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Treatment of bovine serum albumin with formaldehyde would result in cytotoxicity to SH-SY5Y cells

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Abstract: Bovine Serum Albumin (BSA) is a well know globular protein having the tendency to form aggregates in the macromolecular assemblies. Its three dimensional structure is composed of three domains, each one is formed by six helices, and its secondary structure is essentially alpha helix. The structure of BSA shows stability at room temperature but as temperature increases results in formation of soluble aggregates through disulphide and non-covalent bonds. The simplest of the aldehydes is formaldehyde (FA). It is a bioactive and fatal compound, naturally present in human habitation. Formaldehyde, existing in all kinds of cells, may have some different and unknown physiological functions and may be related with some substantial human diseases and their pathology. The possible role of formaldehyde in the formation of beta-amyloid aggregation related to the pathology of Alzheimer's disease (AD) has been reported. The research further showed the high expression of SSAO co-localized with A beta deposits on the blood vessels in AD brains. Important findings of the study include the significant decrease in intrinsic fluorescence of protein with reference to control BSA both in a concentration and time dependent manner. SDS PAGE results showed some aggregation but there is no significant difference between the control and FA treated group. The important finding of the study is that the BSA monomer is highly cytotoxic to SH-SY5Y Cells. There is significant and higher level of cell cytotoxicity observed in SH-SY5Y cells for both 0 day and 7 days incubation with samples of BSA treated with formaldehyde as compared to BSA and formaldehyde alone. The results of the present study reveals that the BSA treated with formaldehyde would be transformed into cytotoxic protein. However further studies are required to investigate the possible role of lead in affecting the structure and function of proteins.

Keywords: Cytotoxicity, aggregation, formaldehyde, bovine serum albumin, SH-SY5Y cells.
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INTRODUCTION

It is a well-known fact that the native structure of protein plays a key role in its biological and chemical properties in the complex environment of cell. Under some conditions, proteins fail to fold correctly in their native form which results in a wide range of diseases like amyloidoses, which involves the deposition of aggregated proteins in various tissues[1-3]. Neurodegenerative pathologies like Alzheimer's and Parkinson's diseases belong to this category.

Protein aggregation process is going in competition with the normal folding pathways[4,5] and it takes place from misfolded and partially unfolded state[6,7].

Bovine Serum Albumin (BSA) is a well know globular protein that has the tendency to aggregate in the macromolecular assemblies[8]. Its three dimensional structure is composed of three domains, each one is formed by six helices, and its secondary structure is essentially alpha helix[9,10]. The structure of BSA shows stability at room temperature but as temperature increases results in formation of soluble aggregates through disulphide and non-covalent bonds[11,12].

Albumin has a high affinity for fatty acids, hematin, bilirubin and a broad affinity for small negatively charged aromatic compounds. It forms covalent adducts with pyridoxyl phosphate, cysteine, glutathione, and various metals, such as Cu (II), Ni (II), Hg (II), Ag (II), and Au (I).

The simplest of the aldehyde class is formaldehyde (FA). It is a bioactive and fatal compound, present naturally and in human habitation. Formaldehyde, existing in all kinds of cells[13], may have some different and unknown physiological functions and may be related with some substantial human diseases. Based on its binding with proteins, formaldehyde is present in three manners *in vivo*: dissociation, reversible association and irreversible association[13,14]. According to the previous studies, it has been shown that the proportion of formaldehyde existing in these three manners is different in different biological samples.

The dissociative formaldehyde is much more harmful to animals and humans than the associative ones. The persisted exposure of dissociative formaldehyde for days significantly impairs memory, equilibrium and dexterity in histology technicians[15]. Formaldehyde is also able to induce Tau protein misfolding and globular amyloid like aggregation that is toxic to hippocampal neurons[16-18]. Furthermore, it has also been reported that formaldehyde exposure could also induce cancer and disorders of immune function in some cases.

Formaldehyde in concentration of 1mM enhances the apoptosis and reduced mitosis in cultured cells as well as tumor cells in a moderate way. The high concentration of formaldehyde about 10mM results in high degree of cell damage and a total eradication of the cell cultures[19]. The possible role of formaldehyde in the formation of beta-amyloid aggregation related to the pathology of Alzheimer's disease (AD) has been reported by the Canadian researchers[17,20]**.** The research further showed the high

expression of SSAO colocalized with Abeta deposits on the blood vessels in AD brains[21].

It has been reported that formaldehyde exposure leads to formation of DNA/protein cross-links which is a major mechanism involve in DNA damage. The DNA/proteins crosslinks have been used as a measure of dose in drug delivery[22]. Formaldehyde has also been reported as a cross linking agent and can react with thiol and amino groups of various proteins, which could result in protein polymerization[23,24]. According to a previous study, methanol ingestion has been reported as danger to public health concern because of its toxic metabolites, formaldehyde and formic acid which would affect retina, optic nerve and central nervous system[25]. Methanol is oxidized by alcohol dehydrogenase in the liver and retina. It has been shown that in the semicarbazide-sensitive amine oxidase (SSAO)-mediated pathogenesis of Alzheimer's disease, formaldehyde reacts with β-amyloids which results in the formation of irreversible cross-linked neurotoxic amyloid like complexes[23,24,26].

It has recently became evident that glycation is involved in the physiological neurodegenerative disorders like Alzheimer's[27]. It has been reported that glycation affects the biological activity of proteins and their degradation process. Therefore, the study about AGE's has gained importance in the areas of biomedical research. The role of glucose in the glycation of proteins has been studied extensively, and its implication in diabetes[28], cataract[29], renal failure[30] and other disorders[31].

Glycation of serum albumin has been greatly focused in the recent years[32-35], and bovine serum albumin (BSA) has been extensively used a molecular model. Friedman and colleagues have reported brain-penetrant serum albumin including BSA[36,37].

In the present study, based on the information regarding the misfolding and aggregation of BSA leading to many neurodegenerative diseases, a study has been done to investigate the possible role of formaldehyde on BSA to figure out the possibility of the formation of aggregates and polymers which could result in the cytotoxicity to the neuroblast cells.

MATERIALS AND METHODS

BSA used was free from fatty acids and purchased from Sigma (USA). Formaldehyde, ribose and other chemicals were of analytical grade.

Sample Preparation

BSA was dissolved in 20mM Tris-HCl pH 7.4 to yield a stock of 0.1mM. BSA solution was then resuspended with FA solution prepared in 20mM Tris-HCl to prepare four different ratios which were 1: 25, 1:50, 1:100 and 1:200. The final concentration of BSA was 0.01mM and that of formaldehyde were 0.25mM, 0.5mM, 1mM and 2mM. BSA alone and four controls of formaldehyde were also used. The samples were then incubated at 37^0C for 0 to 7 days. All solutions were filtered with 0.22uM membrane (Millipore USA)

Cell Viability Test (CCK-8)

SH-SY5Y cells were seeded in 96 well plate at a concentration of 10^4 cells per well and either exposed or not exposed to the FA treated BSA (0 and 7 days) for 24 hours. After adding the FA treated BSA, the plates were incubated at 37^0C, 5% CO2 and O.D. was measured at 450nm by Multi Scan MK3.

SDS PAGE

The samples used were of 0 to 7 days incubations and subjected for SDS PAGE. BSA final concentration was 0.01mM. One control of BSA alone (0.01mM) was used. The samples were subjected to electrophoresis using Bio-Rad (USA), electrophoresis equipment. BSA monomer was separated by using ultra centrifugation and subjected to SDS PAGE after treatment with formaldehyde (2mM).

Native PAGE

The samples used were of 0 to 7 days incubations and subjected for SDS PAGE. BSA final concentration was 0.01mM. One control of BSA alone (0.01mM) was used. The samples were subjected to electrophoresis using Bio-Rad (USA), electrophoresis equipment.

Fluorescence Measurements

Fluorescence of FA treated BSA was measured on an F-4500 fluorophotometer (Hitachi, Japan) with a circulating water bath at 37^0C. The fluorescence spectra were measured at a fixed protein concentration of 0.01mM. Excitation and emission slits were set at 5 nm. The spectra were recorded in 290 to 450nm range and the excitation wave length was set at 280nm.

Absorption Measurements

Absorbance of FA treated BSA was measured on U-2010 spectrophotometer (Hitachi, Japan) with a circulating water bath at 37^0C. The absorption spectra were also measured at a fixed protein concentration of 0.01mM. The range of the spectra was from 200-350nm.

Phase Contrast Microscopy

SH-SY5Y cells were seeded in 96 well plates at a concentration of 10^3 cells per well and either exposed or not exposed to the FA treated BSA (0 and 7 days) for 24 hours. After adding the FA treated BSA, the plates were incubated at 37^0C, 5% CO2. After incubation Hoechst staining was performed according to standard protocol and cell pictures were taken under 10X magnification.

RESULTS

Cell Toxicity Assay

The cell culture study revealed that BSA alone is showing decreased number of viable SH-SY5Y cells. The formaldehyde alone at higher concentration>0.1mM showed significant cytotoxicity to SH-SH5Y cells for both 0 day and 7 days incubation. Interestingly, the BSA along with formaldehyde is even highly cytotoxic to the SH-SY5Y cells as compared to FA alone at same concentrations.

SDS-PAGE Results

The result from SDS PAGE shows that no significant difference in formaldehyde treated BSA samples as compared to BSA control. Interestingly, the BSA monomer treated with formaldehyde shows no signs of aggregation.

Native PAGE Results

The result from the NATIVE PAGE also confirms the results obtained from SDS-PAGE results. It shows the formation of polymers.

Fluorescence Studies

The fluorescence results show that there is a significant change in fluorescence intensity at 335nm both in a concentration dependent and time dependent change. At day 7, the samples of BSA treated with formaldehyde, shows significant decrease in intrinsic fluorescence at 335nm.

Absorbance Studies

The absorbance results show that there is a significant decrease in absorbance at 280nm in samples of BSA treated with formaldehyde with time incubation.

Phase Contrast Microscopy

The cell pictures taken with bright field microscopy and Hoechst shows a significant higher cell death in the FA treated BSA as compared to positive control and formaldehyde alone.

DISCUSSION

It has been reported in previous research studies that eukaryotic proteomes have a significantly higher occurrence of disordered proteins relative to prokaryotes[27]. The role of many proteins in the neurodegenerative disorders, particularly Alzheimer's and Parkinson's diseases have been investigated in the past research. Previous research showed that partially or fully disordered proteins are prevalent in complex disorders like neurodegenerative diseases[28], cancer, cardiovascular disease or diabetes[29].

It has been reported by the Chinese colleagues in our research group that rapid glycation of BSA with ribose could result in the formation of Advance Glycation End (AGE's) Products, which would result in the cytotoxicity to the neuroblast cells like SH-SY5Y[30]. It has also been reported that formaldehyde in low concentrations can react with protein Tau to form globular amyloid like aggregates both in vivo and in vitro, which showed cytotoxicity to SH-SY5Y & HEK 293 cells[16].

In the present study it is observed that BSA alone at a concentration of 0.01mM showed decreased number of viable neuronal cells. The formaldehyde at a concentration above 0.1mM significantly proved to be cytotoxic to SH-SY5Y cells. The interesting result of this study has shown that the mixture of BSA and formaldehyde in the given concentrations showed more significant cytotoxicity as compared to the BSA and formaldehyde alone with same concentrations for both 0 day and 7 days incubation (Figure 1a, b) The important finding of the present study is the cytotoxic effect of BSA monomer after its reaction with formaldehyde. It is possibly because of a fast reaction between free amino groups of BSA with formaldehyde forming protein adducts which would result in high cytotoxicity to SH-SY5Y cells (Figure 1c).

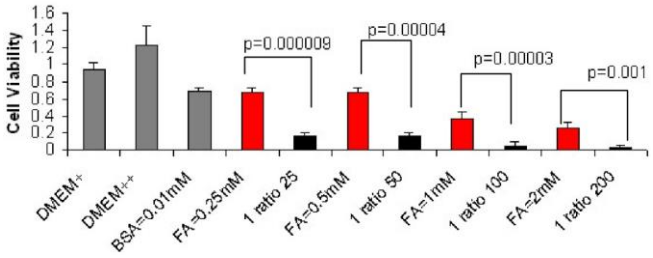

Figure 1a: Cell viability measured by CCK-8. BSA alone or incubated with different concentrations of formaldehyde at 37^0C for 0 day, was added to SH-SY5Y cells for 24 hours and cell viability was measured by using the CCK-8 assay.

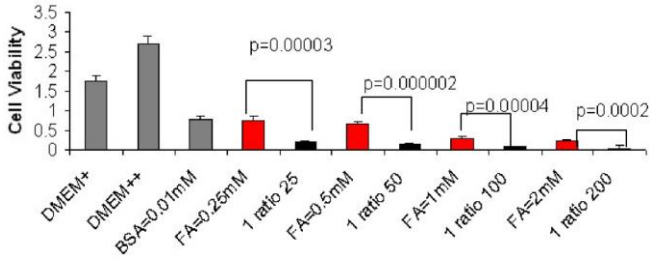

Figure 1b: Cell viability measured by CCK-8. BSA alone or incubated with different concentrations of formaldehyde at 37^0C for 7 days, was added to SH-SY5Y cells for 24 hours and cell viability was measured by using the CCK-8 assay.

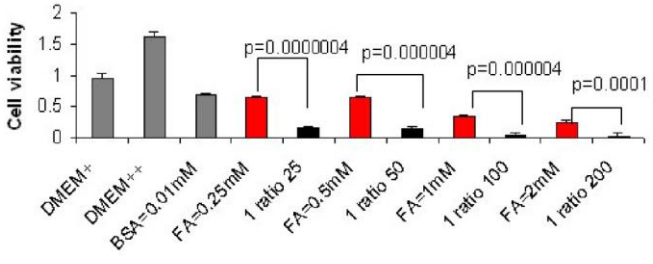

Figure 1c: Cell viability measured by CCK-8. BSA (monomer) alone or incubated with different concentrations of formaldehyde at 37^0C for 0 day, was added to SH-SY5Y cells for 24 hours and cell viability was measured by using the CCK-8 assay.

The SDS PAGE results also correlate with the above mentioned finding. There is no significant difference in formaldehyde treated BSA and the control. The results of the study revealed that probably the aggregation or polymerization has observed in both control and formaldehyde treated BSA (Figure 3a).

It is important to mention that when BSA monomer was separated by using centrifugation at high speed and treated with formaldehyde shows no aggregation, hence it is concluded that it is BSA monomer which resulted in the cytotoxicity to the SH-SY5Y cells (Figure 3b).

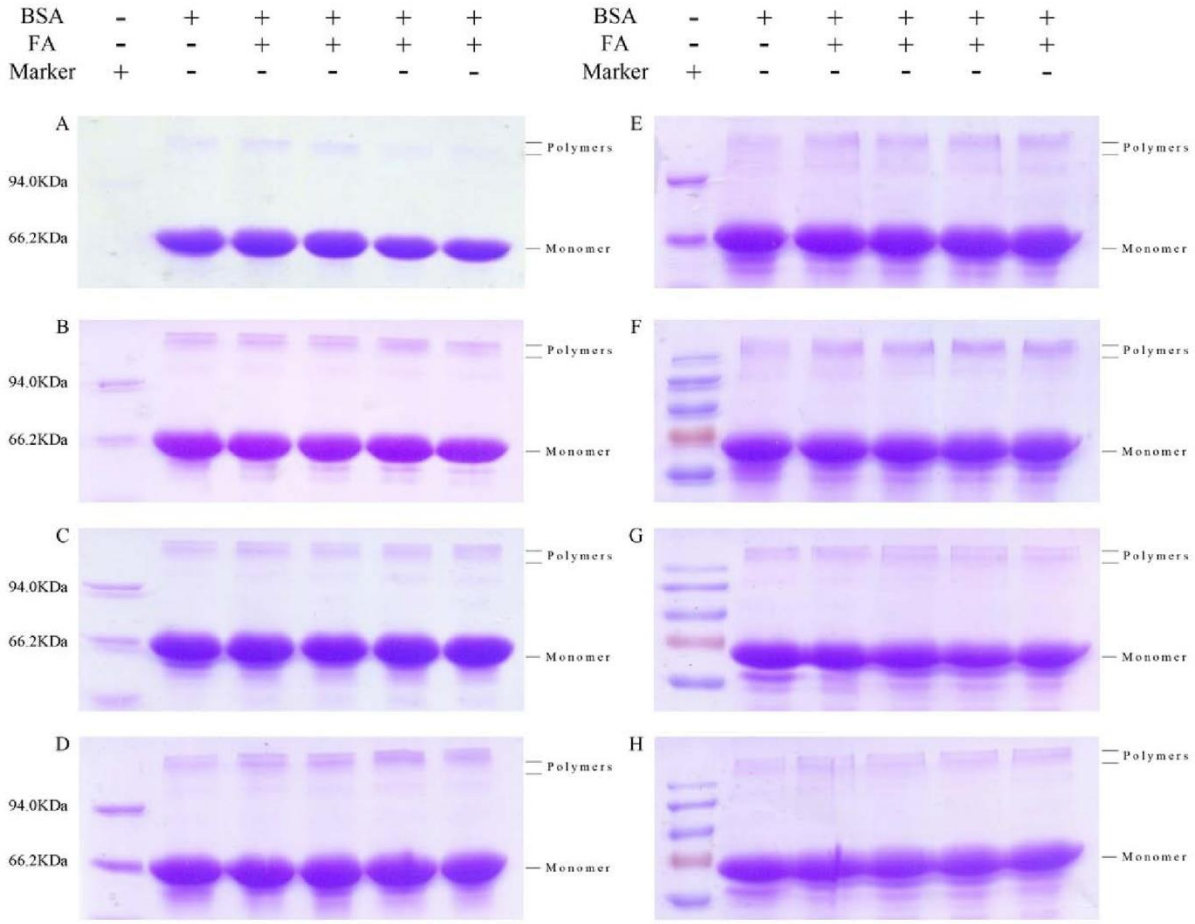

Figure 2a: 10% SDS-PAGE of the products of BSA incubated with different concentrations of formaldehyde for 0-7 days at 37^{0}C. BSA (final concentration 0.01mM) in the presence of different concentrations of formaldehyde (0.25, 0.5, 1 & 2mM). Aliquots were taken for SDS-PAGE electrophoresis. (A-H refers to 0-7 days).

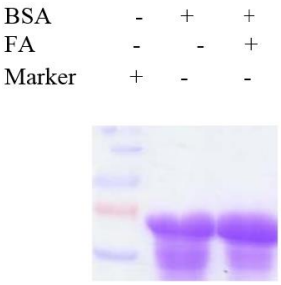

Figure 2b: 10% SDS-PAGE of the product of BSA incubated with formaldehyde for 1 day at 37^{0}C. BSA (final concentration 0.01mM) in the presence of formaldehyde 2mM. Aliquots were taken for SDS-PAGE electrophoresis.

The Native PAGE results also confirm the results of SDS PAGE experiment showing polymer formation in both control and formaldehyde treated BSA (Figure 3c).

To investigate that BSA treated with formaldehyde results in any structural change in BSA, we performed the fluorescence studies at the excitation wave length of 280nm. The results showed that there is significant decrease in the intrinsic fluorescence at 335nm for the samples of BSA treated with formaldehyde as compared to control and this decrease is even more significant at day 7 (Figure 4a and b). In order to further elaborate these changes, we have performed absorbance studies. The results of the absorbance studies also showed a significant decrease in absorbance at 280nm with time incubation but not in the concentration dependent manner (Figure 4c).

Phase contrast microscopy has been done to further elaborate and support the above findings. The results show a significant decrease in the number of viable cells and their morphology in BSA treated with formaldehyde group as compared to the control and BSA alone group (Figure 4a-d).

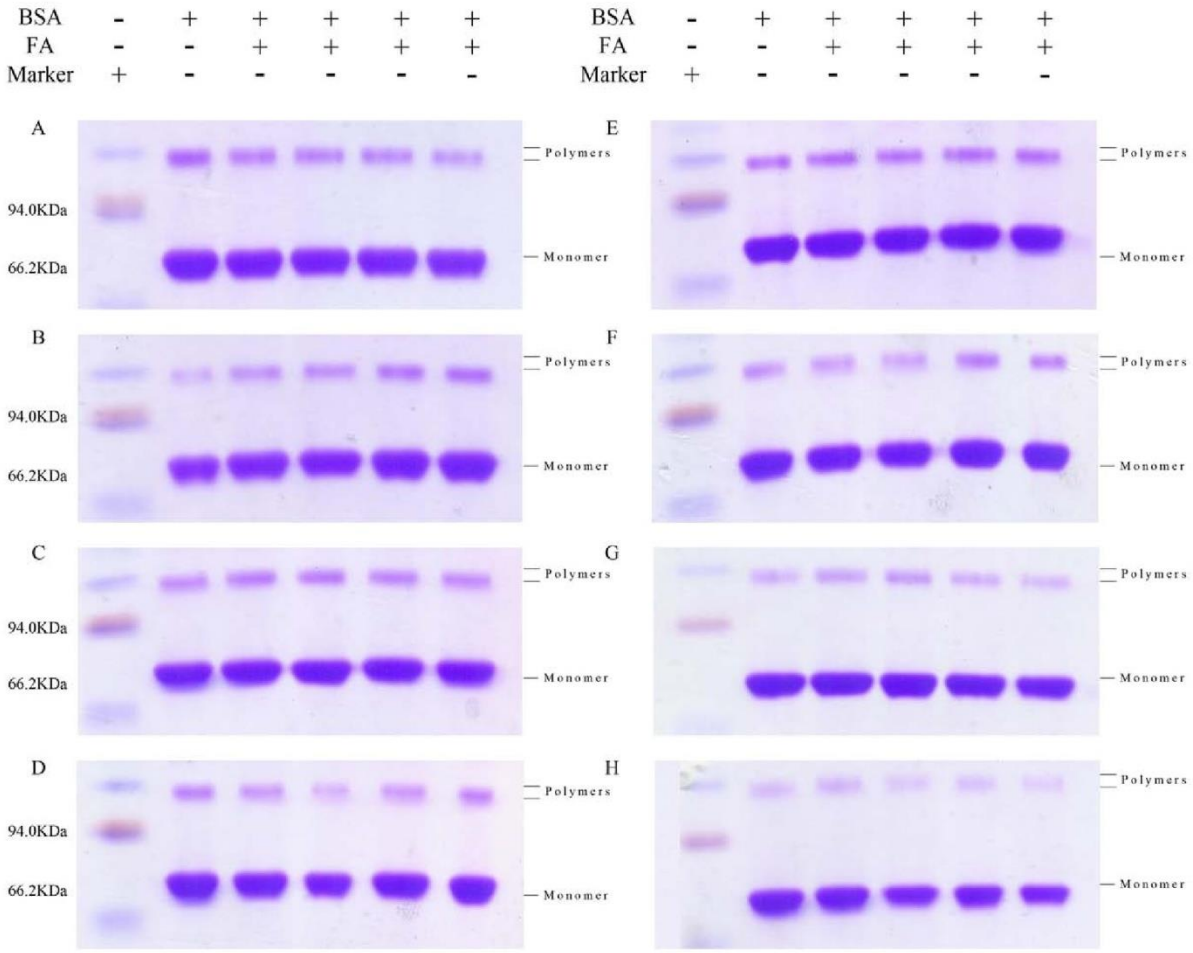

Figure 2c: 10% NATIVE-PAGE of the products of BSA incubated with different concentrations of formaldehyde for 0-7 days at 37^{0}C. BSA (final concentration 0.01mM) in the presence of different concentrations of formaldehyde (0.25, 0.5, 1 & 2mM). Aliquots were taken for NATIVE-PAGE electrophoresis. (A-H refers to 0-7 days).

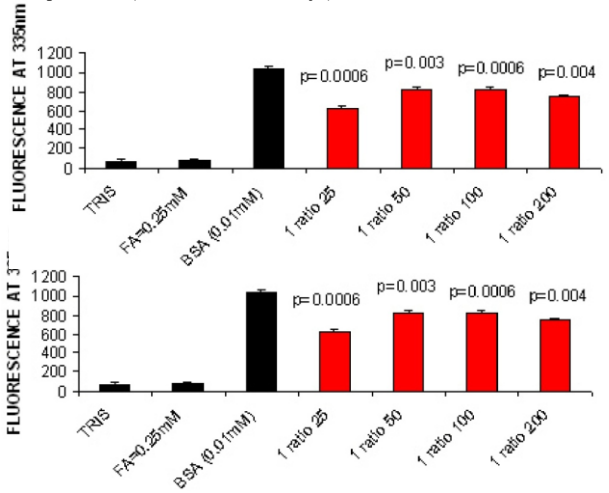

Figure 3 a and b: Time and concentration dependant changes in the fluorescence of BSA treated with formaldehyde. BSA (final concentration 0.01mM) in the presence of different concentrations of formaldehyde (0.25, 05, 1 & 2mM). Aliquots were taken for measurement of fluorescence (λex 280nm; λem 335nm).

CONCLUSIONS

In conclusion, we have demonstrated that BSA monomer in presence of formaldehyde (FA ≥ 0.1mM) results in the cytotoxicity to the neuronal cell lines like SH-SY5Y cells. We concluded that the free amino group of BSA possibly reacted with formaldehyde forming protein adducts which resulted in the cytotoxicity. However, further studies are required to figure out the detailed mechanism of how formaldehyde reacted with BSA and what changes in the structure of BSA resulted in the cytotoxicity.

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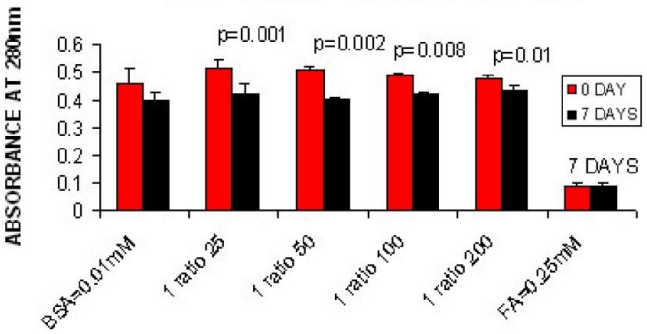

Figure 3c: Time and concentration dependant changes in the absorbance of BSA treated with formaldehyde. BSA (final concentration 0.01mM) in the presence of different concentrations of formaldehyde (0.25, 0.5, 1 & 2mM). Aliquots were taken for measurement of absorbance at 280nm.

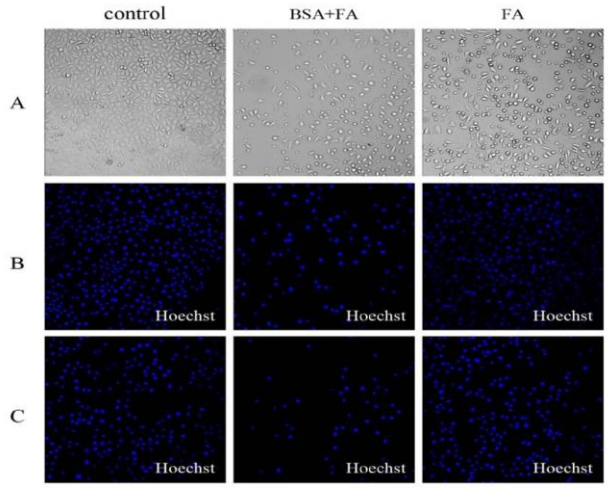

Figure 4A Shows bright field pictures of control (DMEM), FA treated BSA and FA alone (0 day samples). **B** Shows Hoechst staining of control (DMEM), FA treated BSA and FA alone (0 day samples). **C** Shows Hoechst staining of control (DMEM), FA treated BSA and FA alone (7 days samples).

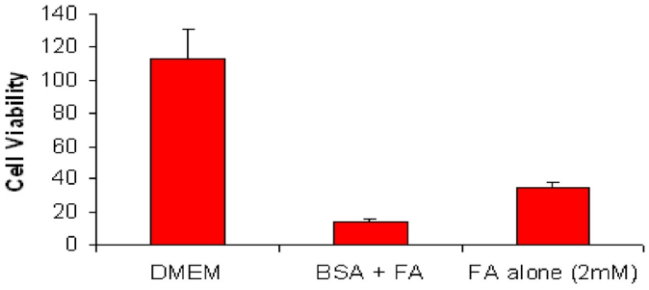

Figure 4D: Shows Cell Viability Count of DMEM, BSA treated with formaldehyde and formaldehyde alone of day 7 sample.

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