**Research Article**

# Dissimilar Response of 2D and 3D Astrocyte Cell Cultures to Protective Effect of Recombinant Human Erythropoietin Against Amyloid-β$_{25-35}$ Toxicity

Gholamhossein Riazi*[1], Hooman Kaghazian[2], Mohammad Reza Lornejad[3], Farzad Mokhtari[4], Saeedeh Farhadinejad[5], Leila Shahriari[6], Nima Allahyari[7]

*[1]Institute of biochemistry and biophysics (IBB), University of Tehran, Iran.
[2]Institute of biochemistry and biophysics (IBB), University of Tehran, Iran.
[3]BioMed-zet Life Science GmbH, Austria.
[4]Institute of biochemistry and biophysics (IBB), University of Tehran, Iran.
[5]Department of Biological sciences, Kharazmi University, Tehran, Iran.
[6]Institute of biochemistry and biophysics (IBB), University of Tehran, Iran.
[7]Yazd University, Yazd, Iran.
*Corresponding author's E-mail: ghriazi@ibb.ut.ac.ir

**ABSTRACT**

Human erythropoietin (Hu-Epo) is a glycoprotein growth factor that involved in regulation of blood erythrocytes population.[1,2] Besides of erythropoiesis, variety of studies have shown that Hu-Epo and its receptor (Hu-EpoR) are expressed in central nervous system(CNS). The protective properties of recombinant form of Hu-Epo (r-Hu-Epo) in different neural cell cultures have been established previously. These protective effects of r-Hu-Epo could play an important role in therapeutic strategies of neurodegenerative disease such as Huntington, Parkinson and Alzheimer's disease.[3-5] Pathological studies indicate that neurodegenerative diseases link to astrocyte cell death in addition to neural degeneration. Recently, astrocytes-specific pathology in neuro degeneration has been clarified. Therefore, protecting astrocyte cells from emergent toxins is important.[6-7] In present study, we investigated the protective effects of erythropoietin in monomer (r-Hu-Epo) and dimer (r-Hu-Epo)$_2$ formson Amyloid-β$_{25-35}$ induced astrocytes toxification in 2D and 3D culture. 3D culture of astrocytes cell led us to investigate a realistic behavior model of natural tissue and a reliable cell response under the toxic conditions. Cell vitality and cytotoxicity in2D and 3D cultures were studied by conventional MTT and LDH assays. Results showed dissimilar response of amyloid-β-induced 2D and 3D cultures through protective effect of r-Hu-Epo and (r-Hu-Epo)$_2$. Both r-Hu-Epo and (r-Hu-Epo)$_2$ increased cell vitality and decreased cell death by 11±3% in 2D cultures compared to 40±5%in 3D cultures. Analysis of cell vitality and toxicity in treated and untreated cells after 24, 48 and 72 hours proposed that elimination of amyloid-β$_{25-35}$ from medium could be considered as possible protection mechanism of these proteins. Interaction of r-Hu-Epo with amyloid-β$_{25-35}$ was studied *in vitro*. Structural changes and formation of amyloid fibrils in presence of r-Hu-Epo and (r-Hu-Epo)$_2$ were measured by circular dichroism and fluorescence. Results showed that dimer form of r-Hu-Epo interacted more than monomer with amyloid-β aggregates.

**Keywords:** Alzheimer's disease, Amyloid-β$_{25-35}$, Astrocytes, Erythropoietin, Neuro degeneration, 3D Culture.

## INTRODUCTION

Human Erythropoietin (Hu-Epo) is a 30 to 40-kDa glycoprotein hormone involved in the erythropoiesis. The primary function of Hu-Epo is erythrocytes concentration conservation at physiological level. Hu-Epo is produced mainly by kidneys and has been purified from patients with aplastic severe anemia.[1-3] In spite of proliferation and differentiation of erythroid precursor cells a wide variety of experimental studies have shown that Hu-Epo and its specific receptor (Hu-EpoR) are expressed in the central nervous system. Some evidence revealed that Hu-Epo can cross the blood-brain barrier; also, the Hu-Epo content of peripheral and central nervous system depend on each other.[4]

Recently, this fact has cleared that Hu-Epo provided remarkable neuro protection properties in both cell and animal models of neurological diseases. The models of hypoxia, ischemia, cerebral hemorrhage and multiple sclerosis have been tested before for investigating of Hu-Epo protection properties.[5-8]

According to Hu-Epoprotection properties, pharmaceutical perspectives of this protein become stronger nowadays and haveled to a sharp increase in production and consumption of r-Hu-Epo. Thus, recombinant form of this glycoprotein is coming up to pass the clinical pharmacological trial phases for new therapeutic drug for neurodegenerative diseases.[2]

Approximately, 40% of r-Hu-Epo weight corresponds to the weight of bounded oligosaccharides. Biological activity and stability of r-Hu-Epo is depending upon oligosaccharide bounded regions. Dimerization of r-Hu-Epo is an undesirable by product that take place in purification process.[4,5,10]

As noted above, the protective role of r-Hu-Epo in central nervous system is crucial. Therefore, new therapeutic strategies in neurodegenerative diseases would be proposed. Neural protection depended on r-Hu-Epo occurred in the brain through a series of cellular pathways. Ability of Hu-Epo to regulate Janus tyrosine kinase 2 (JAK2), protein kinase B, glycogen synthase kinase 3b (GSK-3b) and many cellular factors preventing

inflammatory activation and ultimately leads to cellular protection.[9]

On the other hand, the importance of astrocytes-specific pathology in neurodegenerative disease and the essential roles of these cells have been extensively studied. Numerous studies have tried to find novel therapeutic targets for neurodegenerative disorders. Astrocyte cell death may cause impairment of glutamate transporters and many destructive effects on neurons. Some consequent effects of astrocyte cell death and dysfunctions are: up-regulation of glial fibrillary acidic protein, releasing of glutathione and mutant superoxide dismutase1 and also altering the amount of ATP released and calcium signaling in astrocytes. Undesirable calcium signaling causes by amyloid-β toxicity in Alzheimer's disease. The importance of astrocytes is known for their housekeeping functions in neural environment, including neuronal metabolism, regulating cerebral blood flow, maintaining synaptic function and contributing in neural communication. Understanding these functions has revealed that astrocyte cell population shortage has driven researchers to study on protection of astrocyte in neurodegenerative diseases.[13-14]

One of the main disrupting molecules that led to astrocyte cell death is Amyloid-βaggregates particularly, in Alzheimer's disease. Amyloid-βmotivates disruptive processes through the altering calcium signaling via interacting to gap junctions and increasing ATP released from astrocytes. Amyloid-β is the major component of senile plaques (SPs) and considered to have an important role in incidence and progress of Alzheimer's disease. Although the mechanism of Amyloid-βneurotoxicity remains obscure but, protection of brain neurons and glia cells from Amyloid-β toxicity considered as an important strategy in AD curing.[8]Therefore, molecules which may have a protective role for astrocytes toxification of amyloid-β structures would be important candidate for Alzheimer's diseases therapeutic research.

Neuro protective effect of r-Hu-Epo against Amyloid-β induced neural cell apoptosis in primary hippocampal culture has been studied before.[5]Although the neuro protective effects of r-Hu-Epo have been described in different models of neural cells *in situ*, there is no enough evidence regarding the protective value of r-Hu-Epo on astrocyte cells.

In this research we investigated the protective effect of r-Hu-Epo and (r-Hu-Epo)$_2$ on Amyloid-β$_{25-35}$induced primary astrocytes in 2D and 3D cell cultures *in situ* and *in vitro*. Based on pharmaceutical approaches, two types of recombinant Epo namely, r-Hu-Epo and (r-Hu-Epo)$_2$ were selected for this study. We applied Amyloid-β$_{25-35}$ to induce cell death and apoptosis to astrocytes which poses functional domain of Amyloid-β for toxic effect. This peptide is highly cytotoxic to neural cell and is widely used in both *in vitro* and *in vivo* neuroscience research. It is obvious that 3D cultures of primary cell would show

more realistic behavior that would be close to apparent natural response under the experimental conditions.

## MATERIALS AND METHODS

### Recombinant r-Hu-Epo Expression

### celllines

pKepo/pSV2dhfr-Infected Chinese hamster ovary (CHO) cells obtain from Pasture Institute of IRAN. CHO cells were cultured in DMEM medium, Gibco, (α-nucleosides10% FBS 100 Units/mL, penicillin 2 mM, L-glutamine2 mM) at 37° C in 5% $CO_2$. In growing stage, cells were treated with trypsin and placed in 60 mm tissue culture plates (Nunc, Denmark).After 24 h, cells were transfected by using the precipitation of DNA with $Ca_3(PO4)_2$. The pKepo and pSV2dhfr plasmids transferred CHO cells with the proportion 9:1. These plasmids contained information of Hu-Epo and murine dihydro pholate reductase enzyme under the control of SV40 virus promoter respectively. Cells after 48 h were treated again with trypsin and cultivated in the same medium and supplements but without nucleosides. From this pool of cells, a dilution method was performed to isolate individual cells that produce r-Hu-Epo. Cells allowed to forming a monolayer. Formation of this monolayer was observed after 5 days. Then bottles were incubated with trypsin in order to remove cells from the flask surface and cells were harvested in the adequate recipient to be inoculated in the next step of the process. After two cell expansions, cells were inoculated in10rotation bottles with the volume of 1062cm$^2$with 5% fetal bovine serum, at 37° C for 5 days more. The harvests were performed with the DMEM supplemented with insulin and three recuperation cycles were done with 5% FBS (DMEM/Gentamycin (20 mg/ml) Gibco,/sodium bicarbonate (1 g/l)). Finally, 0.01% Tween 20 was added to harvests and filtered for eliminating cellular debris and stored at 4 $^O$C until releasing.[11]

### Size-exclusion Chromatography

A chromatographic column BPG 300/950 was packed with 56.5 ml of Sephadex-G25 coarse (Pharmacia) for buffer exchange. The mobile phase used was 50mMNaAc/0.01% Tween 20, pH 4.5. 30% of column volume applied with the supernatants of cell cultures. This column was always operated at30 m/h as linear flow rate and the absorbance was measured at 280 nm.

### Ion Exchange Chromatography

A BPG 300/500 column packed with 10.5ml of Q-Sepharose Fast Flow (Pharmacia) was equilibrated with 50mMNaAc/0.01% Tween 20, pH 4.5. Previous evaluation from size-exclusion chromatography were applied to the column at room temperature, which was subsequently washed with 50mMNaAc/0.01% Tween 20. pH 4.0 to eliminate basic isoforms of r-Hu-EPO. Elution was performed using 50mMNaAc/75mMNaCl, pH 5.0 and absorbance was measured at 280 nm. The flow rate used during the whole chromatography was 100 m/h.

## Size Exclusion high Performance Liquid Chromatography

An HPLC-GF column TSK G3000 PW (5.5 mm/60 mm) was used for eliminating aggregates of ther-Hu-Epo and to separate monomers and dimmers of the r-Hu-Epo. The chromatographic mobile phase employed was 150 mM PBS, pH 7.0. The working volumetric flow rate used was 0.24 mL/ min and the sample volume applied was 85 mL in each chromatographic cycle.

## r-Hu-Epo Peptide Mapping

Peptide mapping with RP-HPLC (Column C18 (Vydac 4.6 x 250 mm, 10 µm) , used for confirming the primary structure accuracy of purified r-Hu-Epo, the protein hydrolysis enzymatic with Trypsin solution (Dissolve 20 µg in 100 µL of enzyme buffer (acetic acid 2%) and incubate at $30^{\circ}C$ during 15 minutes. Dissolve the standard Epo STD NIBSC code: 11/170 in 50 µl of reaction buffer (0.1 M Tris/Acetic pH 8), to obtain a protein concentration of 1 mg/mL.then5µl of trypsin (1µg) added. The ratio trypsin/EPO is 1:50.RP-HPLC Solution A was 1 mL of TFA in 1 L of Milli Q water and RP-HPLC Solution B was 0.5 mL of TFA in 1 L of Acetonitrile and the elution was measured with a UV detector.[11,12]

## Primary Cultures of Astrocytes

Fetal human astrocytes originally obtained from BONYAKHTEH Lab in Tehran. All cells were cultured in 160 mL sterile flask with DMEM medium with 10 % Fetal Bovine Serum (FBS). The medium was changed every two days, and cells were plated at an appropriate density. Expanded astrocytes stayed in culture for 11 days to reach confocal shape. Following shaking, the resulting cultures contained more than 90% astrocytes. The medium was removed and 1ml trypsin–EDTA solution was added to each flask for 5 min at 37∘C and 5% $CO_2$flow. The trypsin was inactivated by the addition of 1 ml FBS/flask supplemented with penicillin/streptomycin and 10% FBS. The cells were centrifuged at 300Xg for 4 minutes. Next, the cells were counted by using a haemocytometer. Then cells rinsed with sterile PB to remove any trypsin.[16]

For 3D astrocyte cell culture system, 12 Well cell culture insert (ThinCert[TM], Greiner Bio-One) with translucent membranes (polyester, 0.4 µm pore size) used. Cells were seeded at a density of 1.5 million cells/ml. For Trypsin inactivation, the Membrane–DMEM mixture was gently mixed with the cell suspension and transferred to 12-well plates before placing at 37∘Cto set 5% CO2 flow.

## Amyloid Peptide Preparation and Cell Treatments

Amyloid-$\beta_{25-35}$ (SigmaCAS No.131602-53-4) was dissolved in de ionized distilled water at concentration of 2mM/mL. The stock solution was diluted to desired concentrations before use. For production toxic form of aggregated A$\beta_{25-35}$ the solutions (0.5, 5 and 50 µM) incubated in $37^{\circ}C$ with shaking for 72 hours, then added to mediums of cell culture. For study the effects of r-Hu-Epo the mediums has been prepared in presence and absence of r-Hu-Epo and (r-Hu-Epo)$_2$in 24,48 and 72 hours. [15]

## Cell vitality and Cytotoxicity

Cell vitality was determined using the conventional MTT reduction assay. 96-multiwell plates were seeded at 10,000 cells/well for 2D culture and 60,000 cell/well for 3D culture. Cells were serum-starved for 4 h, and then were pre-incubated with0.2 µg/ml about 6µMr-Hu-Epo, and (r-Hu-Epo)$_2$ for 24,48 and 72 h before adding A$\beta_{25-35}$(50µM). After 24, 48 and 72 hours cells were incubated with 5mg/mL MTT for 4 h at 37 ∘C. Next, the medium was removed and the cells were dissolved with 100 µL dimethyl sulfoxide with 15min shaking. The formazan reduction product was measured by reading absorbance at 570 nm in a plate reader. For measuring cell lysis and toxicity, we used LDH (Lactate dehydrogenase) assay, the cell culture supernatant were centrifuged at 300Xg for 3 min then pellet removed and 10µL of (1:4 NADH/Pyruvate) added to 100 µl of supernatant the incubated in $37^{\circ}C$ for 1 minute and measured the density of 340nm for in duration of 10 minute with plate reader.

## Amyloid Fibrillation Detection with Fluorescence

Thioflavin T (ThT), is a dye that binds to amyloid structures, we used this dye for quantification ofamyloid-$\beta_{25-35}$fibrils.The Amyloid-$\beta_{25-35}$samples, (50µM, pH7.4) were incubated in 37 $^{\circ}C$ with shaking for 72 hours in presence and absence 0.2 µg/ml of r-Hu-Epo and (r-Hu-Epo)$_2$. Then, 30µl of ThT was added to 200µl of each sample. Next, Fluorescence intensity was monitored immediately sing a Varian-spectrofluorometer (Model: Cary Eclipse) with the excitation and emission wavelengths of 440 and 490 nm, respectively.

## Circular Dichroism

Circular dichroism (CD) spectroscopy used for investigating the secondary structure changes of Amyloid-$\beta_{25-35}$ aggregation in presence and absence of r-Hu-Epo and (r-Hu-Epo)$_2$. CD spectra were recorded on an Aviv model 215 Spectro polarimeter (Lakewood, NJ, USA). Measurements were performed in the spectral range of 195–260 nm using a quartz cuvette (Helma) with a path length of 1 mm and a scan rate of 20 nm/min. This test was performed at 50µM Amyloid-$\beta_{25-35}$and0.2 µg/mlr-Hu-Epo concentration. A composite buffer containing 10 mM PB at pH 7.4 was prepared and its contribution was subtracted in the CD spectra of A$\beta_{25-35}$, r-Hu-Epo and (r-Hu-Epo)$_2$ to give a normalized spectrum. Spectra were converted from machine units in milli degrees to delta epsilons.

## RESULTS AND DISCUSSION

### r-Hu-Epo Expression and Purification

Previous studies had shown that active r-Hu-Epo produced in mammalian cells need glycosylation. So, in this study we used CHO cells with pKepo infected vector that contained the information of Human erythropoietin.

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At first place expression of Hu-Epo gene in CHO hosts was tested by loading the cultured CHO lysate on 12%SDS-PAGE.Figure.1-a, shows that CHO cell lysate electrophoresis and confirmed a considerable dense bond in 30-40 kD. Next, purification procedures were conducted by three chromatographic steps as described in material and methods section. Columns used for purification were size exclusion (BPG 300/950), ion exchange (BPG 300/500) and HPLC-GF(TSK G3000 PW) respectively. All columns had the same recovery yield of target proteins and the elution detection of r-Hu-Epo and (r-Hu-Epo)$_2$ were conducted by SDS-PAGE and UV spectroscopy (Figure 1-b-c). The purified Monomer r-Hu-Epo elute after 120min from HPLC-GF (TSK G3000 PW) column. Aggregate and dimer form of r-Hu-Epo were eluted earlier (Figure 1-c). Finally, the structure of expressed r-Hu-Epo confirmed by peptide mapping technique.Figure.1-d-e shows that peptide maps of purified r-Hu-Epo from the final chromatography column compared to standard r-Hu-Epo (NIBSC code: 11/170). The concentration of purified r-Hu-Epo was measured by Bradford test and 280nm spectroscopy. Achieved concentration of r-Hu-Epo and (r-Hu-Epo)$_2$ were 0.38 mg/ml with extinction coefficient22,600 M$^{-1}$ cm$^{-1}$.

## Astrocytes Cultures and Toxification by Amyliod-β$_{25-35}$

After expanding the confocal astrocytes for 11 days, both 3D (translucent insert) and 2D cultures were detected by Axiovert 25 inverted microscopy. The images showed the most cultured cells after 24, 48 and 72 h had the morphology of natural astrocytes, according to the astrocyte morphology that previously tested by *Vitali Matyashet al.(2010)*.[16] (Figure 2-b-c-d)

To obtain an appropriate concentration for toxic effect of Amyloid-β$_{25-35}$, we characterized the response of confocal astrocyte cells to 0.5, 5 and 50 μM concentration of Amyloid-β$_{25-35}$. Astrocyte cell vitality and toxicity were measured by using MTT reduction method.[5] in first 24 hrs in 2D and 3D cultures. Results showed that exposure of 50 μM amyloid-β$_{25-35}$significantly, reduced cell vitality by 50.5±8 % compared to control cells in both culture systems (P <0.01)(Figure2- a). The results led us to select 50 μM of Amyloid-β$_{25-35}$ for making toxic sample in next tests.
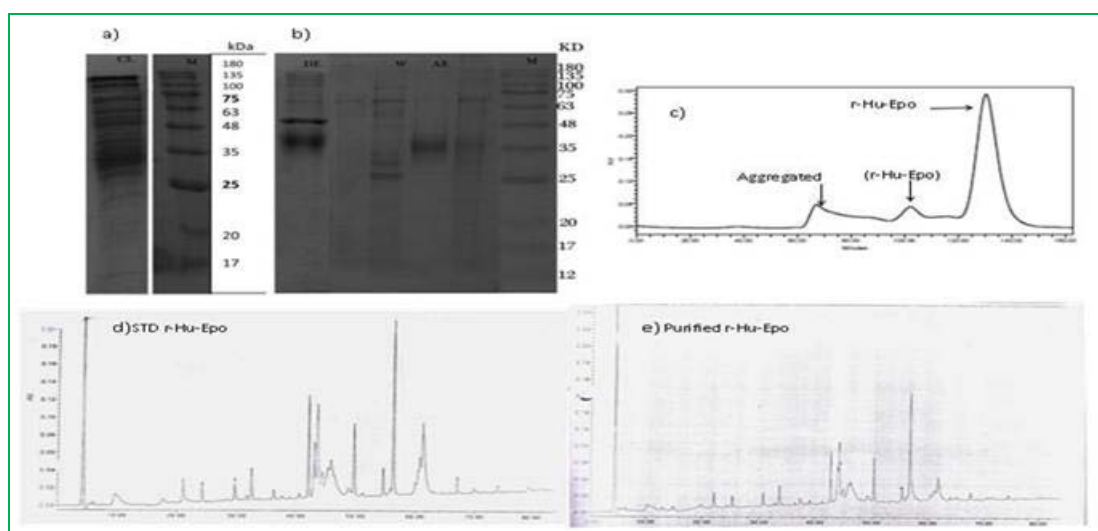


**Figure 1:** r-Hu-Epo expression and purification; Expression of Hu-Epo vectors (pKepo) in CHO hosts tested with 12% SDS-PAGE. a) M show the marker and (CL) indicated CHO cell lysate.b) (M) Molecular weight Marker. (AE) elution from the last HPLC-GF column TSK G3000 PW.W (washing) and DE second eluted proteins that show the dimer of r-Hu-Epo. C) The chromatogram of HPLC-GF column TSK G3000 PW detection for r-Hu-Epo and (r-Hu-Epo)$_2$ with UV spectroscopy. D) Peptide mapping of standard Epo STD NIBSC code: 11/170 and Ph. Eur. Reference e) Peptide mapping of our purified r-Hu-Epo.

**Table 1:** 2D and 3D astrocytes cell culture response to different conditions

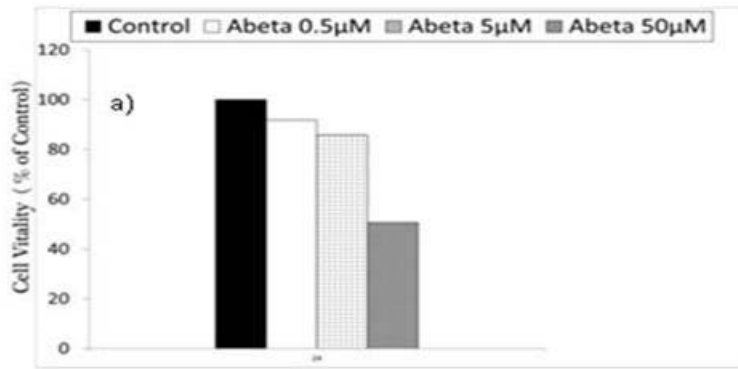| | | 2D culture | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Time interval | Expose to | | | | |
| | | Aβ$_{25-35}$ | r-Hu-Epo | (r-Hu-Epo)$_2$ | r-Hu-Epo +Abeta25-35 | (r-Hu-Epo)$_2$ + Abeta$_{25-35}$ |
| Cell Vitality In contrast to Control | 24 h | 54±3% | 72±2% | 75±3% | 63±3% | 61±4% |
| | 48 h | 52±4% | 82±8% | 70±5% | 65±4% | 67±3% |
| | 72 h | 60±1% | 85±5% | 72±4% | 55±5% | 50±5% |
| | 3D culture | | | | | |
| % Cell Vitality In contrast to Control | 24 h | 50±4% | 90±4% | 100±2% | 97±3% | 87±4% |
| | 48 h | 65±3% | 85±4% | 86±4% | 88±4% | 105±4% |
| | 72 h | 80±4% | 115±3% | 110±4% | 74±3% | 100±4% |

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**Figure 2)Amyloid-β$_{25-35}$ toxicity**
Astrocyte cell vitality by exposure to 0.5, 5 and 50 µM of Amyloid-β$_{25-35}$ measured by MTT reduction method in first 24 h of in 2D cultures. Data were expressed as the percentage of values in untreated control cells and each value represent the mean ± S.D. (n = 3)(a). Visualization of 2D cultured astrocytes in control groups (CNTL) in 24,48 and 72 h (b,c,d,)and in toxic groups (e,f,g) apoptotic cells showed with arrows
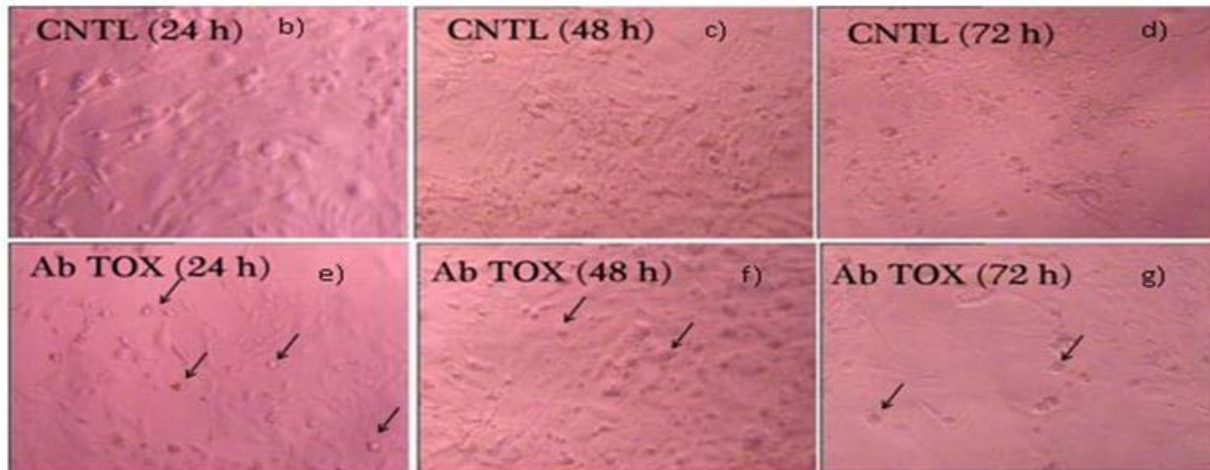
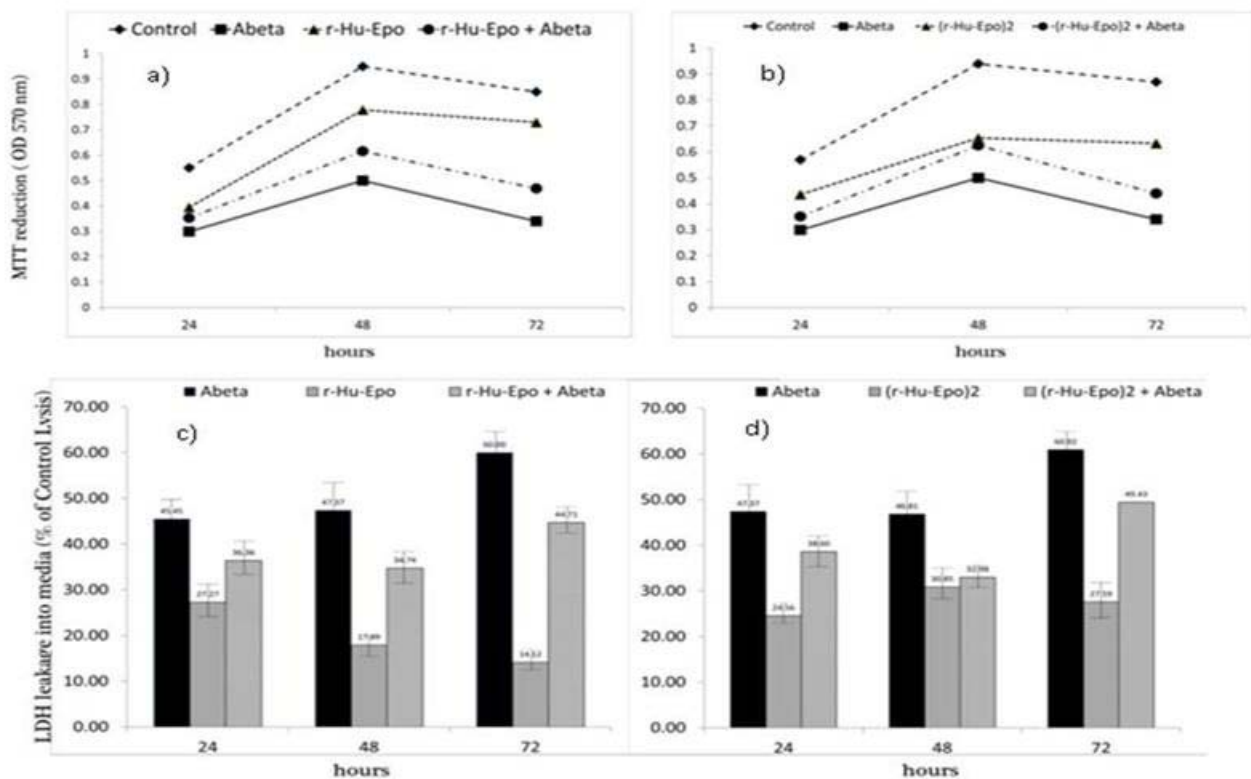**Figure 2:** Amyloid-β$_{25-35}$ toxicity



**Figure 3:** r-Hu-Epo and (r-Hu-Epo)$_2$ protection pattern in 2D astrocytes cell culture; Cell vitality and toxicity of 2D cultures in control, Amyloid-β25-35 (50µM) , r-HU-Epo and r-Hu-Epo (0.2µg/mL)+ Amyloid-β25-35 groups at intervals of 24,48 and 72 h was determined using MTT (a, b) and LDH (c, d) assays. The data in LDH were expressed as the percentage of values in untreated control cells and each value represent the mean ± S.D. (n = 3). In the case of the LDH release assay. Significantly different from Aβ25–35 control (P < 0.01).
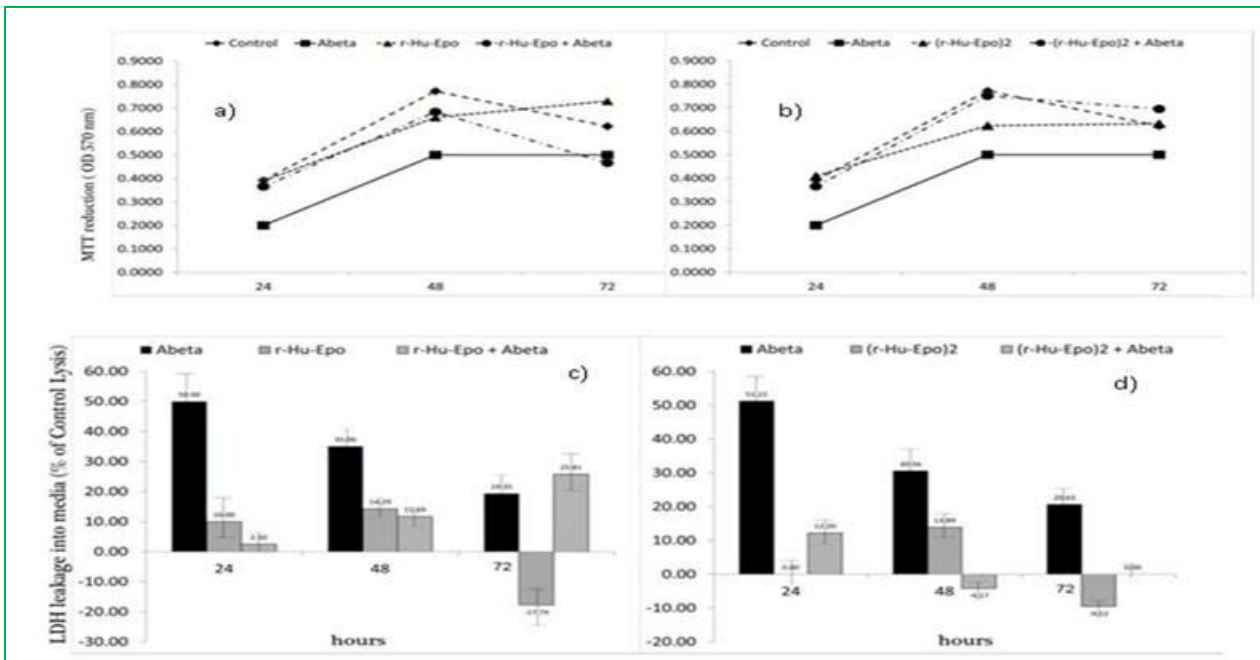
**Figure 4:** r-Hu-Epo and (r-Hu-Epo)$_2$ protection pattern in 3D astrocytes cell culture; Cell vitality of 2D cultures in control, Amyloid-β25-35 (50μM) , r-HU-Epo and r-Hu-Epo (0.2µg/mL)+ Amyloid-β25-35 groups at intervals of 24,48 and 72 h was determined using MTT (a, b) and LDH (c, d) assays. The data in LDH were expressed as the percentage of values in untreated control cells and each value represent the mean ± S.D. (n = 3). In the case of the LDH release assay. Significantly different from Aβ25–35 control (P < 0.01).
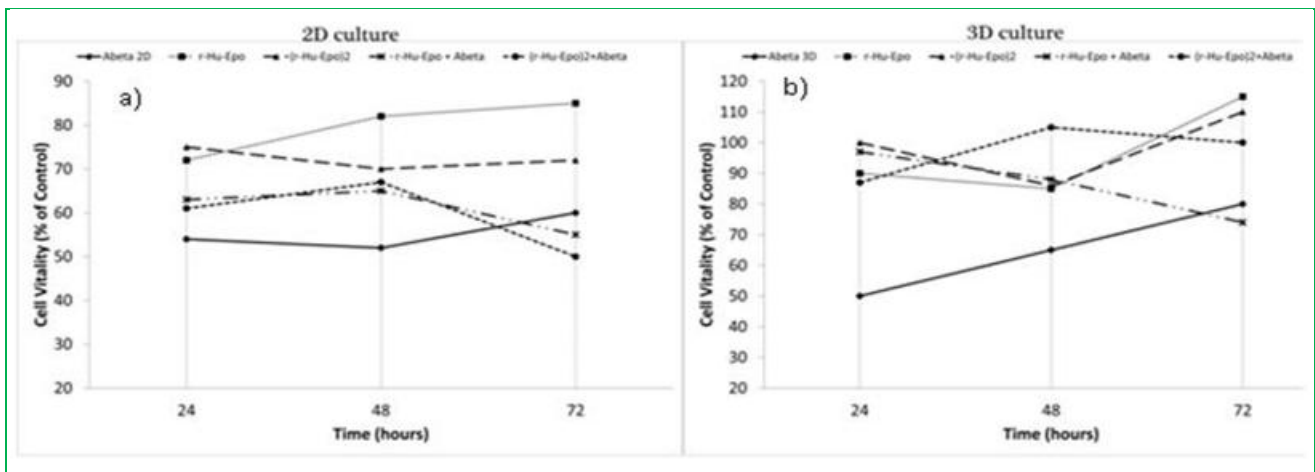


**Figure 5:** r-Hu-Epo and (r-Hu-Epo)$_2$ protection pattern in 3D astrocytes cell culture; Cell vitality rate of control, Amyloid-β$_{25-35}$, r-Hu-Epo and r-Hu-Epo+ Amyloid-β$_{25-35}$ groups after 24,48 and 72 h in 2D cultures(a) and 3D cultures (b)
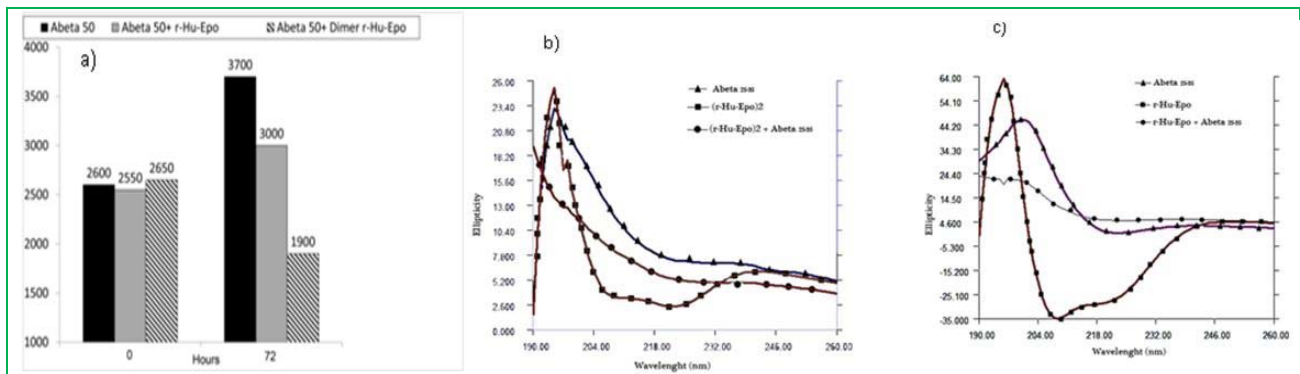


**Figure 6:** Florescence and Far-UV CD spectra of Amyloid-β, r-Hu-Epo and (r-Hu-Epo)$_2$; Amyloid-β 25-35 fibril formation measured by ThT fluorescence. Amyloid-β 25-35 samples (50 µM) incubated in time intervals t$_0$ and after 72 hours thenThT solution was added (a). In Far-UV CD spectra samples at t$_0$ and after a 72 h after incubation measured in expose to r-Hu-Epo (b) and (r-Hu-Epo)$_2$ (c). All the samples were measured at a concentration of 50µM Amyloid-β$_{25-35}$ and 0.2µg/mL of r-Hu-Epo and (r-Hu-Epo)$_2$. Data represent the mean ± S.D. (n = 3).

For confirming MTT results were viewed the microscopic images of 2D culture. The microscopic images showed, reduction of astrocytes population in 24hours after exposure to amyloid-$\beta_{25-35}$.Meanwhile, apoptotic-like cells were characterized by the appearance of rounded shape cells in compare to cells in control group. (Figure 2 e-f-g) In control group apoptotic shape cells were composed 10% of total number of normal cells, however in toxic group, apoptotic like cells increased to more than 60% of the total number of the cells (P < 0.01) in first 24 hours. (Figure 2 e-f-g)

### Protective Effects of r-Hu-Epo and(r-Hu-Epo) 2 on Amyloid-β25–35-induced Cell Vitality

Protective properties of r-Hu-Epo and (r-Hu-Epo)$_2$ in both 2D and 3D cell culture systems were examined separately. After reaching maturity after 11 days and 21 days for 2D and 3D cultures respectively, cells transferred to 96well plate and treated by 0.2 µg/mL of r-HU-Epo and (r-Hu-Epo)$_2$ separately. Next, after 24, 48 and 72h intervals each sample was exposed to amyloid-$\beta_{25-35}$aggregates. The cell vitality and the content of LDH released in medium were measured by MTT reduction and LDH assays.

Results revealed that cell death was prevented by the addition of both r-Hu-Epo and (r-Hu-Epo)$_2$ in 2D and 3D cultures(P < 0.01)(Figure.3- a-b-c-d). Interestingly, more accurate results showed unlike response of 2D and 3D cultures to protective effect of r-Hu-Epo and (r-Hu-Epo)$_2$.

### r-Hu-Epo and (r-Hu-Epo)$_2$ protection pattern in 2D astrocytes cell culture

As shown in Figure.3-a-c, when astrocytes cells were exposed to Amyloid-$\beta_{25-35}$, the cell death was increased 11±3% in compare to toxic groups after 24 hrs. The diagrams indicate that the amyloid-βprevent the cell growth in first 48 hours and later caused cell death. (Figure.3- a-b-c-d).Results indicated that both r-Hu-Epo and (r-Hu-Epo)$_2$ slightly, increased the cell vitality in contrast to toxicated groups. The observed protective effect was about 11±3% in all time intervals. Protective effect and increasing in cell vitality didn't change after 48 and 72 hours notably (Figure 3 and Table 1).

Changing in rate of cell death in (r-Hu-Epo)$_2$ treated 2D cultures after 48 hours is another significant point of MTT diagrams. This results showed that (r-Hu-Epo)$_2$ decreased the cell death with different rate compare to the rate of control and toxicated groups. Changing the rate of cell population can introduce different mechanism (Figure.3 a-b). Slope)decline in cell treated by (r-Hu-Epo)$_2$ compare to r-Hu-Epo is an indication of toxin elimination from cultured media (Figure 5-a-b).

### r-Hu-Epo and (r-Hu-Epo)$_2$protection pattern in 3D astrocytes cell culture

At first glance, dissimilarity was observed in r-Hu-Epo and (r-Hu-Epo)$_2$ treated astrocytes in 3D and 2D cultures. 3D cultures had more than four times cell vitality comparing to 2D culture. The results of MTT and LDH assays

indicated that about 40±7% increase in vital cells compared to toxicated groups in 2D cultures (P < 0.01)(Figure 4 and Table 1).

We studied on cell vitality slopes change in all stages. Some stages that nominate in figure.5showed that the rate changes. Toxin elimination by protective molecules could be a proper reason for changing the cell vitality slopes (Figure 5). We investigated the probable interaction of r-Hu-Epo and (r-Hu-Epo)$_2$ as a protective molecule with Amyloid-$\beta_{25-35}$ aggregates as toxins.

We tried to study the possible interaction of r-Hu-Epo and (r-Hu-Epo)$_2$ with Amyloid-$\beta_{25-35}$ aggregates.

### In-vitro Studies of Amyloid-β Aggregation in Presence of r-Hu-Epo and (r-Hu-Epo)$_2$

#### Fluorescence and Circular Dichroism

To examine the interaction of r-Hu-Epo and (r-Hu-Epo)$_2$ with Amyloid-$\beta_{25-35}$aggregates,Amyloid-$\beta_{25-35}$aggregates was tested in 72 hours with and without r-Hu-Epo and (r-Hu-Epo)$_2$. Aggregations were measured with florescence by use of ThT as dye that interact with beta amyloid aggregate structures. As figure.6-a shows the amyloid-β aggregates in presence of (r-Hu-Epo)$_2$reduced significantly in compare to r-Hu-Epo which revealed that (r-Hu-Epo)$_2$ could inhibit the Amyloid-βaggregation (Figure 6-a). To study the structural change of Amyloid-β aggregates in presence of (r-Hu-Epo)$_2$ the structural changes investigated by circular dichroism. As shown in Figure.6-b-c, while Amyloid-βwas exposed to (r-Hu-Epo)$_2$, the intensity of elipcity of aggregates forms was significantly reduced after 72 hours. Therefore, the results revealed an interaction between Amyloid-$\beta_{25-35}$ aggregates and (r-Hu-Epo)$_2$ might have been occurred.

Previous studies have shown that r-Hu-Epo protects neurons against Amyloid-β aggregates induced oxidative stress, mitochondrial dysfunction, and neurotoxicity (Li et al., 2008). In the present study, we showed the protective effect of r-Hu-Epo and its dimer on astrocytes cells. Our findings indicated that r-Hu-Epo and its dimer can rescue astrocyte cells from Amyloid-β induced apoptosis. Furthermore, we found that in addition of signaling pathways which activated in presence of r-Hu-Epo, possible interaction of (r-Hu-Epo)$_2$ with amyloid-βaggregates could eliminate toxic forms of amyloid-β and might be another essential mechanism responsible for protection.

### CONCLUSION

It is now clear that erythropoietin possesses biological activities in addition to the erythropoietic effects. Many cell types such as brain cells have been demonstrated to produce Epo and its receptor Epo-R. Previous studies showed that astrocytes produce Epo in response to many stresses and could protect neuronal cells from this condition.[7,8,17]In the present study, we have provided evidence of protective effect of r-Hu-Epo and its dimer (r-Hu-Epo)$_2$ on Amyloid-$\beta_{25-35}$induced primary astrocytes in

2D and 3D cell cultures *in situ* and *in vitro*. Different responses of 3D culture showed protective effect of r-Hu-Epo and (r-Hu-Epo)$_2$ depending on cellular interaction. Different concentrations of oxygen, nutrients, and signaling molecules, and to other environmental factors could effect on the cell response to toxicity. Besides of signaling pathway activation, byr-Hu-Epo, the direct interaction between (r-Hu-Epo)$_2$ with Amyloid-βmaybe reduce the toxicity of Amyloid-βaggregates. The structural change in Amyloid-β$_{25-35}$ aggregates may be another protective mechanism of erythropoietin in neurodegenerative diseases.

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