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Original Article

Cytotoxic effects of 4-methylimidazole on bone marrow mesenchymal stem cells in vitro

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Abstract: 4-Methylimidazole (4-MI) is found in a great number of food products. The National Toxicology Program (NTP) revealed that 4-MI is carcinogenic and can also cause anemia and weight loss. Mesenchymal stem cells (MSCs) are able to support hematopoiesis and migrate to the site of tumors. To investigate whether 4-MI has an impact on MSCs, we have measured the ability of cell (osteoblast, adipocyte) proliferation, apoptosis, cell cycle, gene expression, migration and differentiation between control group and the 4-MI group. The results showed that higher concentrations of 4-MI (≥150 μg/ml) had significant effects on BMSCs viability while lower concentrations (≤100 μg/ml) had no significant effects on cell proliferation, apoptosis, migration, differentiation, and expression of relevant marker genes of hematopoietic cytokines, including TPO, SCF, VEGF and FLt3. The results also indicated that 4-MI (≤100 μg/ml) may have no significant effect on the biological characteristics of MSCs. Low concentration of 4-MI in foods and beverages have no toxic effect on BMSCs. The anemia and weight loss of animals caused by 4-MI may not be due to its effect on BMSCs.

Keywords: Mesenchymal stem cells, 4-methylimidazole, biological characteristics

Introduction

According to the National Toxicology Program (NTP), a division of the National Institute of Environmental Health Sciences (NIEHS), 4-Methylimidazole (4-MI) has been identified as undesirable by-products in several food products, including caramel coloring, wine, soy sauce, Worcestershire sauce, ammoniated molasses, caramel-colored syrups, and in mainstream and sidestream cigarette smoke [1-5]. It is also used in the manufacture of pharmaceuticals, cleaning and agricultural chemicals, photographic chemicals, dyes and pigments, and rubber [6]. Its toxicity and carcinogenesis studies were conducted because of widespread human exposure. According to NTP, 4-MI is carcinogenic and induces alveolar/bronchiolar adenoma and carcinoma in mice, and may also induce mononuclear cell leukemia and mammary tumors in female rats [6-11]. Animals exposed to 4-MI exhibited anemia and convulsant activity including restlessness, bellowing, frothing at the mouth and paralysis [12]. Besides, body weight gains in male and female mice or rats with 4-MI were significantly reduced compared to controls [6, 11-13].

In recent years, mesenchymal stem cells (MSCs) have become an attractive therapeutic tool because of their unique characteristics, including their ability to self-renewal, ease of their isolation and expansion [14]. MSCs possess a broad spectrum for regenerative medicine due to their potential to repair tissue [15] and to differentiate into osteoblasts, chondroblasts, adipocytes and myoblasts [16, 17]. MSCs and the cytokines secreted by them are important components of the hematopoietic microenvironment [18]. It is well-known that tumor cells secrete cytokines, chemokines and growth factors that are able to recruit and activate MSCs, and the MSCs could migrate to the site of numerous types of tumors in vivo [19, 20]. Given its clear association with cancer and anemia, we speculate that 4-MI may also have an impact on MSCs. In this study, we examined the effects of 4-MI on the biological characteristics of MSCs, especially their potentials relating to cancer progression. The effect of 4-MI on inducing differentiation to different cell lineag-

es, including adipocyte, osteoblast and myoblast were described. The underlying mechanisms of 4-MI on anemia and weight loss relating to cell maintenance and differentiation of BMSCs were also discussed.

Materials and methods

Isolation and characterization of BMSCs

Bone marrow mesenchymal stem cells (BMSCs) were isolated and cultured according to standard protocols [21]. The cells from 80-100 g Sprague Dawley rats were collected by flushing the femurs and tibiae with phosphate-buffered saline (PBS) and then cultured in low glucose Dulbecco's modified Eagle's medium (L-DMEM; GIBCO-BRL, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The experimental procedure was approved by the Institutional Animal Care Committee of Jiangsu University. To remove non-adherent cells, the culture medium was changed at day 4. Whole medium was subsequently replaced at three-days' intervals. When adherent cells reached 80-90% confluence, they were trypsinized with 0.25% trypsin-EDTA (Invitrogen) and subcultured in new flasks for further expansion. The cells in passage 3-5 were used for the experiments.

The phenotype of BMSCs was analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson). The cells were stained with monoclonal antibodies against CD29, CD44, CD45 and CD90 (FITC-conjugated) (Becton-Dickinson, San Jose, CA, USA) for 30 min at 4°C. FITC-IgG1 isotypic immunoglobulins were used as isotype controls.

MTT assay

The BMSCs viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BMSCs (3×10^3) were plated in 96-well plates containing 200 µl 10% FBS L-DMEM, and allowed to attach overnight. Then cells were treated with various concentration of 4-MI (50 to 500 µg/ml) for 24-96 h. MTT (20 µl) was added to each well for the last 4 h. When the reaction was terminated, all the solution was discarded and 150 µl dimethyl sulfoxide (DMSO) was added to each well. The 96-well plate was subjected to shaking for 10 min to ensure complete solubilization of the purple formazan crystals. Absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay reader.

Cell colony formation assay

BMSCs (1000 cells/well) were plated in 6-well plates in 10% FBS L-DMEM and allowed to attach overnight, then the medium was replaced with fresh L-DMEM supplemented with 10% FBS containing 100 µg/ml 4-MI for 14 days. There was no 4-MI in the control group. The medium was changed every three days. At the end of the growth period, cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 20 min. The cell colonies were photographed and the number of colonies was counted for statistical analysis.

Morphological analysis

BMSCs were seeded in 24-well plates in 10% FBS L-DMEM. After overnight incubation, cells were treated with various concentrations of 4-MI (50 to 400 µg/ml) for 72 h. Cell images were analyzed using bright field microscope under 40× magnifications.

Transwell migration assay

Migration assays were performed based on the manufacturer's instructions (Corning Inc, Corning, NY, USA) with slight modifications. There was conditioned medium (control, 100 µg/ml 4-MI) in the bottom of the transwell. BMSCs (3×10^4) were seeded in 100 µl of serum-free L-DMEM in the top of the chamber and incubated for 16 h. Cells remaining on the top side of the filter were wiped off with cotton swabs. The cells migrating to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. We selected six random fields (magnification, 100×) in each chamber to observe the cells and used Cell Counter software (Borland Software Corporation, Scotts Valley, CA, USA) to count the migrated cells. Each experimental group was repeated three times.

Cell cycle assay

BMSCs were plated in 6-well plates for each data point. After overnight incubation, cells were treated with 4-MI (100 µg/ml) for additional 72 h. Cells were then harvested and washed twice with cold PBS and stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4°C in dark. The stained

Table 1. Specific primers for control and target genes

ID	Gene	Primers sequence, 5' to 3'	Size, bp	Annealing, °C
1	TPO-F	ATTGCTCCTCGTGGTCAT	220	56.0
	TPO-R	CTCCTCCATCTGGGTTTT		
2	SCF-F	TGGATAAGCGAGATGGTA	189	54.0
	SCF-R	TTCTGGGCTCTTGAATGA		
3	VEGF-F	CCTTGCTCTACCTCCAC	280	61.0
	VEGF-R	ATCTGCATCCTGTTGGA		
4	FLt3-ligand-F	CTGGAGCCCAACAACCTATC	353	60.0
	FLt3-ligand-R	TCTGGACGAAGCGAAGACA		
5	GAPDH-F	GAGTCTACTGGCGTCTTCAC	272	58.0
	GAPDH-R	GTCTTCTGAGTGGCAGTGAT		

cells were analyzed by flow cytometry (Accuri C6; BD Biosciences, USA).

Apoptosis assay

BMSCs were plated in 6-well plates and cultured overnight. Cells were then treated with 4-MI (100 μg/ml) for 72 h. Following treatment, the cells were harvested and washed twice with cold PBS and stained with PI and Annexin V-fluorescein isothiocyanate (FITC) for 20 min at RT in dark according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry (Accuri C6; BD Biosciences).

Multi-differentiation capacity

Adipogenesis: Cells were plated in 24-well plates in L-DMEM with 10% FBS. When cells reached 70% confluence, The medium was changed into adipogenic induction medium (culture medium with 10 μg/ml insulin, 0.5 mM IBMX, 200 μM indomethacin and 1 μM dexamethasone) for 3 days and then switched to maintenance medium (supplemented with 10% FBS and 10 μg/ml insulin) for 1 day. A final concentration of 100 μg/ml 4-MI were added to the experimental group. Staining with Oil Red O was carried out when intracellular lipid droplets were observed under the microscope.

Osteogenesis: BMSCs were seeded at 1×10^4 cells/cm^2 in 35-mm plates. After overnight incubation, cells were treated with a modified osteogenic induction medium [0.1 μM dexamethasone, 10 mM glycerophosphate, 4 μg/ml basic fibroblast growth factor (bFGF) and 50 μg/ml ascorbic acid] for two weeks. The experimental group were treated with 4-MI at a final concentration of 100 μg/ml. At the end of induction, the cells were fixed with fixing agent and stained with alkaline phosphatase (ALP) based on the manufacturer's instructions (Biotech, China).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction (Fermentas, Waltham, MA, USA). RNA was processed for cDNA synthesis with Superscript II reverse transcriptase, using Oligo (dT) primer (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The cDNA samples were subjected to PCR with specific primers synthesized by Invitrogen Life Technologies (**Table 1**). The conditions of PCR were as follows: Initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55-70°C for 30 sec (see **Table 1** for temperatures used), extension for 30 sec at 72°C by 30-35 cycles and a final extension at 72°C for 10 min. The PCR products were checked by electrophoresis on a 1.5% agarose gel, with ethidium bromide staining, photographed under UV transillumination and analyzed using the Gel Image Analysis System (Tanon 2500R, Gene, USA). GAPDH was used as an internal control. The cDNA samples were also used for quantitative real-time PCR analysis.

Statistical analysis

All data were expressed as means ± standard deviation (SD). The statistically significant differences between groups were assessed by analysis of variance (ANOVA) with two-way classification, or ANOVA with Student-Newman-Keuls multi-comparison test using the GraphPad Prism V.5 software program. *P* value <0.05 was considered as a statistically significant difference.

Results

Characterization of BMSCs

The BMSCs displayed a polygonal, spindly and fibroblast-like morphology. They had clear cell boundaries and strong refraction, and grew in

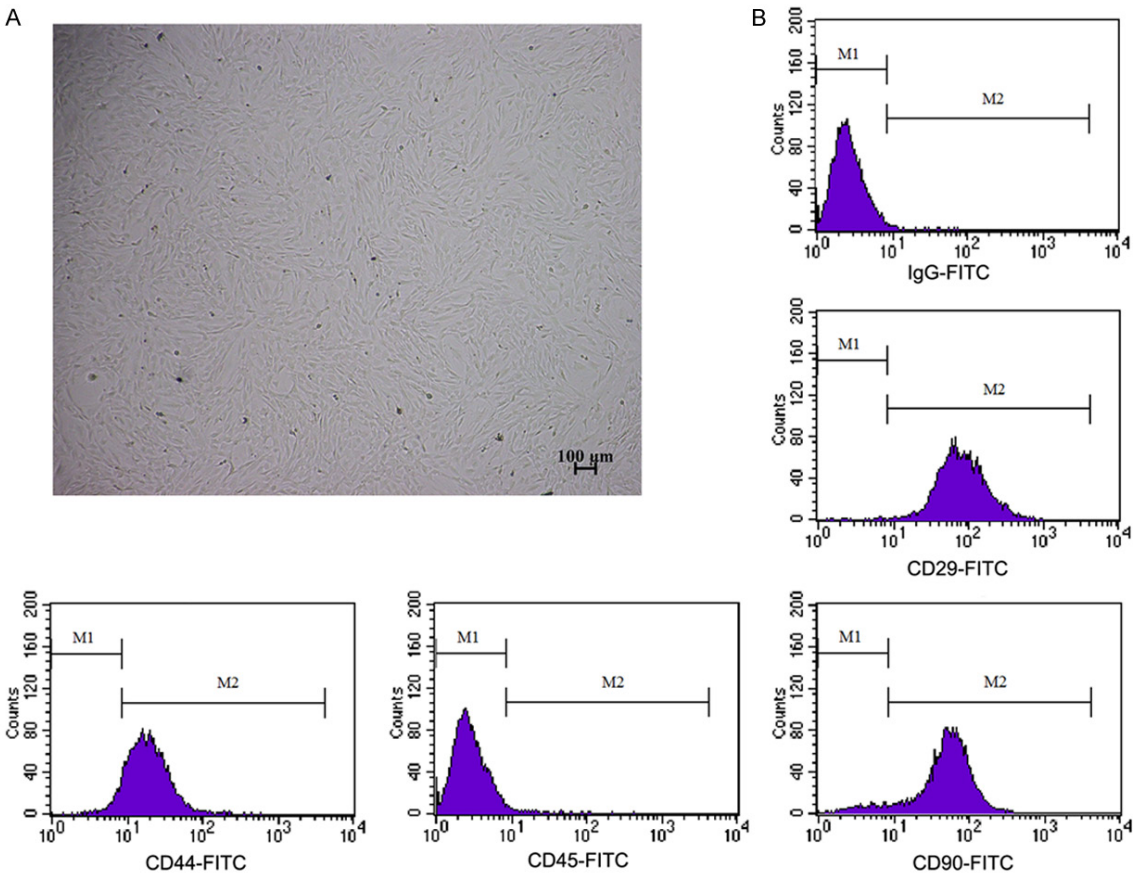


Figure 1. Characterization of BMSCs. A. The cells presented polygonal, spindly and fibroblast-like. Magnifications, 40x. B. BMSCs were positive for CD29, CD44 and CD90, but negative for CD45. BMSCs, bone marrow mesenchymal stem cells.

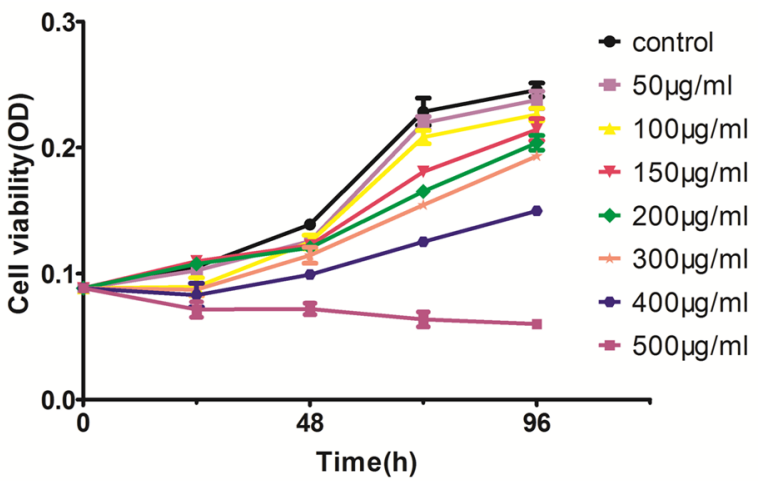
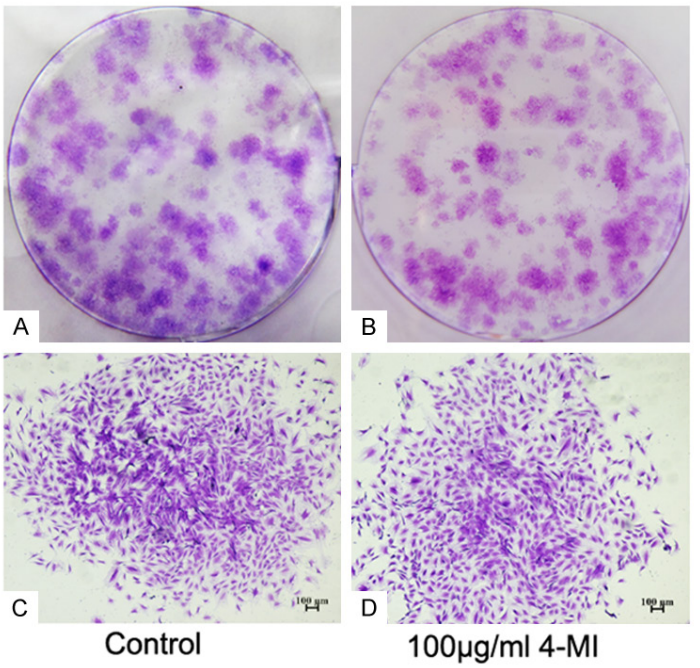


Figure 2. Effects of cell viability of 4-MI on BMSCs. All cells were incubated with various concentrations of 4-MI (50-500 μg/ml) for 24-96 h and cell viability was determined by MTT assay. 4-MI, 4-methylimidazole; MTT, 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide.

colonies (**Figure 1A**). On flow cytometric analysis, the cells were positive for CD29, CD44, and CD90, but negative for CD45 (**Figure 1B**).

Effect of 4-MI on cell proliferation

Cell viability by MTT assay: On MTT assay, 4-MI treatment affected the viability of BMSCs in a dose and time-dependent manner. Results showed that BMSCs remained insensitive towards 4-MI treatments at lower concentrations (<100 μg/ml) for the period of 24-96 h, whereas at higher concentrations (≥150 μg/ml) for the period of 72 and 96 h, a significant loss in cell viability over the control cells was observed (**Figure 2**). There was no significant difference between the control group and 100 μg/ml 4-MI, so we



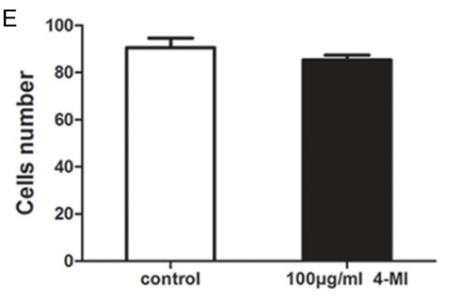


Figure 3. Effect of 4-MI on colony formation. A, B. Representative images of cell colony formation assay. C, D. The morphology of colonies under microscope was shown. Magnifications, 40x. E. Histogram of the number of colonies. Data were listed as mean ± SD of three wells.

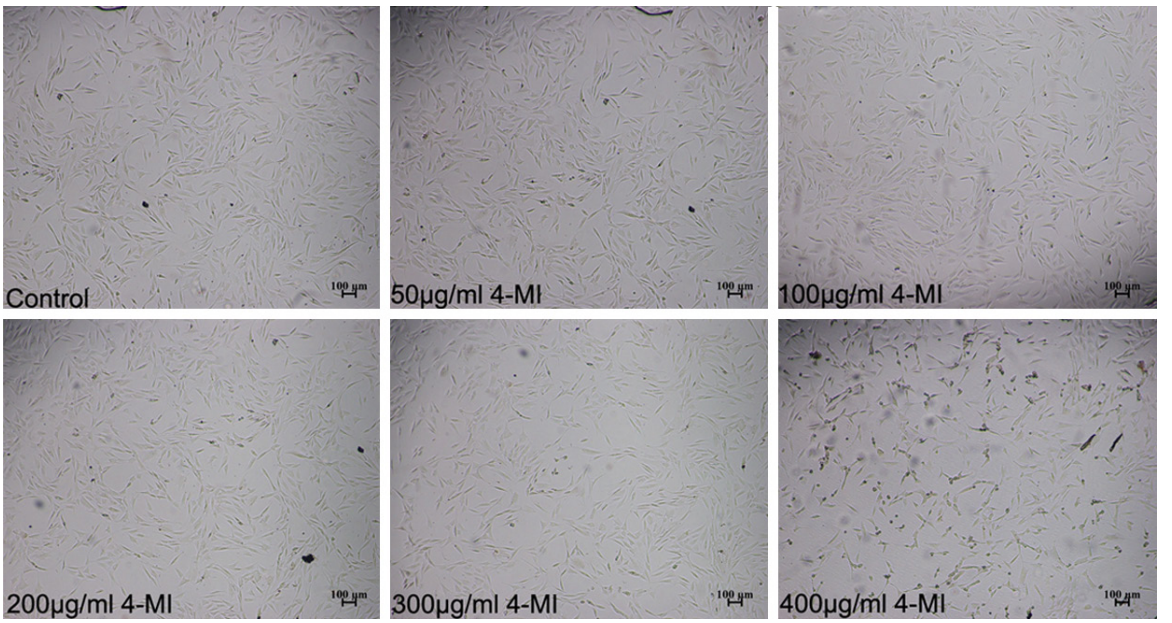


Figure 4. Effect of various concentrations of 4-MI on the morphology of BMSCs. The morphology of BMSCs changed in a dose-dependent manner of 4-MI which ranges from 0 up to the concentration of 400 μg/ml.

chose the concentration of 100 μg/ml for following experiments.

Cell proliferation by colony formation assay: BMSCs in both control group and 4-MI group formed colonies. At lower concentrations (100 μg/ml), 4-MI had no obvious impact on the number or the morphology of colonies, and statistical results show that there was no significant difference between the control group and 4-MI group (**Figure 3**).

Morphological analysis: **Figure 4** demonstrated detached BMSCs morphological view by microscopy. The morphology of BMSCs changed in a dose-dependent manner. In 4-MI treated

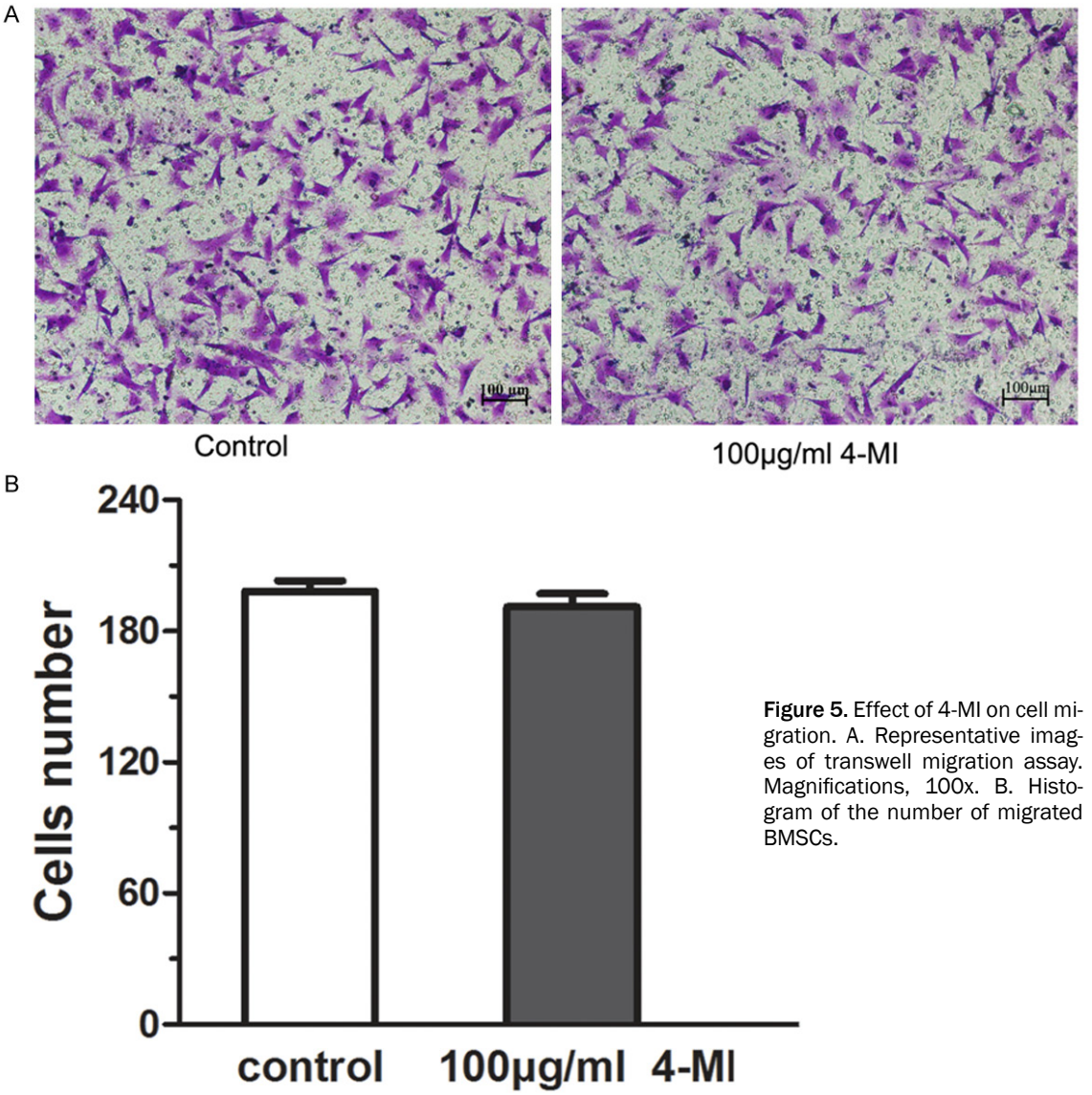


Figure 5. Effect of 4-MI on cell migration. A. Representative images of transwell migration assay. Magnifications, 100x. B. Histogram of the number of migrated BMSCs.

group, the most conspicuous changes were observed in 400 μg/ml 4-MI group including the loss of cell-cell interaction and became round and rough membrane bearing detached cells, whereas these changes were absent in control group or minimal at low treatment level (≤100 μg/ml) of 4-MI.

Effect of 4-MI on cell migration

In this study, we set out to determine whether 4-MI affect the migration potential of the BMSCs. The migrated cells were slightly reduced by 4-MI compared with that of the control group (**Figure 5A**). Statistical analysis showed that there was no significant difference between the control group and the experimental group (**Figure 5B**).

Effect of 4-MI on cell cycle

Flow cytometry was used to determine whether the effect of 4-MI on BMSCs proliferation was mediated, at least in part, by affecting cell cycle progression. The results demonstrated that the percentages of cells pretreatment with 4-MI in G2-M or S phase were marginally increased compared with the untreated BMSCs (**Figure 6A**). It suggested at lower concentration, 4-MI had no obvious effect on cell cycle profile.

Effect of 4-MI-induced apoptosis in BMSCs

To further study the effect of 4-MI on BMSCs apoptosis, cells were stained with Annexin

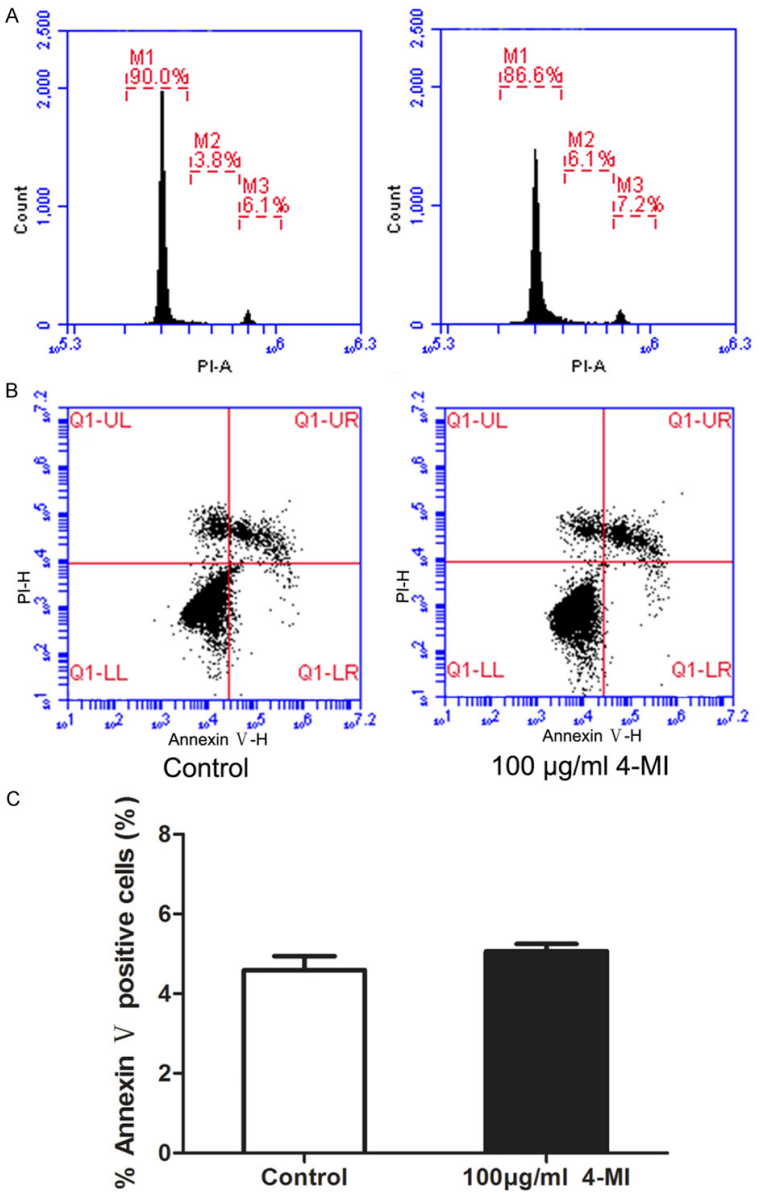


Figure 6. Effect of 4-MI on the cellcycle and apoptosis of BMSCs. A. The DNA content analysis indicated the percentage of cells in the G2/M or S phase were marginally increased compared with the untreated BMSCs. B. Representative scattergrams from flow cytometry profile represents Annexin V-FITC staining in the x axis and PI in the y axis. C. Percentages (%) of Annexin V-positive cells among control or 4-MI treated BMSCs.

V-FITC and PI and then analyzed by flow cytometry. The results showed that the percentage of BMSCs undergoing apoptosis treated with 4-MI were no obvious change compared with that of the control cells (**Figure 6B**), and histogram analysis showed no statistical significance (**Figure 6C**). Thus implying that low concentration of 4-MI probably had no significant effect on cell apoptosis.

Effect of 4-MI on BMSCs differentiation

In order to investigate the effect of 4-MI on the BMSCs differentiation potential, cells were induced to undergo adipogenic or osteogenic differentiation. After differentiation induction, cells were stained with Oil red O or ALP respectively. The percentage of Oil red O positive or ALP positive cells had no significant differences between 100 μg/ml 4-MI group and control group (**Figure 7**). These results indicated that low concentration of 4-MI had no significant effect on BMSCs adipogenic or osteogenic differentiation.

Effect of 4-MI on certain genes of hematogenesis

qPCR was applied for characterization of expression of cytokines in BMSCs, which participated in the process of hematopoiesis. In **Figure 8A**, there had no obvious difference in the control group and experimental group. Real-time PCR results showed that when treated with 100 μg/ml 4-MI, the expression of TPO, SCF, VEGF and FLt3 was slightly decreased, but no statistically significant difference compared with the control group (**Figure 8B**), suggesting that low concentration of 4-MI (≤100 μg/ml) probably had no significant effect on the expression level of above hematopoiesis-supportive genes in BMSCs.

Discussion

Recently, 4-MI has raised great concern among federal and state regulatory agencies because of its toxicity, carcinogenicity and presence in foods and beverages [22-26]. At high doses,

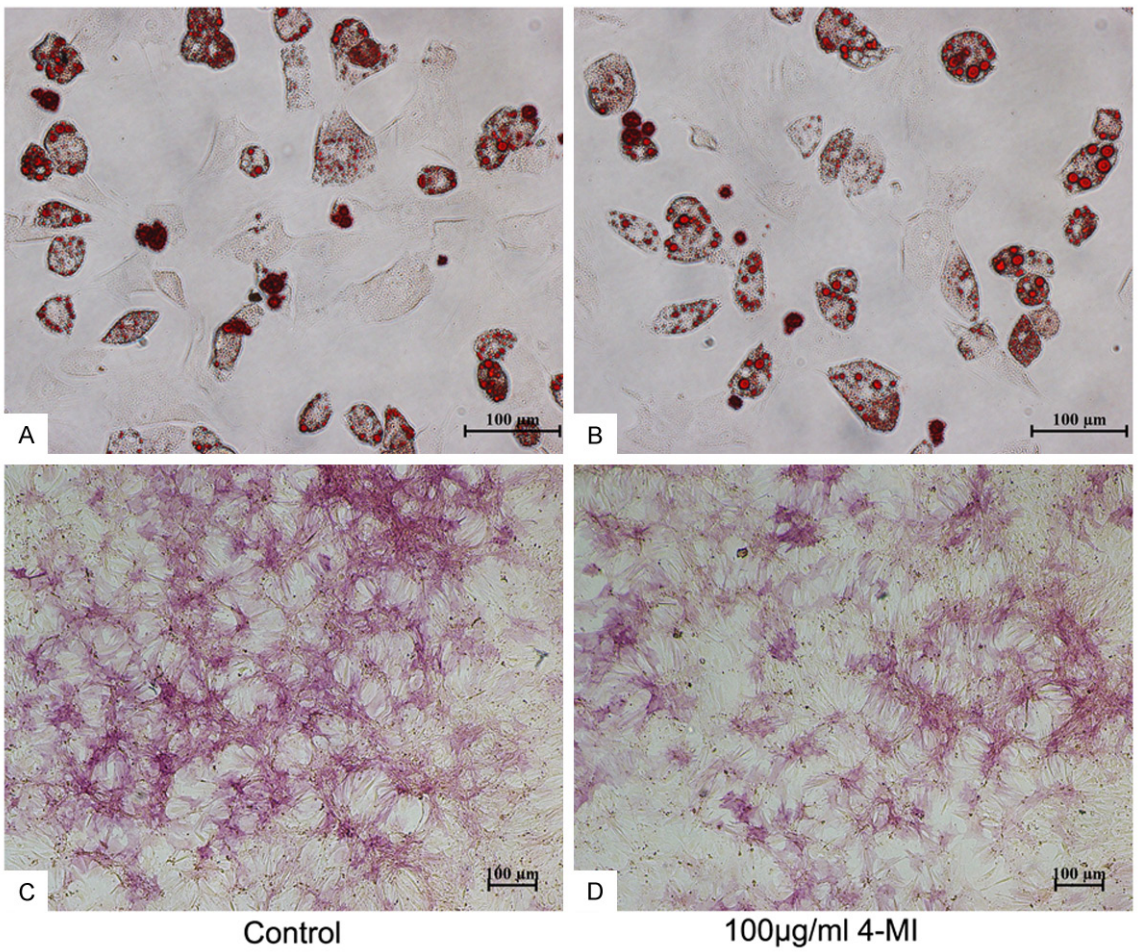


Figure 7. Effect of 4-MI on BMSCs differentiation. Cells were induced towards adipogenic (A, B) and osteogenic (C, D) differentiation, in the presence (B, D) or without (A, C) 100 μg/ml of 4-MI. The presence of Oil Red O and ALP positive was assessed by microscopic observation of histochemical staining. ALP, alkaline phosphatase.

4-MI is neurotoxic in rabbits, mice, cattle and chicks [27-30]. Previously, studies conducted by NTP have provided clear evidence of carcinogenic activity of 4-MI in B6C3F1 mice and F344/N rats [6-8]. Therefore, 4-MI was listed as possible carcinogen and 16 μg per day was set as the "No Significant Risk Level" (NRSL) intake [12]. A 70-year life time exposure beyond 16 μg/day of 4-MI may cause one extra death out of 100,000 people, although 4-MI has been shown to exhibit tumor preventive activity in the rat based upon the results of the NTP bioassay [12, 13, 31, 32].

Whether 4-MI in foods causes damage to human body is currently inconclusive.

4-MI in colas was estimated at a level of 0.36 to 0.76 μg/ml. According to a survey, average American consumption of carbonated soft drinks is about 14 ounces per day [11], and a 12-ounce serving of those drinks would contain 130 μg of the contaminant, which is 8 times higher than the NSRL [33]. However, how this may affect the health of Americans is apparently no conclusion yet.

In this study we aimed to test the biological effects of 4-MI on BMSCs at the concentration between 50 to 500 μg/ml. To investigate whether 4-MI had effects on BMSCs proliferation, we did MTT and colony formation assay. We have shown that 4-MI at the concentration of 100 μg/ml, which is a 100 times higher than drinks, had no obvious impact on BMSCs proliferation and the number or the morphology of colonies. We also performed cell apoptosis and cell cycle detection assay, compared with the

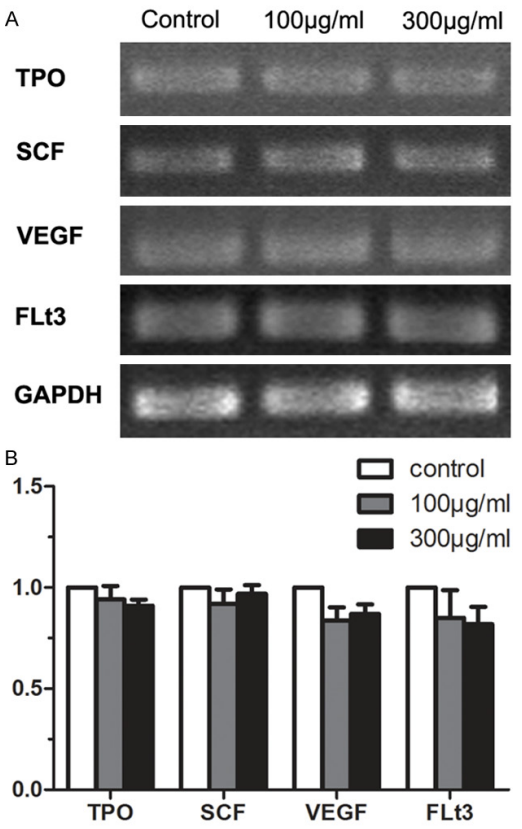


Figure 8. Effect of 4-MI on gene expression. A. Cells were treated with 100, 300 μg/ml 4-MI for 72 h. The mRNA expression of TPO, SCF, VEGF and FLt3 were determined by PCR. B. Real-time PCR analyses of mRNA expression. *P<0.05.

control group, the proportion of apoptotic cells in the treatment group showed slight change and the percentages of cells in G2-M or S phase were marginally increased. These results suggest that relatively low concentrations of 4-MI had no significant effect on BMSCs proliferation and apoptosis. We have also analyzed the cell migration and differentiation ability, and the results showed that low concentrations of 4-MI had slight impact on BMSCs migration. The osteogenic and adipogenic capacity of BMSCs did not diminish either, suggesting that animal weight loss caused by 4-MI was not due to decreased production of adipocytes that derived from BMSCs.

MSCs can produce a number of cytokines, extracellular matrix proteins and express cell adhesion molecules, all of which are critical for hematopoiesis [34], we therefore investigated the effect of 4-MI on hematopoiesis by determining marker expression for a number of the hematopoietic cytokines. No significant difference was detected for genes TPO, SCF, VEGF and FLt3 in cells with or without treatment of low concentration of 4-MI, demonstrating that the anemia caused by 4-MI was not due to inhibition of the hematopoiesis-supportive function of BMSCs.

In conclusion, at lower concentrations, 4-MI (≤100 μg/ml) has no significant effect on the biological characteristics, including proliferation, apoptosis, migration and genes of hematogenesis expression and differentiation of BMSCs. Therefore, relatively low concentration of 4-MI in foods and beverages may have no toxic effect on BMSCs, although further investigation is needed to determine whether prolonged exposure of 4-MI causes cytotoxic effect on BMSCs in vivo, and whether 4-MI influence other aspect of BMSCs.

Disclosure of conflict of interest

None.

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