



# The Synergistic Effect of Co-delivery of Anticancer Drugs Into Astrocytes Isolated From Human Glioblastoma Multiforme

Mansoureh Hashemi<sup>1\*</sup>, Alireza Zali<sup>1</sup>

<sup>1</sup>Functional Neurosurgery Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

## Abstract

**Background:** Chemotherapy drugs are not effective in the treatment of primary brain tumors due to the low efficacy of these drugs and drug transfer from the blood-brain barrier (BBB) toward the tumor site. Our purpose in this study was to assess the co-delivery of anticancer drugs to increase drug permeability from BBB.

**Methods:** In this study, two chemotherapy drugs, namely methotrexate (MTX) and paclitaxel (PTX), were inserted into polyvinyl alcohol and poloxamer188-conjugated nanoparticles (NPs). Astrocytes were treated with different concentrations of 0-50 µg/ml from MTX, PTX, the MTX-PTX mixture, PTX-loaded NPs, MTX-loaded NPs, and PTX-MTX co-loaded NPs for 48 hours. The tumoricidal effect was assessed using the survival rate, Hoechst staining, and western blotting.

**Results:** The results indicated significant reduction of the survival rate in astrocytes treated with PTX-MTX co-loaded NPs. In addition, apoptosis hallmarks consisting of fragmented DNA, overexpression of Bax, and expression reduction of Bcl-2 were in the cultured astrocytes.

**Conclusions:** Our study proposes that the PTX-MTX co-delivery to NPs could be used as a possible approach for anti-cancer drug delivery to glioblastoma multiforme.

**Keywords:** Paclitaxel; Methotrexate; PEGylated nanoparticle; PLGA; Drugs co-delivery; Synergistic effect; Apoptosis; Glioblastoma multiforme; Anticancer drugs

## \*Correspondence to

Mansoureh Hashemi,  
Functional Neurosurgery  
Research Center, Shahid  
Beheshti University of Medical  
Sciences, Tehran, Iran.  
Tel: +98 2122724214,  
Email: mansoureh.hashemi@  
sbmu.ac.ir

Published online 28 September,  
2020



**Citation:** Hashemi M, Zali A. The Synergistic Effect of Co-delivery of Anticancer Drugs Into Astrocytes Isolated From Human Glioblastoma Multiforme. Clin Neurosci J. 2020;7(4):185-189. doi:10.34172/icnj.2020.23.

## Introduction

Glioblastoma multiforme is the most aggressive and deadliest of malignant brain cancers in humans. Glioblastoma can grow very fast and spread quickly into the surrounding normal brain. This property causes tumor recurrence and decreases the patient survival rate. The current therapies including surgery, radiation therapy, and chemotherapy are not effective in the treatment of glioblastoma patients due to the tumor's biological function in the brain. The median survival rate of patients was reported to be 15 months after prognosis.<sup>1</sup> Despite the advancement of chemotherapy drugs for growth inhibition of cancer cells, drug entrance into the brain is restricted due to the blood-brain barrier (BBB) structure.<sup>2</sup> To overcome this problem, studies have indicated that nanotechnology can help chemotherapy in drug delivery from BBB and increase chemotherapy efficacy through drug transfer to brain tumor cells. Recently, drug delivery carriers with targeting ligands have appeared to increase passage efficiency across BBB.<sup>3,4</sup> However, different targeting ligands have been introduced for drug delivery improvement, and among them is 188 (Pluronic F-68) or polysorbate. Poloxamer

188 (Pluronic F-68) can coat nanoparticles (NPs) to facilitate drug delivery that cannot cross BBB.<sup>5</sup> NPs are synthesized from natural or synthetic polymers with a size of about 100 nm. NPs can deliver various drugs including hydrophilic and hydrophobic small drugs, vaccines, and biologic macromolecules.<sup>6</sup> Among different types of NPs evaluated as delivery vehicles in various studies, poly(lactic-co-glycolic acid) (PLGA) is one of the best polymers due to its non-immunogenic biocompatibility and biodegradability. Moreover, hydrolyzed PLGA has low toxicity in the body.<sup>7-9</sup> Assessing the synergistic effect of methotrexate (MTX) on NPs is a suitable method for reducing drug toxicity.<sup>10,11</sup> In the present study, poloxamer188-conjugated PLGA NPs were synthesized to simultaneously deliver MTX and paclitaxel (PTX or taxol) for brain tumor treatment. PTX and MTX have specific solubility characteristics and various tumoricidal functions due to their antitumor efficiency, and thus, have been used to treat different brain tumors.<sup>12,13</sup> PTX is a drug that inhibits cell growth, and also, is a highly hydrophobic drug that can cause apoptosis via disorganization of cyto-skeleton and inhibit the cell cycle in the late G2 and M phases, thereby blocking cell replication.<sup>14</sup> MTX

is another chemotherapy drug that can inhibit a major enzyme of folic acid synthesis including dihydrofolate reductase. MTX is able to inhibit production of purines and pyrimidines, contributing to growth inhibition of tumor cells<sup>15</sup>. The purpose of this study was to co-deliver two hydrophobic (PTX) and hydrophilic (MTX) drugs to astrocytes isolated from human glioblastoma. Moreover, the synergic effect of PTX and MTX-loaded NPs was evaluated through cytotoxicity and induced apoptosis in the isolated astrocytes.

## Materials and Methods

### Primary Astrocytes Isolated From Human Glioblastoma

Glioblastoma tissue was collected from patients under surgical operation whose glioblastoma was confirmed by a pathologist. Astrocytes were isolated from glioblastoma tissue through digestion with 0.05% trypsin-EDTA for 10 minutes at 37°C water bath. Centrifugation was performed at 180×g for 5 minutes. Then, the astrocytes were cultured in DMEM/F12 medium consisting of 1% (100 U/mL) antibiotic/antimycotic and 2% fetal bovine serum (FBS) and maintained in an incubator. The FBS volume in the culture medium was progressively enhanced from 2% to 10% for 2 weeks.

### MTT Assessment

The toxicity level of the drugs was evaluated through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The astrocytes were seeded in a 96-well culture plate at the density of  $7 \times 10^3$  per well and incubated for 12 hours. The cells were treated with MTX, PTX, the MTX-PTX mixture, PTX-loaded NPs, MTX-loaded NPs, and PTX-MTX loaded NPs at various concentrations of 0, 10, 20, 30, 40, and 50 µg/mL for 48 hours. An amount of 100 µL of DMEM containing MTT was added to each well into cells and incubated at 37°C for 4 hours. The MTT solution was eliminated from the wells, and 100 µL of DMSO was used to dissolve formazan crystals. The absorbance was assessed at 570 nm using a microplate reader.

### Hoechst Staining

The astrocytes were cultured in DMEM/F12 medium containing 10% FBS overnight. Then, the astrocytes were treated with the drugs for 48 hours. Subsequently, the astrocytes were washed with phosphate buffered saline three times, cell fixation was performed with 4% paraformaldehyde, and staining was done with Hoechst 33342 (5 µg/mL) for 20 minutes at 25°C. Finally, the cells were assessed using a fluorescence inverted microscope.

### Western Blot

The astrocytes (density of  $1 \times 10^6$  per well) were cultured and treated for 48 hours. The cells were lysed and

homogenized with RIPA lysis buffer. Protein was loaded in 12% SDS-polyacrylamide gel electrophoresis. Blotting was performed using the wet blotting system, and the membrane blocking was carried out in 5% non-fat milk in TBST for 1 hour. The membrane was treated with the rabbit polyclonal Bax primary antibody (1:200; cat number: sc-493, Santa Cruz Biotechnology Inc., USA), the rabbit polyclonal Bcl-2 primary antibody (1:200; cat number: sc-492, Santa Cruz Biotechnology Inc., USA), and the rabbit polyclonal β-actin primary antibody (1:200; cat number: sc-10731, Santa Cruz Biotechnology Inc., USA) overnight at 4°C. After washing, the membrane was incubated with HRP conjugated anti-goat IgG and HRP-conjugated anti-rabbit IgG (1:10 000; cat number: sc-2004, Santa Cruz Biotechnology Inc., USA) for 1 hour at room temperature in the dark. Finally, protein was exposed on film using a chemiluminescence kit (Amersham Biosciences, Orsay, France).

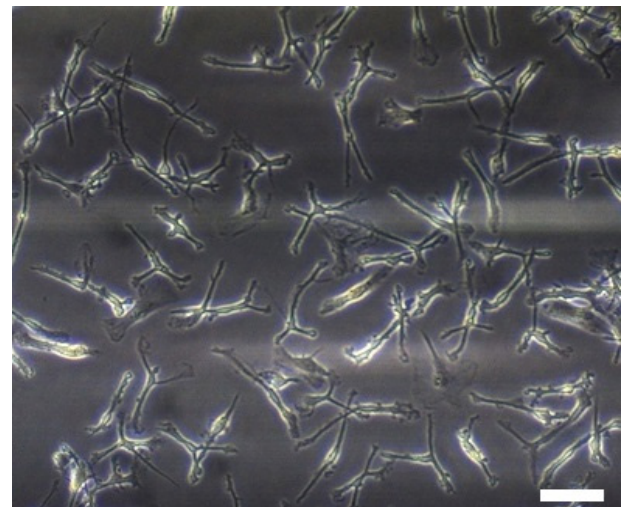
### Statistical Analysis

The data were presented as mean ± standard error of the mean. All the assessments were repeated in three separate experiments. The statistical analysis of the data was performed with SPSS 20 statistical software using one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values less than 0.05 were considered statistically significant.

## Results

### Cell Rate Survival

The astrocytes were isolated from human glioblastoma tissue and treated with different drugs after 14 days (Figure 1). MTX and PTX were the two chemotherapeutic drugs loaded in a similar carrier to assess the co-delivery synergistic effect on the primary astrocytes.



**Figure 1.** Astrocytes Morphology Isolated From Human Glioblastoma.

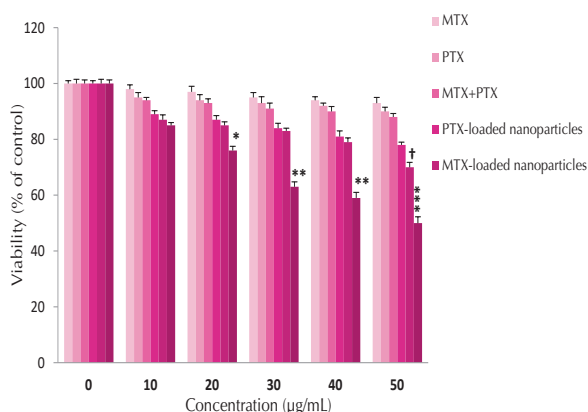
The cytotoxicity level of MTX-PTX co-loaded NPs was compared with those of PTX-NP, MTX-NP, PTX, MTX, and the MTX-PTX mixture at the concentrations of 1-50 µg/mL and the treatment time of 48 hours. The results indicated that the viability of cells was dependent on formulation and concentration of the drugs. As presented in Figure 2, the co-delivery of PTX and MTX significantly decreased the cell survival rate compared to NPs containing free single drug and single drug suggesting carrier entrance into cell and induction of the synergic effect. PTX-NP, MTX-NP, PTX, MTX, and the MTX-PTX mixture induced approximately the same toxicity in the primary astrocytes. Moreover, the IC50 of the co-delivery of PTX and MTX, PTX-NP, MTX-NP, PTX, MTX, and MTX-PTX mixture was 30 µg/mL for 48 hours, which was also selected for the other tests of the study.

### Apoptosis Induced Into Astrocytes With Co-delivery of Tumoricidal Drugs

Cell programmed death was investigated using Hoechst staining, and the results are indicated in Figure 3. The highest amount of apoptosis was observed when the astrocytes were exposed to PTX-MTX co-loaded NPs. The cell membrane was permeable to Hoechst fluorescent stain, and the cell nuclear was observed in red dye indicating apoptotic cells. However, the induced apoptosis level was significantly higher in the treatment groups of PTX-NP, MTX-NP, and the MTX-PTX mixture, as compared to the control.

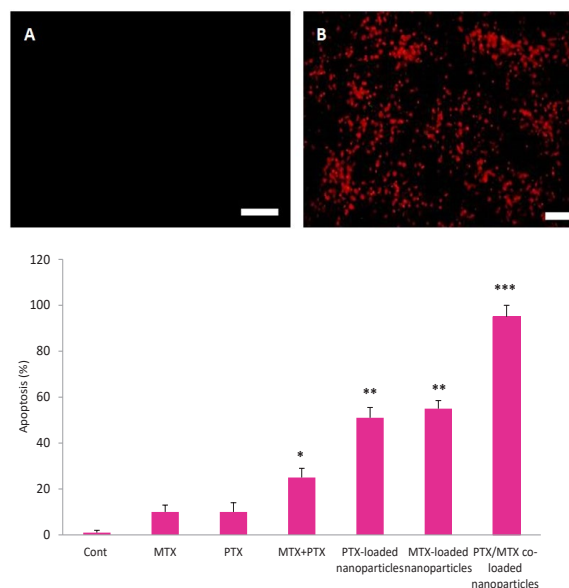
### Western Blot Analysis

We hypothesized that the PTX-MTX co-loaded NPs might reduce cell survival and induce apoptosis. Thus, we assessed the protein expression levels of Bcl-2 and Bax. The results revealed that the Bcl-2 protein expression significantly decreased, but the Bax protein expression

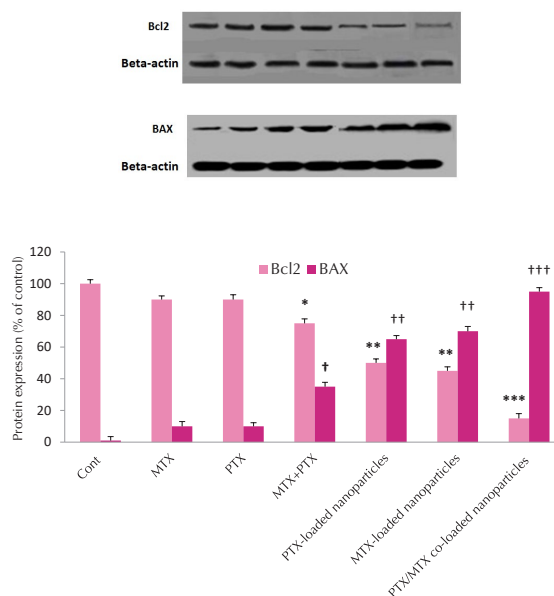


**Figure 2.** Survival Rate of Astrocytes. Viability of the astrocytes reduced with concentration increase of different drugs. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , differences versus control. † $P < 0.05$ , differences versus control.

significantly increased in the astrocytes treated with PTX-MTX co-loaded NPs, PTX-NP, MTX-NP, and the MTX-PTX mixture, as compared to the control (Figure 4).



**Figure 3.** Presentation of Apoptotic Astrocytes by Hoechst Staining. Apoptotic cells were observed red. A) Control: cells are without treatment. B) Apoptotic astrocyte was treated to PTX/MTX co-loaded nanoparticles. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , differences versus control. (scale bar 100 µm).



**Figure 4.** The Proteins Expression Level of Bcl-2 and BAX in the Astrocytes. Apoptotic proteins rate in astrocytes treated with different drugs were assessed in astrocytes through western blot assessment. The expression level of Bcl-2 (A), and BAX (B) were calculated. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , differences versus control. † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$ , differences versus control.

## Discussion

PTX and MTX as two chemotherapeutic drugs were applied in the current study. Chemotherapeutic drug administration is limited to mediate their side effects in the body. The purpose of present study was to perform PTX and MTX co-loading in PLGA NPs conjugated to poloxamer 188 to increase the drug delivery efficiency according to the current formulation. It is critical to select a good nano-carrier according to physiochemical characteristics of chemotherapeutic agents. Therefore, nano-carriers conjugated to poloxamer 188 play a major role in the successful pass of drugs from BBB<sup>16</sup>. Moreover, this nano-carrier can control drug release to increase bioavailability and reduce drug toxicity to normal tissues.<sup>17,18</sup> Combinational therapy was selected for this study to mediate the synergic effect of the tumoricidal drugs. Therefore, a co-delivery system was considered in this study, which was able to simultaneously transfer the two drugs in a nano-carrier toward the primary astrocytes. In addition, PAX and MTX were co-loaded in a nano-carrier to induce the synergic effect. Dominant reduction was observed in the viability of the astrocytes. NPs may pass across the cell membrane through endocytosis or phagocytosis to induce the cytotoxic effect.<sup>19</sup> Several tumoricidal mechanisms might be involved in the synergic effect. MTX appears to stop DNA replication and follow trigger intracellular signaling pathways consisting of apoptosis in astrocytes. PAX is able to inhibit assembly of microtubules and destroy the microtubule network in the cell, thereby leading to cell apoptosis.<sup>20</sup> The results of this study showed apoptosis indexes including DNA fragmentation through Hoechst staining, downregulation of the anti-apoptotic protein, Bcl-2, and overexpression of the apoptotic protein-BAX in the primary astrocytes isolated from human glioblastoma.<sup>21,22</sup>

## Conclusion

In the present study, poloxamer188-conjugated PLGA NPs loaded with MTX and PTX were capable of co-delivery of drugs into cells. Effects of biocompatibility, cytotoxicity, and apoptosis induction were observed in the present study. It appears to apply as a suitable carrier in the drug co-delivery system for brain tumor treatment.

## Conflict of Interest

The authors declare that they have no conflict of interests.

## Acknowledgements

The authors would like to thank from the Functional Neurosurgery Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

## Authors' Contribution

MH done experiments and wrote the manuscript draft. AZ edited and approved the manuscript.

## Funding/Support

This study was supported by Functional Neurosurgery Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

## Ethical Statement

The study was performed according to the principles of the Declaration of Ethical Committee of Shahid Beheshti University of Medical Sciences with ethical code: IR.SBMU.RETECH.REC.272 for molecular and Cellular study.

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