Effect of homocysteine on survival of human glial cells

Henrieta Škovierová[‡], Silvia Mahmood, Eva Blahovcová, Jozef Hatok, Ján Lehotský, Radovan Murín[‡]*

Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Mala hora 4, 036 01 Martin, Slovakia

*Corresponding author: Radovan Murín**,** Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Medical Biochemistry, Mala hora 4, 036 01 Martin, Slovakia

Tel.: +421 43 2633 407

e-mail:murin@jfmed.uniba.sk

[‡] These authors contributed equally

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Abstract

Several neurodegenerative conditions, such as Alzheimer's disease and Parkinson's disease, or vascular dementia and cognitive impairment, are associated with mild hyperhomocysteinemia. Hyperhomocysteinemia is defined as an increase of the homocysteine (Hcy) level beyond 10 μM. Although the adverse effect of Hcy on neurons is well documented, knowledge about the impact of this amino acid on glial cells is missing. Therefore, with the aim to evaluate the neurotoxic properties of Hcy on glial cells, we used a glioblastoma cell line as a study model. The viability of cells was assayed biochemically and cytologically. At a concentration around 50 μM in the culture medium D,L-Hcy induced cell death. It is noteworthy that Hcy induces cell death of human glial cells at concentrations encountered during mild hyperhomocysteinemia. Therefore, we propose that Hcy-induced impairment of neuronal functions along with damage of glial cells may contribute to the etiopathogenesis of neurodegenerative diseases associated with hyperhomocysteinemia.

Introduction

Hyperhomocysteinemia is a condition characterized by an elevation of the concentration of L-homocysteine (Hcy) in plasma above its physiological level, which varies in range between 5 − 15 μM (mean 10 μM; Kang et al. 1992). Even ~~moderate~~ mild hyperhomocysteinemia is considered as a risk factor for several neurodegenerative diseases (Mattson and Shea 2003; Neeman et al. 2005; Sachdev 2005; Herrmann and Obeid 2011), such as Alzheimer's (Clarke et al. 1998) and Parkinson's diseases (Allain et al. 1995; Kuhn et al. 1998), dementias (Gottfries et al. 1998, Seshadri et al. 2002), and brain atrophy (den Heijer et al. 2002, Sachdev et al. 2002). Under these conditions the amount of Hcy in blood, serum and cerebrospinal fluid reaches a range of concentrations in the tens of micromolar. The mechanism by which Hcy may exert its effects on brain cells is not fully understood (Obeid and Herrmann 2006). An early theory on the role of Hcy in neurodegeneration suggested an indirect effect of Hcy on brain tissue mediated via its capability to affect the properties of blood vessels and therefore to influence the exchange of compounds between blood stream and brain parenchyma. Several recent studies performed on cultured neural cells revealed that Hcy can also directly influence neural cells by several distinct mechanisms (Obeid and Herrmann 2006). Hcy is considered to exert an excitotoxic influence on neurons due to its role as a glutamate agonist acting on both the ionotropic (Olney et al. 1987; Lipton et al. 1997) and the metabotropic (Folbergrova et al. 2001, Zieminska et al. 2003, Lazarewicz et al. 2003) type of glutamate receptor, with an effect on intracellular calcium signaling. In addition, Hcy enhances formation of free radicals (Welch et al. 1997) and promotes oxidative stress (Streck et al. 2003).

Glial cells are the most numerous cellular constituent of brain parenchyma. They are heavily involved in sustaining physiological roles of this tissue (Allen and Barres 2009). In addition,

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they are considered to play an active role in the etiopathogenesis of neurodegenerative diseases (Vila et al. 2001; Teismann et al. 2003; Rodriguez et al. 2009). While the neurotoxic effect of Hcy on neuronal cells has been studied intensively, only a few data exists concerning its effect on glia. It has been shown that at millimolar concentration this amino acid impacts the survival of cultured rat astroglial cells (Maler et al. 2003). This concentration exceeds by more than one order of magnitude its estimated levels in elderly with progressing neurodegenerative disease. When applied to cultured rat astrocytes in micromolar concentrations Hcy compromises their metabolism (Jin and Brennan 2008), and induces cytoskeletal remodeling, oxidative stress (Loureiro et al. 2010) and epigenetic reprogramming (Jin et al. 2011). In spite of the knowledge that Hcy exerts effects on rat glial cells, the vulnerability of human glia remains enigmatic. Therefore, the present study was undertaken to mimic hyperhomocysteinemia *in vitro* to evaluate the hypothesis that Hcy may affect the viability of human glial cells.

Materials and methods

Materials

Dulbecco´s modified Eagle´s medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S) solution, and trypsin/EDTA solution were from PAA (Pasching, Austria). D,L-Homocysteine, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), propidium iodide (PI), and paraformaldehyde were purchased from Sigma (Bratislava, Slovakia). Dulbecco´s phosphate buffer saline (DPBS, without Ca^{2+} and Mg^{2+}) was from Gibco-Invitrogen (Bratislava, Slovakia). VECTASHIELD mounting medium supplemented with 4´,6-diamidino-2-phenylindole (DAPI) were from Vector Laboratories (Peterborough, UK). Sterile plastic material and culture dishes for cell culture were from Greiner (Frickenhausen, Germany).

Cell culture

The human glioblastoma cell line T98G (ECACC, Sigma, Bratislava, Slovakia) was cultivated in culture medium consisting of DMEM supplemented with 10 % (v/v) FBS, 100 I.U. penicillin per ml and 0.1 mg streptomycin per ml in a humidified atmosphere with 10 % CO_2 at 37°C. The culture medium was renewed every third day.

MTT assay

The colorimetric MTT assay, based on the enzymatic conversion of MTT to a violet formazan salt (Mosmann 1983), was used to assess the viability of the T98G cells. The cells in culture medium were seeded in wells (3×10^3/well) of 96-well microtiter plates. On the third day, the medium was changed to culture medium supplemented with Hcy of varied concentration and the incubation continued for the next two days. After treatment with Hcy, the cells were

rinsed once with DPBS (2.7 mmol.l^{-1} KCl; 1.5 mmol.l^{-1} KH$_2$PO$_4$; 138 mmol.l^{-1} NaCl; 8.1 mmol.l^{-1} Na$_2$HPO$_4$) and further incubated in medium supplemented with 0.5 mg/ml MTT in humidified atmosphere for 6 h. During a subsequent incubation for 16 h in medium containing SDS [5 % (w/v)] the precipitated formazan, the amount of which is proportional to the number of live cells, was solubilized. The absorbance of the formazan containing solution was measured at 540 nm using an ELISA plate reader (Bio-Rad PR2100). The absorbance was also determined for the medium of the control cells not exposed to Hcy and the corresponding viability was set as 100 %.

Confocal microscopy

For confocal laser scanning microscopy, 3x10^4 cells were grown on coverslips (15 mm x 15 mm) in a 6-well plate (well diameter: 3 cm). After 3 days, the medium was changed to culture medium supplemented with Hcy and cells were incubated as described in *Cell culture* for another 3 days. Two kinds of treatments were applied to stain the cells for fluorescent microscopic investigation. The cells were (i) washed by DPBS and subsequently fixed with ice cold methanol at room temperature for 5 min or (ii) incubated at 37°C in culture medium supplemented with PI (0.75 µg/ml) for 30 min before washing with DPBS and subsequent fixation with 4 % (w/v) paraformaldehyde for 10 min. Fixed cells were washed twice with DPBS and once with DPBS supplemented with 0.1 % (w/v) glycine. Cover slips were mounted cells down with Mounting medium supplemented with DAPI and the cells were examined on a confocal fluorescence microscope (Olympus FluoView FV10i, Tokyo, Japan). The blue fluorescence of DAPI was observed after excitation with wavelength of 359 nm and emission at 461 nm and red fluorescence of PI was inspected at wavelength excitation and emission of 537 nm and 617 nm, respectively. The cell viability (%) was assessed as the ratio

between the numbers of cells staining positively for PI and the number of nuclei staining for DAPI in an area of 0.16 mm^2.

Statistical analysis

One-way analysis of variance with post-hoc comparisons by Student-Newman-Keuls test was carried out to test for differences among groups. Differences between data sets were considered statistically significant if the corresponding *p* values were <0.05. The statistical analysis was performed with the program InStat (GraphPad Software, USA).

Results

Hcy affects growth of cells.

An earlier study demonstrated that Hcy at a concentration of 2 mM induced cell death in rat astrocytes (Maler et al., 2003). The Hcy concentrations chosen in the present study are in the range used in other *in vitro* studies (Jin and Brennan, 2008). Glioblastoma cells were cultured with Hcy (0.5 mM, 2 mM, and 5 mM) for 72 h. Nuclei were labeled with DAPI and visualized under the confocal microscope (Fig. 1). Cultures treated with Hcy at a concentration of 0.5 mM (Fig. 1b) contained approximately 40 % less cells than the untreated controls (Fig. 2i). At an Hcy concentration of 5 mM (Fig. 1d) the cell number dropped to 25% of the control values (Fig. 2i).

Hcy induced cell death.

In order to determine the influence of Hcy on the viability of the cells, glioblastoma cells were cultured in the presence of Hcy at various concentrations for 72 h. For the visualization

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of dead cells the cultures were exposed to PI for 30 min. This reagent penetrates dead cells due to their compromised plasma membranes but not alive cells with intact membranes. To assess the total number of cells the nuclei of fixed cells were stained with DAPI for inspection by confocal microscopy (Fig. 2). Based on the results obtained from staining with DAPI, in comparison to control cultures (Fig. 2a) the number of cells had decreased by 20 %, more than 40 %, and 75 % during treatment with Hcy at concentrations of 50 µM (Fig. 2c), 0.5 mM (Fig. 2e) and 5 mM (Fig. 2g), respectively.

With increasing concentrations of Hcy the proportion of cells labeled with PI increases (Fig. 2i). In control cultures not treated with Hcy only a few cells were labeled by PI (Fig. 2b). The cultures exposed to Hcy at concentrations of 50 µM (Fig. 2d), 0.5 mM (Fig. 2f) and 5 mM (Fig. 2h) contain 2, 6, and almost 17 times, respectively, more PI labeled cells than the controls. The estimated values of dead cells (Fig. 2i) might be grossly underestimated as a consequence of probable detachment of the dead cells from the surface. This result indicates that the extent of cell death increases with the concentration of Hcy in the culture medium.

Effect of Hcy on cell viability.

After exposure of glioblastoma cells to Hcy (10 µM to 5 mM) for 48 hours their viability was measured by a colorimetric method, the MTT assay. Already at a concentration of 50 µM D,L-Hcy, corresponding to 25 µM of the physiological enantiomer L-Hcy (Poddar et al. 2001) caused a statistically significant decrease in viability to 78 %. This effect was even stronger at 2 mM (50 % viability) and 5 mM D,L-Hcy (36 % viability; Fig. 3).

Discussion

Evidence is provided that D,L-Hcy in concentrations above 50 µM induces the death of cultured human glioblastoma cells. The cell line T98G used in this study was derived from

human glioblastoma cells (Stein 1979) and together with other cell lines such as A172 and U-87MG (De Vries and Boullerne 2012) derived from glioblastoma and astrocytoma, respectively, represents a valuable tool to investigate the properties of human astrocytes in culture.

A plasma Hcy concentration around 10 μM is considered to be physiological, while mild and moderate hyperhomocysteinemia are the conditions when the concentration of L-homocysteine in the plasma are in the ranges from $10 - 30$ or $30 - 100$ μM, respectively. Severe forms of hyperhomocysteinemia, usually associated with the deficiency in the enzymes metabolizing homocysteine, are characterized by the level of homocysteine in plasma exceeding 100 μM (Kang et al. 1992; Mansoor et al. 1993). During neurodegenerative conditions, such as Alzheimer's disease, the levels of homocysteine in serum and cerebrospinal fluid are both increased above the normal value to a mean value above 20 μM (Selley et al. 2002). The present publication reports on the effects on the viability of T98G cells of Hcy at concentrations ranging from physiological levels to levels found under severe hyperhomocysteinemia.

Previous investigations had shown that Hcy has a detrimental influence on human neurons (Ho et al. 2002; Lipton et al. 1997; Oldreive and Doherty 2007), whereas cultured rat astroglial cells sustain Hcy up to concentrations of 2 mM (Maler et al. 2003). The results presented here reveal that in contrast to rat glial cells the human glioblastoma cells of the present study undergo cell death already at D,L-Hcy concentrations in culture medium of 50 μM. Since only the L enantiomer is biologically active (Poddar et al. 2001) and occurs naturally (Ueland and Refsum 1989) this corresponds to a concentration of 25 μM of the L enantiomer of Hcy. Such concentration is comparable with the level of L-Hcy determined in

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the cerebrospinal fluid of patients with Alzheimer's disease (Selley et al. 2002). Thus, the data presented here demonstrate that Hcy is a potent gliotoxic agent capable of inducing the death of human glial cells already at concentrations reached in brain during hyperhomocysteinemia (Selley et al. 2002).

L-Hcy is a non-proteinogenic amino acid containing a sulfhydryl group. In the cells, Hcy is generated by hydrolysis of *S*–adenosylhomocysteine, which is a byproduct of transmethylation processes in which *S*–adenosylmethionine serves as a methyl donor. In the mammalian body Hcy may be enzymatically or spontaneously converted to a number of compounds. Enzymatic conversions lead to compounds such as methionine, cystathionine, homocystine, and homocysteine-thiolactone (Jakubowski 2004). The data available suggest that Hcy, even at moderate levels, can exert its neurotoxic effect by i) several mechanisms that trigger neuronal cell death, *e.g.*, increased oxidative stress (Imamura et al. 2007); ii) acting as an agonist for glutamate receptors; iii) its capability to elicit a rise in the level of cytosolic Ca^{2+} (Olney et al. 1987; Lipton et al. 1997; Folbergrova et al. 2001, Zieminska et al. 2003, Lazarewicz et al. 2003); and iv) inducing DNA damage and changes in energy metabolism associated with reduced availability of ATP (Kruman et al. 2002). In rat astrocytes Hcy i) significantly modulates metabolism by affecting the mitochondrial functions (Jin and Brennan 2008); and ii) causes epigenetic modifications by hypomethylation of global DNA and hyperacetylation of histones (Jin et al. 2011).

The increase in the level of Hcy accompanies the progression of several neurodegenerative diseases (Mattson and Shea, 2003; Sachdev 2005; Herrmann and Obeid 2011). It is well known that Hcy acts as a neurotoxin by exerting several adverse effects on neurons (Mattson and Shea, 2003). Nevertheless, the precise molecular mechanism(s) underling its roles in the

etiopathogenesis of neurodegenerative diseases remain to be fully elucidated. Furthermore, the capability of Hcy to induce the death of cultured human glioblastoma cells indicates its gliotoxicity. Therefore, an increase in the Hcy level in the brain may also cause a decline in physiological functions of glia and a progression of neurodegeneration (Rodríguez and Verkhratsky 2011). In accordance with these pieces of knowledge the present results support the view that damage of both, neuronal and glial cells, may underlie the etiopathogeneses of the neurodegenerative diseases associated with hyperhomocysteinemia.

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Figure legends

Fig. 1 Homocysteine affects the population size of cultures of human glioblastoma cells.

T98G glioblastoma cells were grown in culture media without (a) or with D,L-Hcy [0.5 mM (b), 2 mM (c), and 5 mM (d)] for 3 days. Cells fixed by methanol had their nuclei stained with DAPI before they were inspected with a confocal laser scanning microscope.

Fig. 2 Homocysteine-induced death of cultured human glioblastoma cells.

Confocal laser scanning micrographs of T98G cells after incubation in media supplemented with D,L-Hcy (**c** and **d**, 50 μM; **e** and **f**, 0.5 mM; **g** and **h**, 5 mM;) for 3 days. The control cells were cultivated in the same medium without addition of Hcy (**a, b**). The dead cells were labeled by PI (**b**, **d**, **f**, and **h**) added to the culture media for the last 30 min of the culture time. After fixation of cells by paraformaldehyde the nuclei were visualized by the intercalating fluorescent dye DAPI (**a**, **c**, **e**, and **g**). CTR, control cells without addition of Hcy. Changes in both, cell density (■) and percentage of dead cells in culture (●) were determined (**i**). The results are the average of the mean values ± SD from three independent experiments (n = 15). *$p < 0.05$; **$p < 0.001$; ***$p < 0.005$

Fig. 3 Effect of homocysteine on the MTT-assessed viability of cultured human glioblastoma cells.

For 2 days the cells of human glioblastoma cell line T98G were grown in microtiter plates in culture medium supplemented with D,L-Hcy of varied concentration. Thereafter, the viability of the cells was assessed by the MTT assay described in the Methods section. The amount of the ensuing MTT-formazan was determined spectrophotometrically at 540 nm. The control value (100 % viability) was determined with cells that had been incubated in culture medium

in which no homocysteine had been added to. The results are the average of a mean $\pm$ SEM

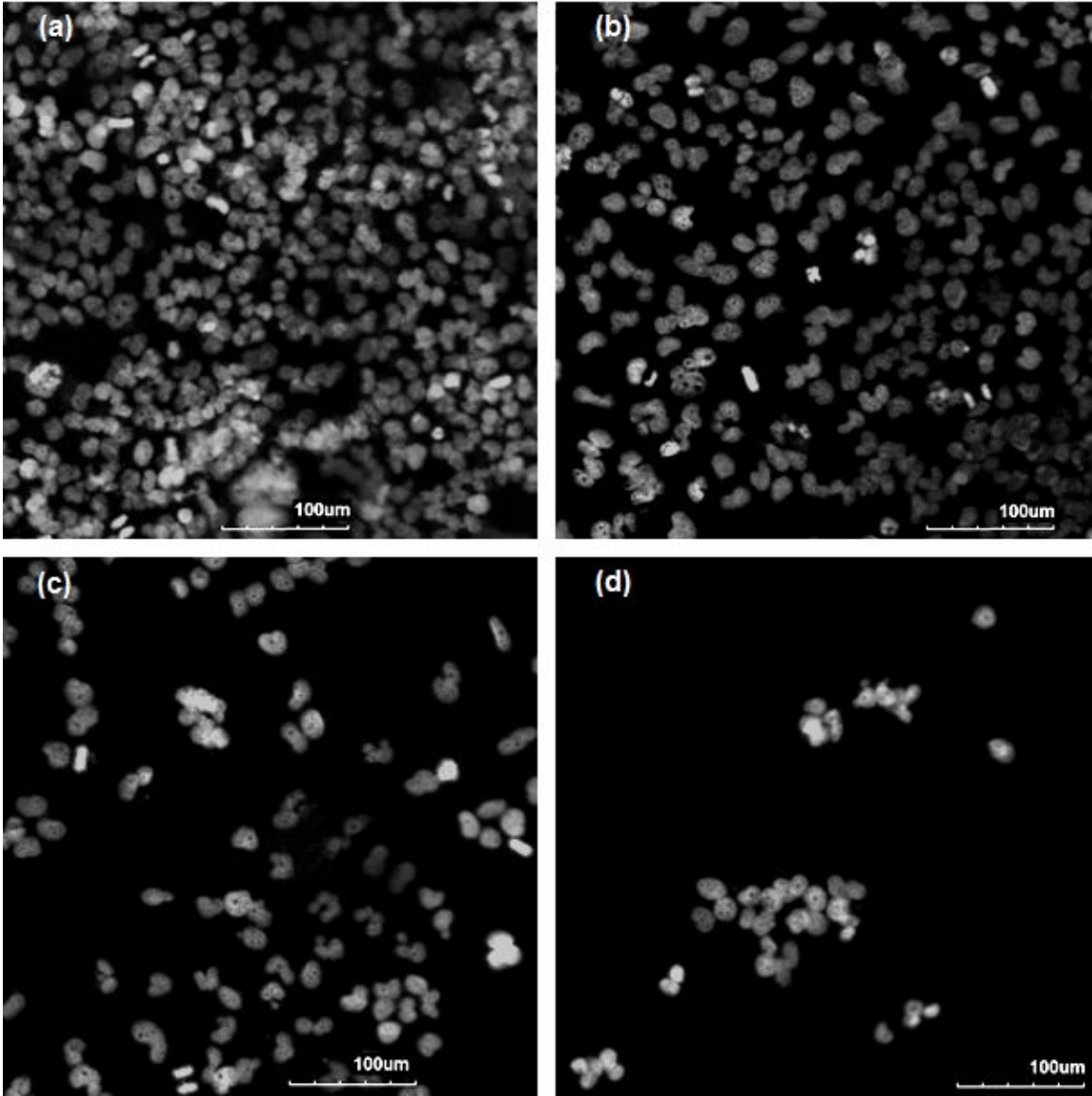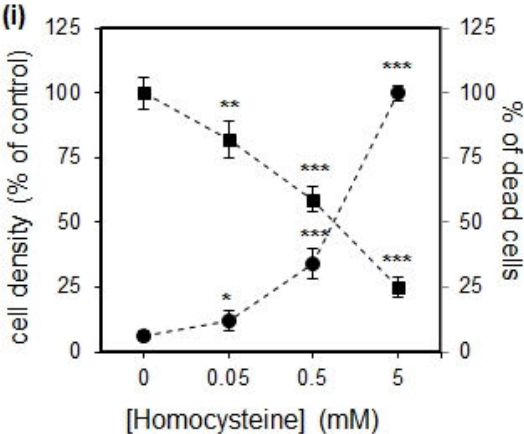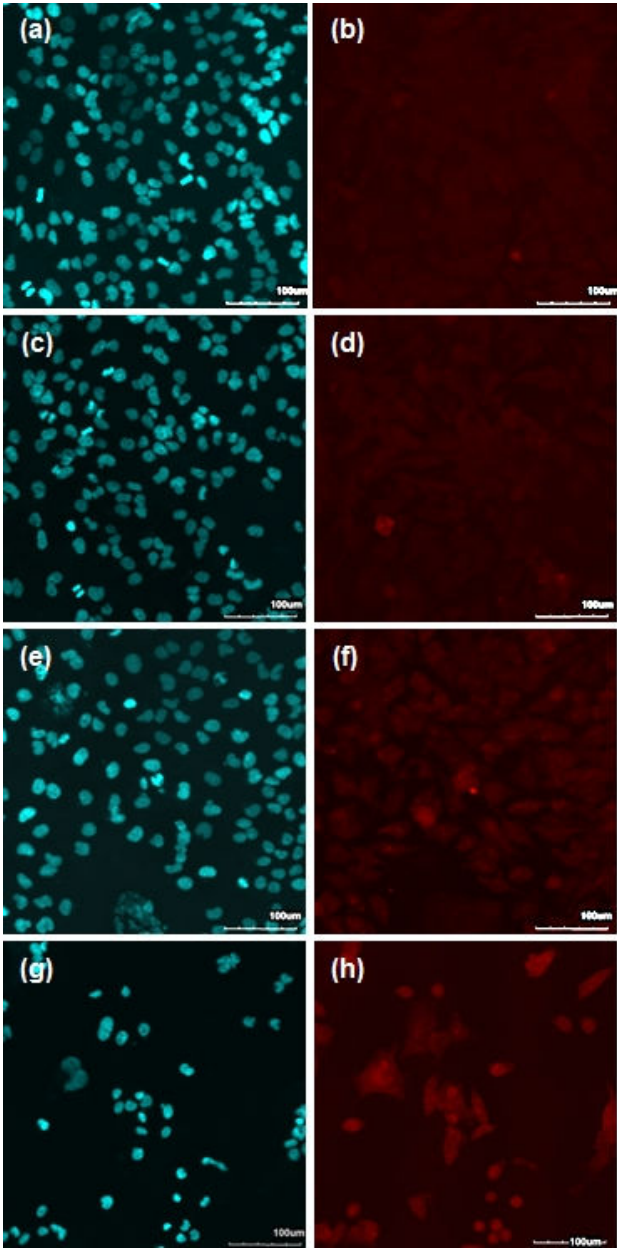values from three independent experiments (n = 18). ***$p < 0.005$

Fig. 1

Fig. 2

Fig. 3