. 2b). However, as seen in the figure, the amyloid burden was considerably reduced in these organs (Fig. 2c) in αB-Crystallin treated mice.

Previous finding suggests that α-1-antitrypsin (AAT) and αB-Crystallin (also called HSP 27) are down regulated in Huntington's disease (HD). Thus maintaining αB–Crystallin availability during the course of HD might prevent neuronal cell death and therefore suggested being useful in delaying the disease progression (Zabel et al 2002).

Considering the abundance of αB-Crystallin expression in the peripheral tissues and possible therapeutic value of αB-Crystallin combating amyloid burden in the brain, transport of αB-Crystallin to brain tissues through the blood brain barrier was investigated. To find out whether αB-Crystallin could pass through the BBB, we injected the radio labeled [^{125}I] αB-Crystallin in control and casein injected mice (mice with systemic amyloidosis). Fig. 5. indicates that the

radiolabeled αB-Crystallin enters into the brain through blood (Fig. 4) of the mice affected by systemic amyloidosis. The αB-Crystallin incorporation in the brain tissue was specific to the SAA in the amyloidotic condition, because the amyloidotic mice when injected with radio labeled [^{125}I] BSA showed negligible accumulation. This observation precludes the possibility of leaky brain endothelial cell capillaries, which will allow most of the proteins to be diffused in the inflammatory conditions. Further, radio labeled [^{125}I] alphaB-Crystallin and [^{125}I] BSA to the control shows no significant incorporation in the control brain (Fig. 5).

There are several reports indicating that in inflammatory condition Aβ protein was increased ~20 fold greater than in the normal condition. The abeta's were done separation and quantification of Abeta41, Abeta43, Abeta54, and Abeta17-45 by RP-HPLC from mice brain respectively, 0.29 ± 0.02 pmol/g, 1.18 ± 0.16 pmol/g, 0.43 ± 0.03 pmol/g and 0.87 ± 0.07 pmol/g (Fig. 6a) the chromatogram represents the elution profile of the control mice brain extract respectively, 5.8 ± 0.48 pmol/g, 23.4 ± 2.1 pmol/g , 8.4 ± 7.8 pmol/g and 17.4 ± 1.6 pmol/g (Fig. 6b) the chromatogram represents the elution profile of the untreated systemic amyloidotic mice brain extract respectively, 0.32 ± 0.02 pmol/g, 1.24 ± 0.11 pmol/g, 0.49 ± 0.04 pmol/g and 0.94 ± 0.08 pmol/g (Fig. 6c) represents the elution profile of the alphaB-Crystallin treated systemic amyloidotic mice brain extract. Synthetic standards were used to the Abeta40, Abeta42, Abeta54 and Abeta17-45 in the RP-HPLC chromatogram. Excess accumulation of Aβ in the systemic amyloidotic mice is shown Table 1.

Severe inflammation was observed in systemic amyloidotic mice together with excess secretion of inflammatory markers and SAA protein. In this work, we have seen that in systemic amyloidotic mice, the Abeta's level come to normal after treatment with αB-Crystallin. The αB-Crystallin treatment is reduced the amyloid burden in the systemic amyloidotic mice brain.

IV. DISCUSSION

The results of the experiment present the following conclusions. The alphaB-Crystallin transported across the BBB in the mice with systemic amyloid disease. The transported SAA along with SAP gets localized in the brain (Asokan 2015). The inflammatory response in pathologically affected regions of amyloid deposition in the brain, particularly in AD, is well-established (Sisodia and Price 1995). As in the case of peripheral degenerating tissues, damaged neurons and neurite provide stimuli for inflammation. This will lead to up-regulation of acute phase reactants and cytokines (Song et al 2001). Thus, the elevation in SAA and SAP level partly attributed to the brain tissue expression of these proteins following inflammatory stimulus (Asokan 2015). The result of the present work suggests that peripheral inflammation significantly contributes to SAA and SAP transport across the brain and associated toxic responses.

Our results also indicate that along with SAA fibrils, SAP also crosses the BBB. The presence of SAP in peripheral and neuronal amyloid deposits is well documented (Hawkins et al 1998). However, the mRNA encoding SAP was not detected in the brain (Kalaria and Grahovae 1990) indicating that cerebral SAP can be sourced from peripheral region. The present work is consistent with the hypothesis that the SAP, which is found in various inflammatory conditions in the brain, may be due to its crossing of BBB from the plasma.

The present observation indicates that the inflammation and associated systemic amyloidosis will also trigger the Aβs deposition in the brain. The neurotoxic fragments of APP, are believed to be located partly in the extracellular compartment (the first 17-20 residues) and partly (the carboxyl-terminal half of Aβ) within the membrane bilayers. Aβ is released from neurons in a soluble form, which circulates in the cerebrospinal fluid at low levels. The generation of Aβ as a proteolysis by-product of an intracellular degradative process is triggered by mutations in the APP sequence. Murine endogenous Aβs and its soluble counterparts were already observed by Wang et al (1996) in the immuno isolates of neuro 2a cell extract using monoclonal antibodies. In the present work, did not observe such a low molecular weight distribution was not seen in the HFIP extract, presumably because of the solubility of low molecular weight fragments on SDS treatment in the procedure that was adopted.

In the present case, the reason for the accumulation of Aβs is not clear. However, a marked decrease in the cerebral glucose levels is observed in the mice with systemic amyloidotic condition, which is due to impaired liver functions (Jessy et al 1990). The stress induced glucose deprivation was known to promote β-secretase cleavage (Furukawa et al 1996). Accumulating evidence demonstrates that inflammatory process plays an important role in AD. Infact, most of the

proposed toxicological mechanisms rely on the ability of Aβ peptide inducing the inflammatory process in AD brain (Paris et al 2000). Aβ was shown to activate glial cells under *in-vitro* conditions to induce expression of various inflammatory products like IL-1β, tumor necrosis factor (TNF-α), nitric oxide (NO) reactive oxygen species, IL-6 and IL-8. However, it is not clear whether the cytokine elevation in the brain due to other inflammatory conditions will lead to accumulation of Aβs. Cytokine elevation is also observed during systemic amyloidosis (Akiyama et al 2000). Guo et al have observed that in inflammatory condition; transgenic mice (containing SAA1 protein in the brain) increase cytokine levels in the brain (Guo et al 2002). This support the idea that elevated cytokine could possibly increase Aβ levels in the mice brain due to systemic amyloidosis.

V. CONCLUSION

In conclusion, from the results of this work, it has been demonstrated that there is an accumulation of Aβ content compared to normal mice brain following the induction of systemic amyloidosis by sub-chronic treatment with casein. The congo red stain and immune flourscence and RP-HPLC results were concludes that the injection of alphaB-Crystallin the amyloid burden brings to the normal level. The study clearly tells that radio labbled alphaB-Crystallin crosses the blood brain barrier preventing their abeta's uncontrolled aggregation in Systemic Amyloidosis condition.

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APPENDIX

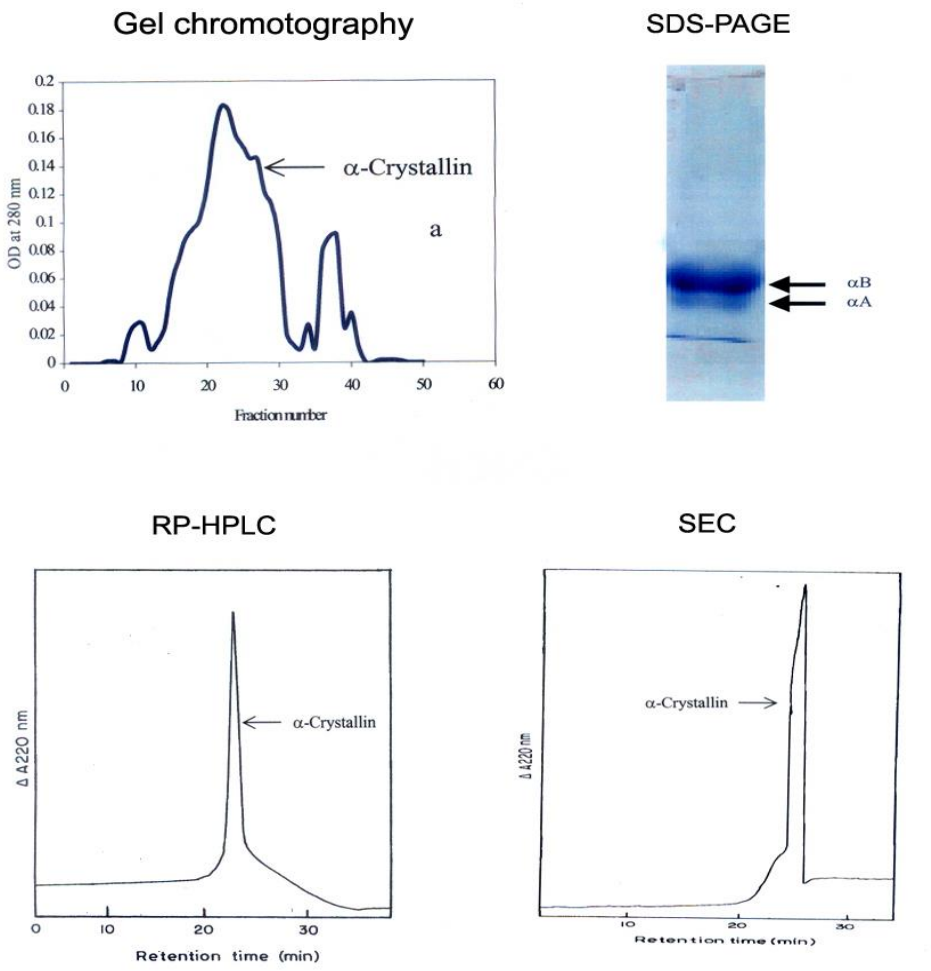

Fig.1. Gel filtration chromatographic profile of alphaB-Crystallin (a) SDS-PAGE profile of alphaB-Crystallin (b) RP-HPLC profile of the alphaB-Crystallin (c) and SEC profile of the alphaB-Crystallin

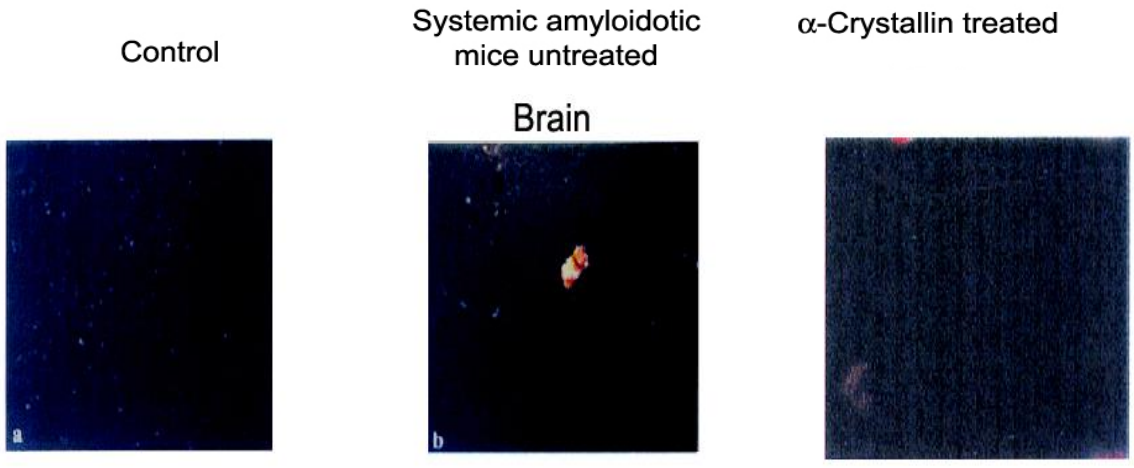

Fig.2. Congo red stained sections of amyloid deposits with cross-polarized light (a) brain control mice (b) brain section of untreated systemic amyloidotic mice (c) brain of a-Crystallin treated sysytemic amyloidotic mice. Magnification: 40.

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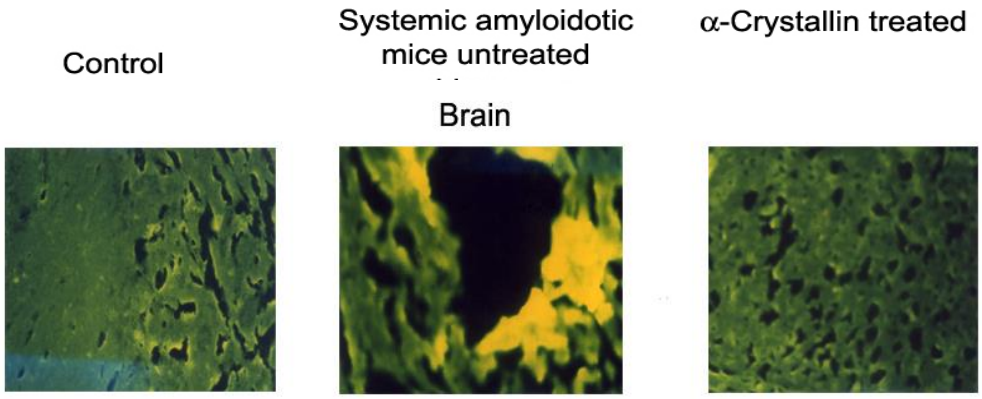

Fig.3. Fluorescence microscopic picture of amyloid deposits (a) SAA immunostaining of control mice brain (b) SAA immunostained with untreated systemic amyloidotic mice brain (c) SAA immunostaining of alphaB-Crystallin treated mice brain.

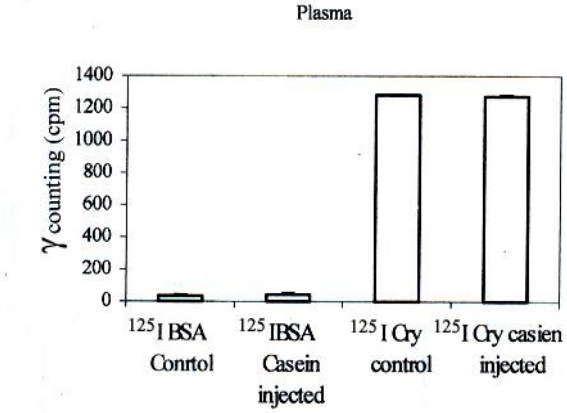

Fig.4. Incorporation of ^{125}I Labelled alphaB-Crustallin and ^{125}I Labelled BSA in the control and Casein injected mice plasma. (a) Accumulation of ^{125}I alphaB-Crustallin in Casein injected mice plasma.

Note: significant accumulation in the control mice. However no significant in BSA incorporation in both control and Casein injected mice.Data show mean γ counting values (±SEM) from single, representative experiments. * p < 0.05; * * p < 0.01: * * * p < 0.001, related to control conditions.

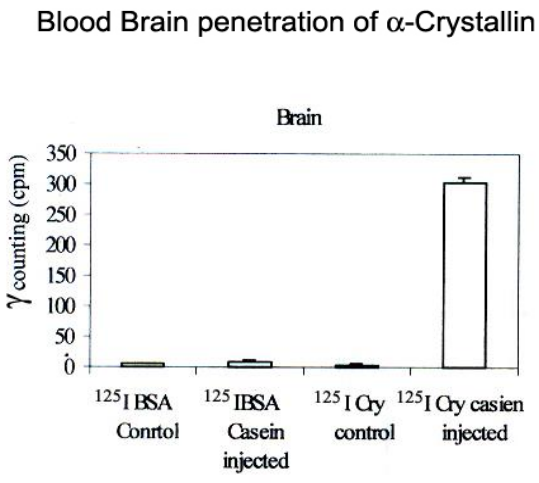

Fig.5. Incorporation of ^{125}I Labelled alphaB-Crustallin and ^{125}I Labelled BSA in the control and Casein injected mice brain. (a) Accumulation of ^{125}I alphaB-Crustallin in Casein injected mice brain.

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Note: less significant accumulation in the control mice. However no significant in BSA incorporation in both control and Casein injected mice. Data show mean γ counting values (±SEM) from single, representative experiments. * p < 0.05; * * p < 0.01: * * * p < 0.001, related to control conditions.

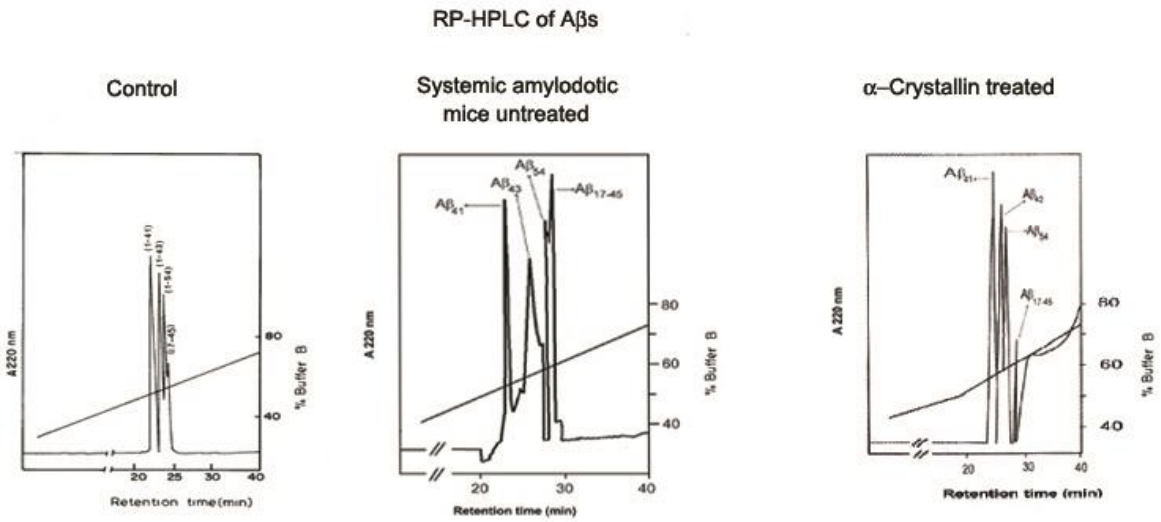

Fig.6. Seperation and quanification of Abeta41, Abeta43, Abeta54, and Abeta17-45 by RP-HPLC from mice brain (a) the chromatogram represents the elution profile of the control mice brain extract, (b) the chromatogram represents the elution profile of the untreated systemic amyloidotic mice brain extract, (c) represents the elution profile of the alphaB-Crystallin treated systemic amyloidotic mice brain extract. Synthetic standards were used to the Abeta40, Abeta42, Abeta54 and Abeta17-45 in the RP-HPLC chromatogram.

Table 1: RP-HPLC quantification of Abeta peptide level in control, systemic amyloidotic condition and alphaB-Crystallin treated mice brain.

Abeta peptides	Control mice brain	Systemic amyloid mice brain	AlphaB-Crystallin treated mice brain	Percentage (%)
$Abeta_{17-45}$	0.29 ± 0.02 pmol/g	5.8 ± 0.48 pmol/g	0.32 ± 0.02 pmol/g	10
$Abeta_{41}$	1.18 ± 0.16 pmol/g	23.4 ± 2.1 pmol/g	1.24 ± 0.11 pmol/g	40
$Abeta_{43}$	0.43 ± 0.03 pmol/g	8.4 ± 7.8 pmol/g	0.49 ± 0.04 pmol/g	15
$Abeta_{54}$	0.87 ± 0.07 pmol/g	17.4 ± 1.6 pmol/g	0.94 ± 0.08 pmol/g	35

Data show mean values (±SEM) from single, representative experiments. * p < 0.05; * * p < 0.01: * * * p < 0.001, related to control conditions.