嘉南藥理科技大學 97 年度教師研究計畫成果報告

計畫名稱：S-腺核苷同半胱胺酸對 RAW264.7 巨噬細胞在 LPS 誘發免疫反應下的機制探討

計畫類別：■個別型計畫　　□整合型計畫

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計畫成果報告

S-腺核苷同半胱胺酸對 RAW264.7 巨噬細胞在 LPS 誘發免疫反應下的影響

計畫編號：CN9737

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ABSTRACT

The limited research has been performed regarding the *s*-adenosylhomocysteine (SAH) or homocysteine (Hcy)-evoked cell damage in hepatic and neuronal cells. In this study we assessed effects of SAH or Hcy in hepatic and neuronal cells on cell cytotoxicity and DNA damage, and attempted to find the underlying mechanism. Cell cytotoxicity and DNA damage were evaluated in murine hepatic cells (BNL CL.2 cell line) and microglia cells (BV-2 cell line) with SAH or Hcy treatment for 48 hours. The influences of SAH or Hcy on lipid peroxidation and DNA methylation were also measured in both cell lines. SAH (5-20 μM) or Hcy (1-5 mM) dose dependently inhibited cell cytotoxicity and enhanced DNA damage in both types of cells. Furthermore, SAH treatment markedly increased intracellular SAH levels and DNA hypomethylation, the effect of SAH was much stronger than that of Hcy. The findings were also obtained that Hcy significantly induced lipid peroxidation, but not SAH. The present results show that SAH might cause cellular DNA damage in hepatic and microglia cells by DNA hypomethylation, further resulting in irreversible DNA damage and decrement of cell cytotoxicity. Additionally, higher Hcy could induce cellular DNA damage through increased lipid peroxidation and DNA hypomethylation. We suggest that SAH is more informative to cell damage than that of Hcy in hepatic and microglia cells.

INTRODUCTION

S-adenosylhomocysteine (SAH) is the immediate precursor of homocysteine (Hcy) in human body. SAH releases adenosine and Hcy by hydrolysis. This reaction is reversible; it tends to favor SAH synthesis over SAH hydrolysis [1]. Epidemiological studies have shown that high serum levels of Hcy is a high risk factor for cardiovascular diseases (CVD) [2, 3], neuronal dysfunction [4], chronic renal failure [5], and hepatic diseases [6, 7]. However, it has been recently found that plasma SAH levels is significantly increased in CVD patients [8], cystic fibrosis [9], and chronic renal failure [10-12]. Most studies have shown that plasma SAH is a better biomarker of atherosclerosis than Hcy and may be causally linked to the pathogenesis of the vascular disease [8, 13, 14].

SAH has also been implicated as a risk factor for hepatic diseases and neuronal disorders. For example, increased SAH levels would enhance the tumor necrosis factor-alpha-induced hepatic toxicity [15, 16]. Kennedy et al. reported that the elevated SAH in plasma and cerebral tissue have negative correlation with the cognitive ability in Alzheimer's patients [17]. Another study suggested that cerebrospinal fluid measurements of SAH are more informative than those of Hcy in neurological disorders [18]. As described above, many accumulated evidences also imply that the elevated SAH levels might have related with pathological development of hepatic diseases and neuronal disorders. To the best of our knowledge, very limited research has yet evaluated SAH-evoked cytotoxicity, especially in hepatic and neuronal cells. In fact, the effects of SAH have been presently evaluated on cytotoxicity in fewer species, such as yeast [19], rodent pheochromocytoma cells (PC12 cells) [20], and mouse fibroblasts (L929 cells) [21]. Indeed, our previous findings showed that SAH promotes hydrogen peroxide-induced DNA damage possibly by impairing of DNA repair in both mouse endothelial and human intestinal cell lines [22].

Despite the fact that both SAH and Hcy are increased in various diseases and that both are biologically active, little is known about the SAH-evoked cytotoxicity. Therefore, we attempted to evaluate the effects of SAH on cell cytotoxicity and DNA damage, as well as determine its possible mechanism by both murine hepatic (BNL CL.2 cell line) and microglia cells (BV-2 cell line). We speculated that SAH might cause cellular DNA damage in hepatic and microglia cells, possibly due to reductions in the SAM/SAH ratio and the intracellular content of 5-methyldeoxycytidine (5-mdc%), further resulting in irreversible DNA damage and decrement of cell cytotoxicity. For comparison, we also tested the effect of Hcy on both cell lines simultaneously.

MATERIALS and METHODS

Reagents

Homocysteine (Hcy, reduced), *s*-adenosylhomocysteine (SAH), dimethylsulphoxide (DMSO), Trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazloium bomide], deoxycytidine (dc) 5-methyldeoxycytidine (5-mdc) were from Sigma Chemical Co. (St. Louis, MO USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, sodium pyruvate, and non-essential amino acids (NEAA) were from GIBCO/BRL (Rockville, MD USA). All chemicals used were of reagent or higher grade.

Cell culture and treatment

BNL CL.2 cells (murine hepatic cells) and BV-2 cells (murine microglia) were used in this study were obtained from Bioresource Collection and Research Center, BCRC (BCRC, Hsinchu, Taiwan). The cells were grown in DMEM containing 10% (v/v) FBS, 0.12% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% CO_2 in an incubator at 37°C. For each cell line, a T-75 flask was seeded with 1x 10^6 cells, and cells were incubated at 37°C. The cells were harvested at ca. 90% confluence (10^6 cells/flask), and the survival rates were always higher than 95% by Trypan-blue assay. Cell were then incubated with Hcy or SAH at 37°C for 24~72 hours. Hcy was dissolved in PBS, and SAH was dissolved in dimethylsulphoxide (DMSO); the final concentration of DMSO was 0.2%, which did not affect cell growth or induce DNA damage. All assays were performed in triplicate by using three flasks for each cell line.

Measurement of cell cytotoxicity

The effect of Hcy or SAH on cell cytotoxicity was estimated by the MTT assay, as described previously [4]. Cells were cultured in 24-well plates at 1×10^4 cells/well in DMEM for 24 hours, and each well was washed and filled with 1 ml of DMEM containing various concentration of Hcy or SAH and incubated for 24~72 h at 37°C in order to observe any cytotoxicity. Each well was then incubated with MTT for 1 h, after which the liquid was removed, and DMSO was added to dissolve the solid residue. The optical density at 570 nm of each well was then determined by using a microplate reader (FLUOstar OPTIMA, BMG Labtechnologies GmbH, Germany).

Measurement of DNA damage

Comet assay was adapted from the method of Singh et al. [23], as we described previously [22, 24]. After incubation, cells were suspended in low-melting-point agarose in PBS at 37°C and pipetted onto a frosted glass microscope slide precoated with a layer of 1% normal-melting-point agarose. After application of a third layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100) for 1hour at 4 °C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH and 1 mM EDTA). Electrophoresis was then performed at 300 mA for 20 min in the same alkaline solution at room temperature. The slides were then neutralized with 0.4 M Tris-HCl buffer (pH 7.4) and stained with ethidium bromide. Comet formation, expressed as tail moment (TM) by the formula: TM = %DNA in tail × tail length, was analyzed by computer using the Image Pro Plus software (Media Cybernetics, USA).

At least 30 images per slide were analyzed and there were three slides for each sample. The six TM values were then averaged and the means of three samples were obtained.

Measurement of lipid peroxidation

Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) released into the DMEM medium from BNL CL.2 cells and BV-2 cells following centrifugation at 1,000 g for 10 min. TBARS were measured by mixing equal volumes of the supernatant with 0.7% TBA reagent and 2.5% TCA. BHT (0.5mM) was added to prevent sporadic lipid peroxidation during heating at 100°C for 10 min. TBARS were extracted with an equal volume (3 ml) of butanol. After a brief centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission [25]. TBARS were expressed as nmol malondialdehyde (MDA) equivalent/mg protein using 1,1,3,3-tetramethoxypropane as MDA standard.

Determination of SAM and SAH levels

The extraction method for SAM and SAH was done according to the method of Wise et al. [26]. When measuring the SAM and SAH in cultured cell, 80 μl of o.1 M sodium acetate buffer, pH 6.0, was added to a soft pellet containing a known number of cells. After the cells were lysed with the sodium acetate, 20 μl of 40% TCA were added. The solution was vortex-mixed and allowed to stand on ice for 30 min. The solution was centrifuged at 25,000 *g* for 10 min at 5^{o}C, and 100 μl of the supernatant were transferred to another 0.5 ml eppendorf tube. The solution was extracted twice with an equal volume of diethyl ether, and was filtered. Then, the solutions were analyzed by HPLC. The HPLC system for measurement of SAM and SAH was carried out as described previously [27].

Determination of DNA methylation

5- methyldeoxycytidine (5-mdc) is a marker for the monitoring of DNA methylation [28]. HPLC is the most commonly used chromatographic method for analysis of the genome-wide methylation. DNA methylation was determined as 5-methyldeoxycytidine specifically in DNA by Cooney and Wise [29].

Data analysis

Values are expressed as means ± SD and analyzed using one-way analysis of variance followed by Duncan's multiple range tests.

RESULTS

Cell cytotoxicity

Incubation with 5-20 μM SAH for 24~72 h led to marked inhibition of cell cytotoxicity in both BNL CL.2 cells (Fig. 1A) and BV-2 cells (Fig. 1B). In both cell types, incubation with SAH for 48 h led to dose-dependent decreases in cell cytotoxicity. The solvent (DMSO) control (the final concentration of DMSO was 0.2 %) did not affect cell cytotoxicity. By contrast, Hcy had only weak effects on cell cytotoxicity in both BNL CL.2 cells (Fig. 2A) and BV-2 cells (Fig. 2B), that is, Hcy significantly inhibited cell cytotoxicity at 5 mM, and the effect was equivalent to that of 10 μM SAH.

DNA damage

We adopted an incubation time of 48 h because SAH at 5 μM significantly decrease cell cytotoxicity in both BNL CL.2 cells and BV-2 cells, whereas at an incubation time of 24 h, 5 μM SAH did not significantly inhibit cell cytotoxicity (Fig. 1A, 1B). Fig. 3 shows that incubation of cells for 48 h with 0.2 % DMSO did not induce significant DNA strand breakage (TM= ~2) in both cell types. Incubation of BNL CL.2 cells with SAH for 48 h led to dose-dependent increases in DNA strand breaks, and the TM of cells incubated with 5 μM SAH already significantly higher than that of the control. By contrast, Hcy had only weak effects on DNA strand breaks, that is Hcy significantly enhanced DNA strand breaks at 5 mM Hcy, and this effect was equivalent of that of 10 μM SAH.

Intracellular levels of SAH and SAM

In order to investigate whether intracellular SAH and SAM are involved in the DNA strand break, we measured intracellular SAH and SAM during incubation with SAH or Hcy. As shown in Table 1, incubation with DMSO (as a solvent control) did not change intracellular SAH and SAM levels in both BNL CL.2 and BV-2 cells. In both cell types, incubation of cells with 10 μM SAH markedly increased intracellular SAH levels, although it did not change intracellular SAM levels. However, only incubation of cells with 5 mM Hcy significantly enhanced intracellular SAH levels (2.7±0.1 nmol/mg protein vs. 1.8±0.1 nmol/mg protein in controls in BNL CL.2 cells). Furthermore, the SAM/SAH ratio serves as a marker for cellular methylation [30]. We also determined the SAM/SAH ratio in cells incubated for 48 h with SAH or Hcy (Table 1). We showed that incubated of BV-2 cells with 20 μM SAH decreased the SAM/SAH ratio by 68% (6.0±1.1 to 18.9±2.1, $p<0.05$), and the effect of Hcy was not as strong as SAH.

Levels of MDA

Table 2 shows lipid peroxidation in both BNL CL.2 and BV-2 cells incubated with SAH or Hcy for 48h. In both cell types, incubation with SAH did not induce significant lipid peroxidation, but incubation with Hcy markedly induced lipid peroxidation, and the effect of Hcy was concentration-dependent.

Levels of 5-mdc

As shown in Table3, incubation of BV-2 cells with 2 mM Hcy for 48h did not affect the background concentration of 5-mdc(~3.8%), but incubation with 5μM SAH significantly decreased the concentration to 2.8% ($p<0.05$). SAH (5-20 μM) decreased

the 5-mdc levels of percentage in both BNL CL.2 and BV-2 cells in a dose-dependent manner. We showed that the effects of SAH were much stronger than those of Hcy. For example, the effect of 5 mM Hcy was roughly equal to or slightly that of 5 μM SAH.

DISCUSSION

Compared with the correlation of SAH and Hcy-related diseases, few studies have been performed to examine SAH-evoked cytotoxicity on hepatic and microglia cells. In this study we found that SAH can induce cytotoxicity in BNL CL.2 and BV-2 cells. Our results showed that SAH at 5-20 μM inhibits cell cytotoxicity and enhances cellular DNA damage in a dose dependent manner, whereas Hcy had a weaker effect, that is, the effect of 5 mM Hcy was roughly equivalent that of 10 μM SAH. Although SAH used here is relatively high (5-20 μM) compared with the plasma SAH levels in vivo (0.02-0.04 μM) [8, 27], but SAH might increased in Hcy-related disease [14, 31, 32]. In fact, measurement of plasma SAH has not been carried out in most studies, mostly because the SAH levels in plasma is about 1-500th that of plasma Hcy [32, 33]. The findings of SAH may be pathologically or toxicologically relevant in Hcy-related disease especially in hepatic and neuronal disease.

The effect of SAH on DNA damage appears to involve decreasing SAM/SAH ratio and 5-mdc% (DNA hypomethylation). SAM/SAH ratio could as an indicator of the methylating capacity of cells [30]. However, elevated SAH may be a better indication of hypomethylation than does the SAM/SAH ratio [34, 35]. Therefore, we further demonstrated that SAH significantly enhances intracellular SAH levels. Methylation is significantly in epigenetic regulation of protein expression via DNA and histone methylation [36]. Besides, methylation of CpG dinucleotides catalyzed by DNA methyltransferase forms 5-mdc whose levels may regulate gene expression [37]. SAH is a powerful inhibitor of methyltransferase [38, 39], and SAH could cause DNA repair imbalance which is related to urical misincorporation [22]. Hypomethylated DNA may inhibit DNA repair because DNA methylation play an important role in strand discrimination during post replication mismatch repair [40]. As a result, SAH might cause cellular DNA damage in hepatic and microglia cells by DNA hypomethylation, further resulting in irreversible DNA damage and decrement of cell cytotoxicity. Furthermore, we suggested that SAH may not be involved in the generation of reactive oxygen species (ROS) because it did not increase lipid peroxidation in both cell lines. Unlike Hcy, SAH is not undergoing autoxidation because its sulfhydryl group is adenylated.

According to our finding, Hcy at 1-5 mM inhibits cell cytotoxicity and enhances cellular DNA damage. The mechanism that may be responsible for Hcy on cellular DNA damage in hepatic and microglia cells is lipid peroxidation and DNA hypomethylation. Although Hcy at 1-2 mM did not produce significant DNA hypomethylation (decrement of SAM/SAH ratio and 5-mdc %), it significantly but increased lipid peroxidation. Our result also showed that increment of intracellular of SAH levels to Hcy at 5 mM has occurred. Hcy has the ability to self oxidize and can oxidize other sulfur containing molecules as well, generating oxidized compounds such as H_2O_2, as well as superoxide anion radicals, which can damage cells [41]. Many experiments have found that to generate ROS and cause subsequent cell damage, Hcy concentrations need to be as high as 1~10 mM [41-43]. Levrand et al [41] indicated that Hcy caused decreased cell cytotoxicity and apoptosis in mouse cardiomyocytes through the generation of peroxynitrite. For L5178Y cells and HL60 cells, 5 mM Hcy will not cause DNA damage and change 5-mdc content [44]. However, in this experiment, the cells were cultured in Hcy for 24 hours and the author have concluded that Hcy-induced oxidative stress is cell-type specific. Furthermore, in our results, we found that when culturing hepatic or microglia cells with 1~2 mM Hcy, although resulting in DNA damage, intracellular SAH content did not significantly increase, while lipid peroxidation level did. When Hcy

concentrations were as high as 5 mM, intracellular SAH levels, together with 5-mdc levels, increased significantly.

Taken together, our results show that SAH might cause cellular DNA damage in hepatic and microglia cells by DNA hypomethylation, further resulting in irreversible DNA damage and decrement of cell cytotoxicity. In addition, Hcy could induce cellular DNA damage through increased lipid peroxidation and DNA hypomethylation. We suggest that SAH is more informative to cell damage than that of Hcy in hepatic and microglia cells.

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Table 1. Effects of various concentrations of SAH or Hcy on the levels of SAH, SAM, and SAM/SAH ratio for 48 h in BNL CL.2 and BV-2 cells[1].

	BNL CL.2 cells			BV-2 cells		
Treatment	SAH[2] (nmol/mg protein)	SAM (nmol/mg protein)	SAM/SAH ratio	SAH (nmol/mg protein)	SAM (nmol/mg protein)	SAM/SAH ratio
Control	1.8±0.1^{a}	31±2	17.2±2.1^{c}	1.9±0.2^{a}	35±3	18.4±3.6^{d}
DMSO[3]	1.7±0.2^{a}	33±3	19.4±3.2^{c}	1.9±0.1^{a}	36±2	18.9±2.1^{d}
SAH						
5 μM	2.1±0.3^{ab}	36±3	17.1±4.3^{c}	2.4±0.4^{ab}	38±3	15.8±3.3^{cd}
10 μM	2.6±0.2^{b}	37±4	14.2±2.4^{bc}	2.9±0.3^{b}	36±4	12.4±2.6^{bc}
20 μM	5.3±0.3^{c}	35±3	6.6±0.9^{a}	6.3±0.5^{c}	38±3	6.0±1.1^{a}
Hcy						
1 mM	1.9±0.1^{a}	35±4	18.4±3.0^{c}	2.0±0.2^{a}	37±3	18.5±3.1^{d}
2 mM	2.1±0.2^{a}	34±3	16.2±2.7^{bc}	2.4±0.3^{ab}	35±3	14.6±2.8^{c}
5 mM	2.7±0.1^{b}	36±3	13.3±1.5^{b}	3.3±0.4^{b}	35±3	10.6±2.5^{b}

[1] Abbreviations are as follows: SAH, S-adenosylhomocysteine; Hcy, Homocysteine; SAM, *S*-adenosylmethionine. For each cell line, a T-75 flask was seeded with 1 × 10^{6} cells, and the cells were incubated at 37°C for 48 hours.

[2]Values are means ± SD of triplicate assays. Data in the same column not sharing a common letter are significantly different ($P < 0.05$).

[3]DMSO was the solvent for SAH (the final concentration of DMSO was 0.2%).

Table 2. Effects of various concentrations of SAH or Hcy on lipid peroxidation for 48 h in BNL CL.2 and BV-2 cells[1].

Treatment	MDA (nmol/mg protein)[2]	
	BNL CL.2 cells	BV-2 cells
Control	3.0±0.1[a]	3.2±0.1[a]
DMSO[3]	2.8±0.2[a]	3.3±0.2[a]
SAH		
5 μM	3.3±0.6[ab]	3.2±0.3[ab]
10 μM	3.1±0.3[ab]	3.3±0.3[ab]
20 μM	2.7±0.7[ab]	3.0±0.4[a]
Hcy		
1 mM	3.5±0.2[b]	3.8±0.3[b]
2 mM	4.7±0.3[c]	5.1±0.5[c]
5 mM	5.2±0.3[d]	6.7±0.7[d]

[1] Abbreviations are as follows: SAH, *S*-adenosylhomocysteine; Hcy, Homocysteine. For each cell line, a T-75 flask was seeded with 1 × 10^6 cells, and the cells were incubated at 37°C for 48 hours.

[2]Values are means ± SD of triplicate assays. Data in the same column not sharing a common letter are significantly different ($P < 0.05$). Lipid peroxidation was expressed in the terms of malondialdehyde (MDA) formed per mg protein.

[3]DMSO was the solvent for SAH (the final concentration DMSO was 0.2%).

Table 3. Effects of various concentrations of SAH or Hcy on content of 5-methyldeoxycytidine (5-mdc) for 48 h in BNL CL.2 and BV-2 cells [1].

Treatment	5-methyldeoxycytidine (%)[2]	
	BNL CL.2 cells	BV-2 cells
Control	1.94±0.01^{d}	3.8±0.4^{d}
DMSO	1.93±0.02^{d}	3.5±0.3^{d}
SAH		
5 μM	1.85±0.02^{c}	2.8±0.2^{c}
10 μM	1.78±0.01^{b}	2.2±0.3^{b}
20 μM	1.31±0.03^{a}	1.7±0.1^{a}
Hcy		
1 mM	1.90±0.01^{d}	3.3±0.4^{d}
2 mM	1.89±0.02^{cd}	3.2±0.1^{d}
5 mM	1.76±0.01^{b}	2.7±0.3^{cd}

[1] Abbreviations are as follows: SAH, S-adenosylhomocysteine; Hcy, Homocysteine. For each cell line, a T-75 flask was seeded with 1×10^{6} cells, and the cells were incubated at 37°C for 48 hours.

[2] 5-methyldeoxycytidine (5-mdc) and deoxycytidine (dc) in cellular DNA were determined by high performance liquid chromatography. The molar 5mdc%, that is, 100 × 5mdc/(dC+5-mdc), was calculated. Values are means ± SD of triplicate assays. Data in the same column not sharing a common letter are significantly different ($P < 0.05$).

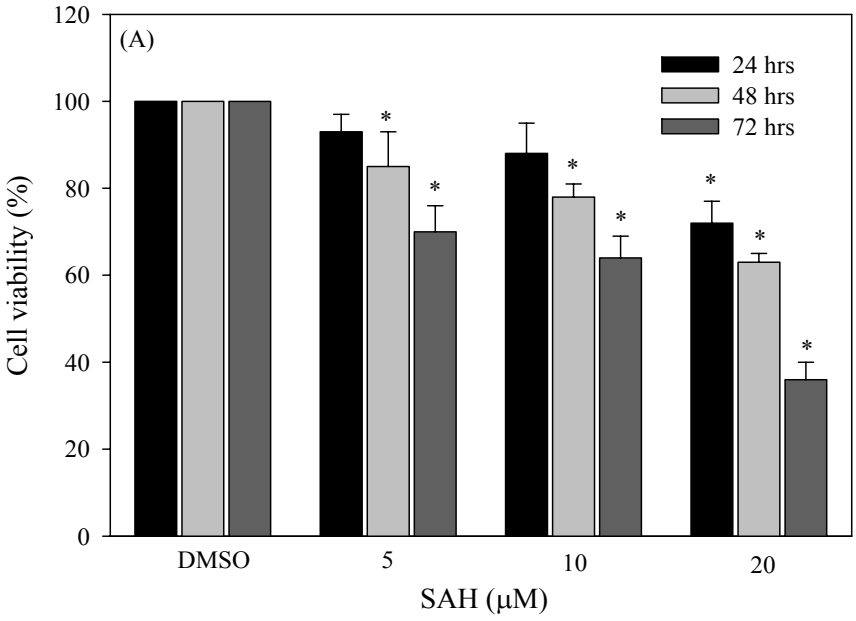
[3] DMSO was the solvent for SAH (the final concentration DMSO was 0.2%).

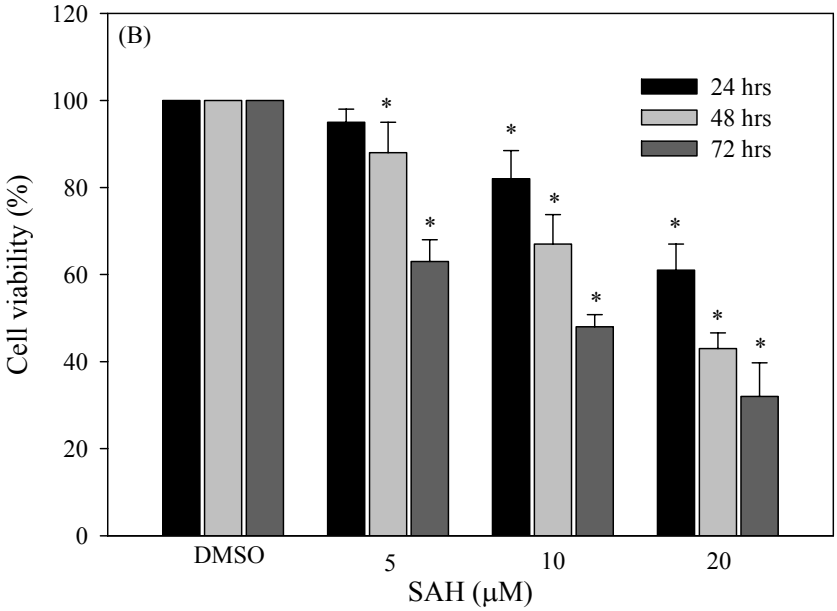
FIGURE LEGENDS

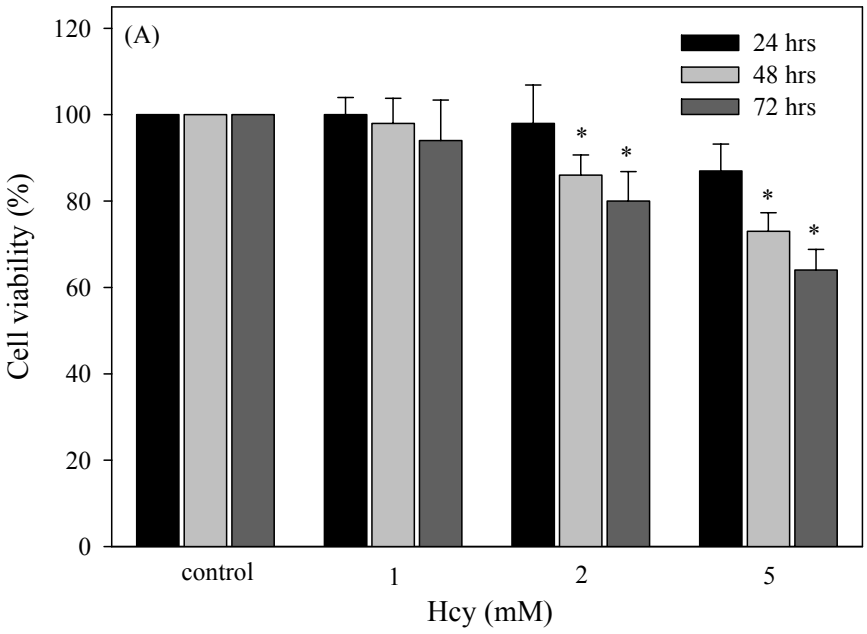
Figure 1. Effects of *s*-adenosylhomocysteine (SAH) on cell cytotoxicity in BNL CL.2 cells (A) and BV-2 cells (B). BNL CL.2 and BV-2 cells were treated with different concentrations of SAH (dissolved in DMSO) for 24, 48, and 72 h. DMSO (0.2%) served as solvent control for SAH. Cell cytotoxicity was determined by MTT and expressed as a percentage of viable cells in the total number of cells counted. Values are means ± SD (n ≥ 3) for each treatment. **P*<0.05 in comparison with solvent control group values.

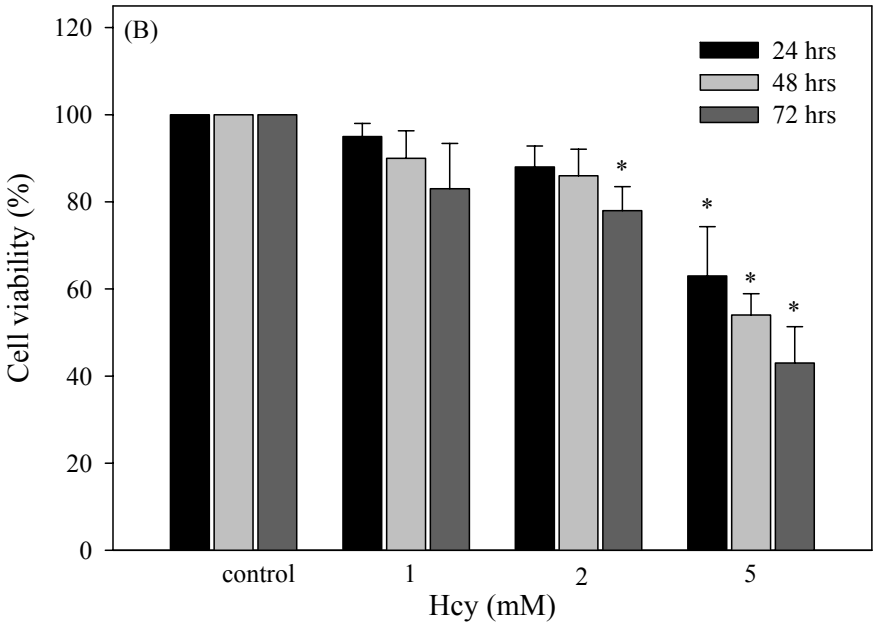
Figure 2. Effect of homocysteine (Hcy) on cells cytotoxicity in BNL CL.2 cells (A) and BV-2 cells (B). BNL CL.2 and BV-2 cells were treated with different concentrate of Hcy (dissolved in PBS) for 24 ~72 h. Cell cytotoxicity was determined by MTT and expressed as a percentage of viable cells in the total number of cells counted. The figure shows means ± SD (n ≥ 3) for each treatment. **P*<0.05 in comparison with control group values.

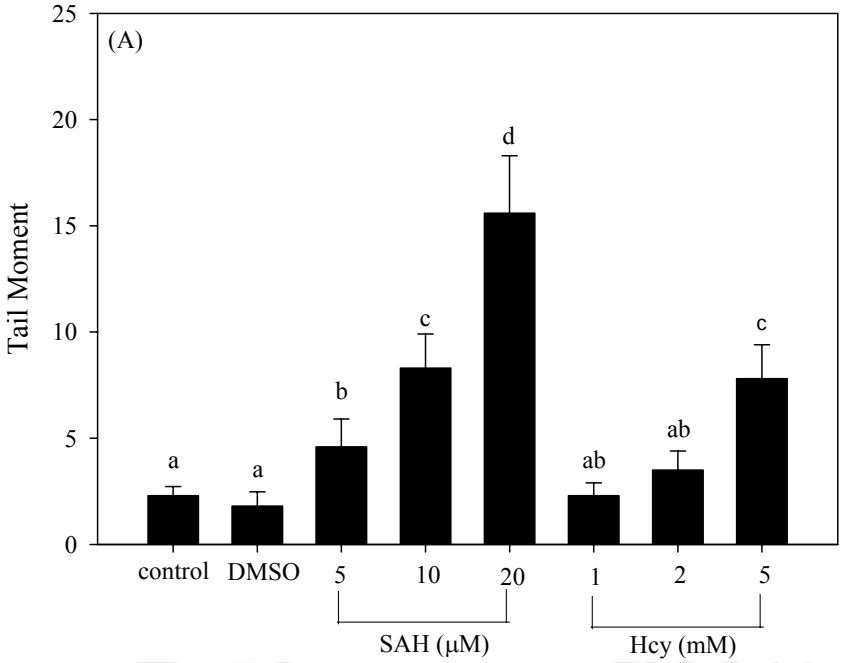
Figure 3. Effects of *s*-adenosylhomocysteine (SAH) and homocysteine (Hcy) on DNA strand breaks in BNL CL.2 cells (A) and BV-2 cells (B). Cells were treated with SAH (dissolved in DMSO) or Hcy (dissolved in PBS) for 48 h. DMSO (0.2%) served as solvent control for SAH. DNA strand breaks were determined using comet assay and expressed as tail moment. Values (means ± SD of triplicate assays) with different letters are significantly different (*P*<0.05).




(B)
24 hrs
48 hrs
72 hrs
Cell viability (%)
120
100
80
60
40
20
0
DMSO
5
10
20
SAH (μM)


(A)
24 hrs
48 hrs
72 hrs
Cell viability (%)
120
100
80
60
40
20
0
control
1
2
5
Hcy (mM)


(B)
24 hrs
48 hrs
72 hrs
Cell viability (%)
120
100
80
60
40
20
0
control
1
2
5
Hcy (mM)
*
*
*
*


(A)
Tail Moment
25
20
15
10
5
0
a
a
b
c
d
ab
ab
c
control
DMSO
5
10
20
1
2
5
SAH (μM)
Hcy (mM)