



Plasma enhance drug sensitivity to bortezomib by inhibition of cyp1a1 in myeloma cells

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Background: Drug resistance is one of the major problems encountered in clinical therapy of multiple myeloma treatment. Combination treatment with several drugs may increase the sensitivity and overcome drug resistance.

Methods: Here, we combined chemotherapy with a newly developed technology, cold atmospheric plasma, to enhance drug sensitivity.

Results: We found that plasma treatment had a synergistic anti-cancer effect with a first line drug (bortezomib). Based on our previous study, we further found that plasma treatment could inhibit Notch pathway and down-regulate cyp1a1 expression and enzyme activity, which contributing to the enhanced drug sensitivity to bortezomib after combination of bortezomib with gas plasma.

Conclusions: Our results showed a new strategy to overcome drug resistance by combination of traditional chemotherapy with cold atmospheric plasma.

Keywords: Cold atmospheric plasma; cyp1a1; drug resistance; multiple myeloma (MM); notch

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Introduction

Multiple myeloma (MM), an incurable B cell disease, is characterized by the accumulation of malignant plasma cells in patients' bone marrow (BM) (1). Patients with MM are at an increased risk of developing infections, anemia, thrombocytopenia, renal failure, and bone disease (2,3). Drug resistance is one of the major problems in MM clinical therapy (4). The interactions between MM cells and the BM microenvironment is considered to play a critical role in MM drug resistance (5). In between, the Notch pathway is important for MM cell growth, migration and drug resistance (6,7). We have demonstrated that Notch activation could induce drug resistance to bortezomib in

murine and human MM cells by up-regulating cyp1a1, which was involved in drug metabolism (8). In this study, we used a new technology, called cold atmosphere plasma (CAP), to treat MM cells (9,10). CAP is mainly consisted of free electron and charged ions, which generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) that could have various biological effects (11,12). Thus, gas plasma has been applied in many biomedical fields such as sterilization, dentistry, cosmetology, wound healing, skin disease and cancer therapy (13-15). Combination of different drugs or therapy for a better anti-cancer effect is a common strategy in cancer therapy. Here, we demonstrated for the first time that gas plasma and bortezomib had a synergistic reduction of cell viability in myeloma cells.

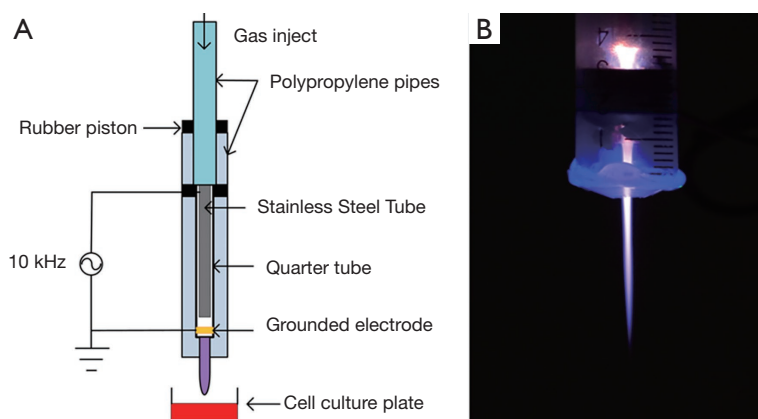


Figure 1 Schematic diagram of gas plasma generation. (A) The structure diagram of a gas plasma jet. (B) The discharging image of He plasma jet.

Gas plasma could enhance the sensitivity to bortezomib in myeloma cells, by inhibiting Notch signaling and *cyp1a1*, which could be applied as a new combination treatment for a better anti-cancer effect and a lower drug side effect.

Methods

Atmospheric pressure plasma jet (APPJ) generation system.

In this study, CAP was produced by a plasma jet device, the schematic diagram of the device structure was shown in *Figure 1A*. It was modified based on a needle. An inner diameter of 4 mm stainless steel tube was used as a high-voltage electrode and gas inlet. The ground electrode was a 10 mm long copper sheet, which wrapped around the quartz tube at a distance of 10 mm from the nozzle. The plasma generation system is consist of a gas flow controller, a high-voltage power supply, oscilloscope, and the above mentioned APPJ device. It was powered by a 10 kHz sinusoidal power supply at 8 kV peak-to-peak voltage and the He gas flow was maintained at 2 SLM. The plasma plume could directly contact with the cell medium surface.

Optical emission spectroscopy

Optical emission spectra (OES) was detected by an Andor SR-750i grating monochromator (grating grooving 1,200 lines mm^{-1}) within a wavelength range of 300–800 nm. The optical fiber was oriented up the APPJ radial direction at a

distance of 1 and 2 cm from the APPJ nozzle.

Cell culture and plasma treatment

LP-1 MM cell line was used in this study and the details could be found in our previous work (16). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, 100 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin (Corning, Ithaca, NY, USA) in an incubator (Thermo Scientific, Waltham, MA, USA) containing a humidified atmosphere of 5% CO_2 at 37 °C. Cells were refreshed 24 h before performing experiments. For plasma treatment, 2×10^5 cells were cultured in 24 well plates in 300 μL RPMI1640 complete medium and were treated with plasma jet 1.5 cm away from the bottom of the plates. After treatment, cells were continually cultured for further experiments. Ethics approval was not required because no clinical experiments were involved in our paper.

Cell viability assay

In this study, we used CellTiter-Glo Assay kit (Promega, Madison, WI, USA) to detect the cell viability, which based on the quantitative determination of ATP in living cells. 100 μL of cells and 100 μL of Cell-Titer-Glo reagent were mixed into the opaque-walled multi-well plate, then cells were incubated at room temperature for 10 min. The luminescence was determined using the microplate reader (Thermo Scientific, USA) with the protocol of “Luminometric” measurement.

Extracellular and intracellular ROS detection

The ROS levels were monitored using CMH2-DCFDA (Invitrogen, USA) following the manufacturer's instruction. After plasma treatment for 24 h, 10 μ M of ROS dye was incubated with cells for 30 min at 37 °C. Extracellular ROS was measured with a microplate reader (Thermo Scientific) with excitation/emission at 485/530 nm using the protocol for "Fluorometric" measurement. Then, the cells were washed three times with PBS and collected for detection of intracellular ROS fluorescence by a fluorescence microscope (Olympus) with blue light motivating.

Real-time PCR analysis

Total RNA was extracted from cells by RNA kit II (Omega Bio-Tec Inc., USA) following manufacturer's instructions and quantified with Nano Drop spectrophotometry (BioTek, USA). We used 2 μ g total RNA to synthesize first strand cDNA by RevertAid first strand cDNA synthesis kit (Thermo Scientific). Real-time PCR was performed on Bio-Rad CFX Connect™ Real-time System (Bio-Rad, USA) and amplified with an optimized cycling condition: 5 min at 95 °C, then 10 s at 95 °C and 30 s at 60 °C for 38 cycles. The total reaction system was 20 μ L: 10 μ L 2 \times QuantiFast SYBR Green PCR MasterMix (Qiagen, Germany), 1 μ L of cDNA templates, 0.5 μ M primer and 8 μ L of DNAase-RNase Free water. The primers were provided by Shenggong Company (Shanghai, China) and the sequences were used in our previous studies (8,17).

Cyp1a1 enzyme activity assay

Twenty four hours after plasma treatment for different time, cells were harvested and washed with PBS for 3 times. Then the cyp1a1 enzyme activity was measured by P450-Glo™ cyp1a1 Assay (Promega, USA) according to the manufacturer's instructions.

Statistical analysis

All values are presented as mean \pm SD of three independent experiments. Differences between groups were evaluated using one-way ANOVA and student T test. $P < 0.05$ was considered statistically significant.

Results and discussion

Generation of He plasma by plasma jet

In this study, we generated the plasma through a device modified by a needle. The profile of the plasma jet device is shown in *Figure 1A*. He plasma was powered by a stainless steel tube with a voltage of 10 kHz/8 kV, which was also used as He gas injection. He gas flow was regulated at a ratio of 2 SLM. *Figure 1B* shows the discharging image of the He plasma jet.

Detection of emission spectra

Since there are many particles with high energy states in the plasma, by detecting their characteristic lines in the emission spectroscopy, the distribution of various particles in the plasma could be understood. The optical fiber was placed 1 and 2 cm away from the plasma jet and the emission spectra was shown in *Figure 2*. Several characteristic lines of N, He and O were marked in the spectra, and the emission intensity is negatively correlated with the distance from the plasma jet.

Plasma enhance the sensitivity to bortezomib

Bortezomib is a first line drug for MM clinical treatment, we investigated whether gas plasma has a synergistic effect with bortezomib in MM cells. The cell cytotoxicity of different concentration of bortezomib was tested by cell viability assay 24 and 48 h after co-incubation (*Figure 3A*). Meanwhile, the reduction of cell viability by plasma treatment for different durations was investigated 24 and 48 h after treatment (*Figure 3B*). We chose 1 and 3 nM concentration of bortezomib and 30 s of plasma treatment for synergistic effects analysis. After 24 h co-treated with bortezomib and plasma, we found that 30 s of plasma treatment could both significantly enhance the sensitivity to 1 and 3 nM of bortezomib treatment (*Figure 3C*).

Plasma increased ROS levels

Biological effects induced by plasma treatment were mostly related to ROS generation. We detected the extracellular and intracellular ROS accumulation after plasma treatment by micro-plate reader and fluorescence

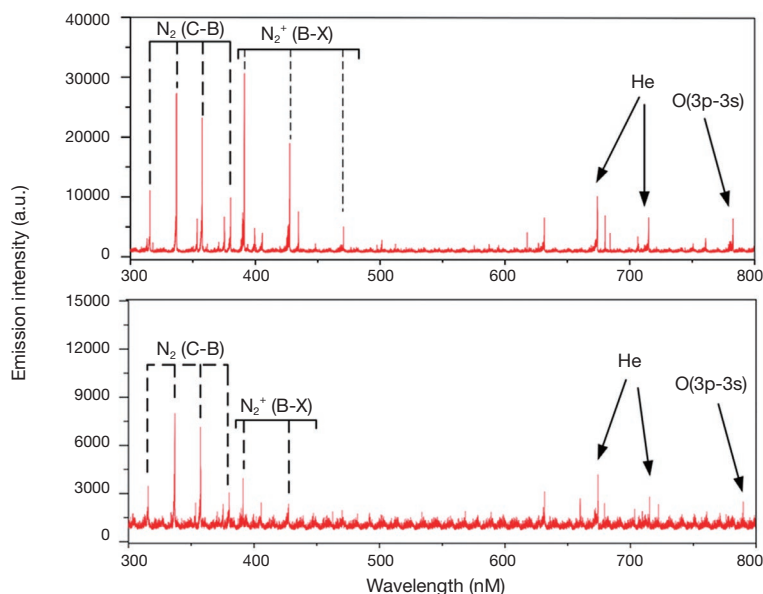


Figure 2 Typical optical emission spectra of the plasma ejected by APPJ at a distance of 1 cm (upper) and 2 cm (lower) from the nozzle.

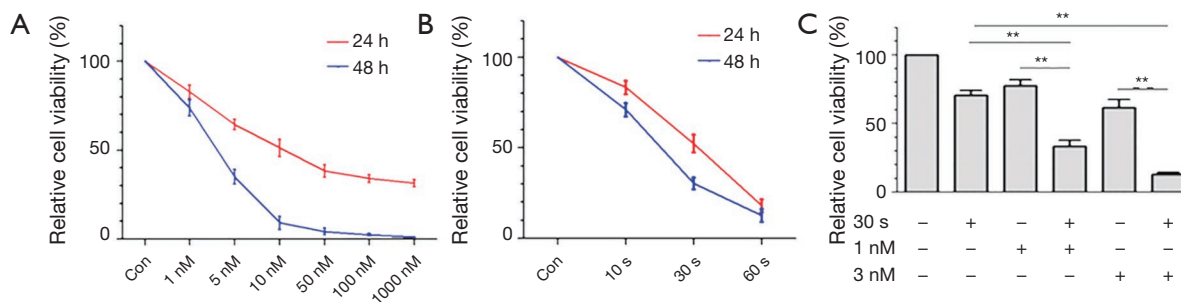


Figure 3 Plasma treatment enhance drug sensitivity to bortezomib in myeloma cells. (A) Cell viability was detected after treatment with different concentration of bortezomib for 24 and 48 h. (B) Analysis of cell viability 24 and 48 h after plasma treatment for 10, 30 and 60 s. (C) Cell viability was determined 24 h after combination treatment of plasma and bortezomib. ** indicates $P < 0.01$.

microscope. It showed that the extracellular ROS level was increased by plasma treatment in a time dependent manner, while the ROS scavenger (NAC) could prevent the ROS accumulation by plasma treatment (Figure 4A). The intracellular ROS was also increased after plasma treatment as the fluorescent intensity was higher than the control and NAC could reverse it (Figure 4B).

Inhibition of Notch and cyp1a1 by plasma treatment

In our previous study, we have demonstrated that cyp1a1, a

Cytochrome P450 enzyme for drug metabolism, is involved in bortezomib resistance in myeloma cells (8). Furthermore, Notch pathway is a critical signaling for regulating cyp1a1 activity. Therefore, we investigated whether plasma treatment had effects on these signaling. By real-time PCR we found that plasma treatment could significantly decrease the expression of Notch downstream target genes such as Hes1, Hes5, Hey1, Hey2 and HeyL (Figure 5A). Meanwhile, plasma treatment could down-regulate cyp1a1 expression in a time depend manner (Figure 5B). Besides, the enzyme activity assay showed that cyp1a1 activity was

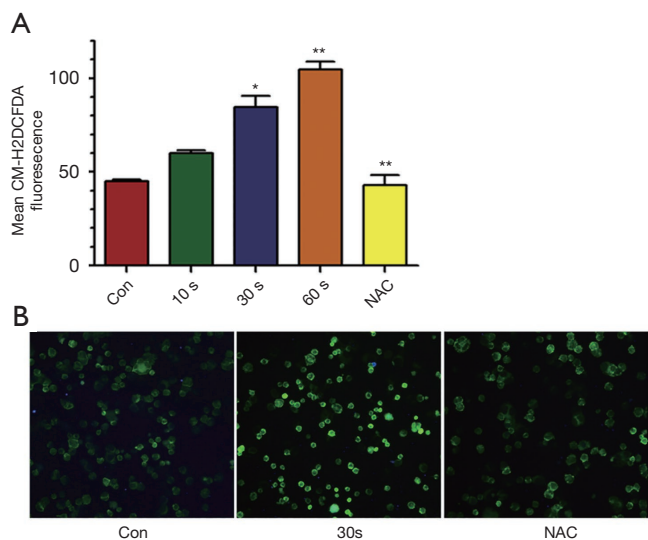


Figure 4 Plasma treatment increased ROS levels. (A) Extracellular ROS level was measured by microplate reader after plasma treatment. NAC is a general ROS scavenger. (B) Intracellular ROS was detected by fluorescent microscope after plasma treatment. *indicates $P < 0.05$, **indicates $P < 0.01$.

suppressed by gas plasma treatment (Figure 5C).

Conclusions

Drug resistance is one of the major problems encountered in clinical therapy of multiple myeloma treatment. Bortezomib is a first line drug used in the standard treatment and has improved clinical outcome. However, some patients do not respond to bortezomib or they eventually relapse after response (18,19). Besides, the 5 years overall survival is still not satisfied and the combination of several drugs to overcome the multiple drug resistance is applied. In this study, we first tried to couple two different kinds of treatment: the chemotherapy and the plasma treatment, to enhance the anti-tumor effects. Gas plasma is a newly developed technology that has been widely applied in biological and medical applications. We found that gas plasma treatment and bortezomib had a synergistic effect on the induction of myeloma cell death, which may lower the drug concentration to avoid the drug side effects in the future. In previous study we found that

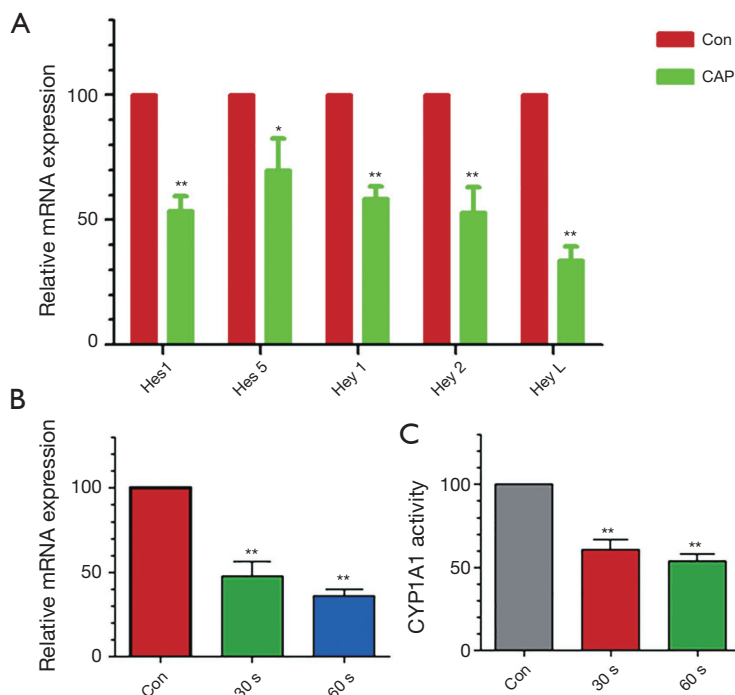


Figure 5 Inhibition of Notch and cyp1a1 by plasma treatment. (A) Expression of Notch target genes by real-time PCR 24 h after plasma treatment for 30 s. (B) Expression of cyp1a1 by real-time PCR 24 h after plasma treatment for 30 s. (C) Measurement of cyp1a1 enzyme activity 24 h after plasma treatment for 30 s. *indicates $P < 0.05$, **indicates $P < 0.01$.

cyp1a1 up-regulated by Notch activation could induce drug resistance to bortezomib in myeloma cells (8), so we detected the Notch signaling and cyp1a1 expression after gas plasma treatment. Interesting, gas plasma treatment did suppress the Notch pathway and down-regulate the cyp1a1 expression and enzyme activity. But how the plasma interact with the Notch pathway remains unknown and it is an interesting work to be further investigated. As we know, plasma treatment induces various biological effects mostly through the generation of many ROS (11). It has been reported that Notch signaling could modulate ROS accumulation especially for H₂O₂ (20,21). To the contrary, ROS could also regulate Notch pathway and affected cell metabolism and cell death (22). In our results, ROS produced by gas plasma discharging could suppress the Notch downstream target genes. Kim *et al.* reported that ROS could down-regulate Notch signaling and induce cell death in breast cancer cells (23). Cao *et al.* demonstrated that inhibition of ROS production could up-regulate intracellular Notch and its downstream effectors (24). These results indicated that Notch might be a downstream target regulated by ROS. Furthermore, we found that cyp1a1, an enzyme involved in drug metabolism, was suppressed by plasma treatment induced Notch inhibition. By promoter analysis we found that there were several classical Notch CSL/RBP-J DNA binding sites (25) in the cyp1a1 promoter region (Supplementary) of either from human species or *Mus musculus* database, indicating that Notch could directly regulate cyp1a1 expression by modulating cyp1a1 mRNA transcription. Our results reported a new strategy to enhance the drug sensitivity by gas plasma treatment, which might be applied in clinical therapy to overcome drug resistance and to reduce side effects during chemotherapy.

In a whole, we used a new technology, the gas plasma, which could generate various ROS, to enhance the sensitivity of myeloma cells to bortezomib treatment. We further pointed out that plasma could inhibit cyp1a1 via Notch signaling and contribute to the synergistic effect with bortezomib.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.10.43>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Ethics approval was not required because no clinical experiments were involved in our paper.

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GAACACTCTTCACTTCAGTCCCTCCTTACAGCCCAAGGTGAGTGCTTGGG 2100
GGGGCTCCTGGATTGCCCTCCCTCCCTCCCTCCCTCCCTCCCTCCCTCC 2150
CAAGAAAAGGAAATTTTGGTGAAGAGTTACTGGGTAGCTCCTTCAAAC 2200
AGGGA ACTACACTTTTATTTATTTATTTATTTATTTATTTATTTAGTGACCAGG 2250
TAAATTCCTTAAGGGAGAACTGAGGCCAGAGGTATCAGCTATTTAGAT 2299

- 1 [37-41] TGGGA
- 2 [176-180] TGGGA
- 3 [477-481] TGGGA
- 4 [656-660] TGGGA
- 5 [951-955] TGGGA
- 6 [1737-1741] TGGGA