EVALUATION OF *IN VITRO* CYTOTOXIC POTENTIAL OF DIFFERENT CARMUSTINE FORMULATIONS AGAINST U-87 MG HUMAN GLIOBLASTOMA CELL LINE

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ABSTRACT

Glioblastoma (GBM) is a frequent as well as violent type of brain tumor. In this research work, different carmustine formulations were prepared and screened for their cytotoxic activity against U-87 MG glioblastoma and normal human fibroblast L-929 cell lines. The flexible liposomes embedded *in situ* nasal gel exhibited maximum percentage of growth inhibition against U-87 MG glioblastoma cell line, and *in situ* nasal gel exhibited lowest percentage of growth inhibition against U-87 MG glioblastoma cell lines. Flexible liposomes embedded *in situ* nasal gel is observed to be safe and biocompatible against normal human fibroblast L-929 cell line. In cellular uptake study, U-87 MG cell line treated with flexible liposomes embedded *in situ* thermoreversible intranasal gel emitted stronger and higher intensity fluorescence. It seems that the presence of flexible liposomes embedded *in situ* thermoreversible nasal gel inside the tumor cells, and would be the best carmustine delivery approach for the management of GBM.

Keywords: Glioblastoma, alkylating agents, carmustine, rhodamine B, U-87 MG, L-929

INTRODUCTION

GBM is the well-known, life-threatening type of brain tumor. Around 60 % of every major brain tumor is GBM, with an occurrence of seventy-four thousand cases around the globe. The present management for GBM is mainly chemotherapy, radiation and surgery. Still, the present management has not extensively proved better for survival as per the statistics[1]. Surgery and radiotherapy are severely constrained by the sensitive nature of brain tissue, and chemotherapy is further constrained due to the BBB. Although there are many traditional chemotherapeutic medications available in the market, most of them are unable to properly cross the BBB, which prevents them from maintaining the preferred therapeutic efficacy in the brain. Also, rigorous dose-related lethal effects related to usual chemotherapy are additional challenges for the effective management of glioma. New drug delivery approaches viz. polymeric micelles, nanoparticles, and nanoliposomes have been explored extensively in recent years to enhance the effectiveness of usual anti-cancer agents, but very few are successful in clinical trials. Amongst a variety of nano-sized lipid-based delivery, nanocarrier platforms have been proved ideal for effective delivery of toxic anti-cancer medicines to the brain. Because of their higher lipophilicity, and extremely small size, these drug delivery systems meet the main requirements needed to cross BBB and enter the brain[2-6].

Many cell viability assays focus on cellular metabolism. For evaluation of cytotoxicity, surviving cells against dead cells in a sample are compared and cell response against cytotoxic drugs is obtained. This assay consists of enzymatic transformation of a dye precursor in living cells against dead cells. The response is further illustrated and quantified by using colorimetric techniques[25].

Carmustine is a cell-cycle phase, nonspecific, alkylating anti-cancer drug and has been used in the management of brain tumors. It inhibits protein synthesis by causing cross-linking in the nucleic acids. In spite of its short half-life with serious harmful impacts viz. bone marrow depression with pulmonary fibrosis, it is used in the management of glioma. Hence, there is a necessity to implement new approaches for impactful delivery of carmustine to the brain, thereby decreasing side effects due to usual drug delivery system of carmustine viz. gliadel wafers (Gliadel®)[7-9]. Carmustine or bis-chloroethyl nitroso urea (BCNU) gliadel wafer is the single FDA-

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approved intracerebral chemotherapeutic medicine for the management of malignant glioma. After the removal of brain tumors by surgery, Gliadel® is placed in that location. But with poor penetration of carmustine, incapability to avoid tumor reappearance along with lack of synergistic activity with additional chemotherapeutic medicines and/or radiotherapeutic drugs, and ineffective therapeutic efficacy, these gliadel wafers are not very successful[7, 10].

The goal of the present work was to study cytotoxicity of carmustine flexible liposomes, *in situ* nasal gel, flexible liposomes embedded *in situ* nasal gel and carmustine API solution towards U-87 MG glioblastoma cell line and normal human fibroblast L-929 cell line. A cellular uptake study of carmustine formulations was performed with encapsulating Rhodamine B dye and qualitatively estimated using fluorescence microscopy (fluorescent microscope of Leica Microsystems). A cellular uptake study was performed to check uptake of carmustine intracellularly, and, thereby, corresponding biological and therapeutic responses.

MATERIALS AND METHODS

U-87 MG human glioblastoma cell line and normal human fibroblast L-929 cell line were obtained from the National Center for Cell Science (NCCS) Pune, Maharashtra, India.

In vitro cytotoxicity study

In vitro cytotoxicity test on human glioblastoma U-87 MG & L-929 cell lines

The *in vitro* cytotoxicity tests of carmustine formulations viz. flexible liposomes, *in situ* nasal gel, flexible liposomes embedded *in situ* nasal gel, and carmustine API solution were performed on human glioblastoma U-87 MG cell line as well as L-929 cell line and assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method. Around 10^3 cells per 96-well plate were seeded in a flat-bottom microplate; later, complete media was added to make the volume 150 µL, and kept at 37 °C using 5 % CO_2 and 95 % humidity overnight[26-28]. Then, 200 µL of different concentrations viz. 100, 50, 25, 12.5, 6.25, 3.125, 1.562 µg mL^{-1} of test formulation were added. The wells were cleaned two times by using PBS (phosphate buffer solution), 20 µL of the MTT solution was mixed to every well, and then the plate was incubated at 37 °C for 4 h. The supernatant was slowly detached and discarded without disturbing the formazan crystals. After 4 h, 100 µL of DMSO (dimethyl sulfoxide) was mixed in every well to liquify the formazan crystals; finally the absorbance was recorded at 570 nm with the help of a microplate reader.

The % cell viability was determined by the below equations:

% cell viability = Mean O. D. of test compound / Mean O. D. of negative control x 100

where, O. D. is the optical density

After that, the half maximal inhibitory concentration (IC_{50}) was determined[11-18].

Cellular uptake study

Rhodamine B was employed as a fluorescent agent which is embedded during the carmustine formulation to estimate the uptake efficiency, localization of formulation and qualitative analysis by fluorescence microscopy. U-87 MG cells were placed into a 12-well plate at a density of 6 x 10^4 cells/well with incubation overnight at 37 °C. The medium was then altered to a free cell culture medium containing different preparations cultured for different times with equivalent quantity of Rhodamine B (1 h, 3 h, and 6 h). The cells in the control sample were left untreated. Following the designated durations, cells were fixed for 10 minutes with 4 % paraformaldehyde before being washed three times with PBS buffer (pH 7.4). By using fluorescent microscopy, cellular uptake of carmustine formulations in human glioblastoma U-87 MG cell line was measured[19-24].

RESULTS AND DISCUSSION

The safety and biocompatibility of flexible liposomes, *in situ* thermoreversible intranasal gel, flexible liposomes embedded *in situ* thermoreversible intranasal gel, and carmustine API solution were tested on human L-929 fibroblast cell line. Based on the viability of normal human L-929 fibroblasts, cell lines were incubated together various concentrations like 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg mL^{-1} and exposed to cytotoxic activity (Table I).

Based on an *in vitro* cytotoxicity study on normal human L-929 fibroblast cell line, the carmustine API solution and flexible liposomes were toxic to normal human L-929 fibroblast cell line at high concentrations whereas *in situ* thermoreversible intranasal gel and flexible liposomes embedded *in situ* thermoreversible intranasal gel were not found to be toxic in the concentration ranges 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg mL^{-1} [Fig. 1(a) and Fig. 1(b)]. The formulations viz. *in situ* thermoreversible intranasal gel and flexible liposomes embedded *in situ* thermoreversible intranasal gel, were observed to be safe on normal

Table I: Effects of carmustine formulations on normal human L-929 fibroblast cell line

Sr. No.	Dose (µg mL^{-1})	% Cell viability of negative control (n=3)	% Cell viability of carmustine API solution (n=3)	% Cell viability of flexible liposomes (n=3)	% Cell viability of *in situ* nasal gel (n=3)	% Cell viability of flexible liposomes embedded *in situ* nasal gel (n=3)
1	100	100	69.8	68.6	75.9	97.4
2	50	100	83.9	85.2	87.6	91.9
3	25	100	98.6	93.5	91.5	81.4
4	12.5	100	87.8	87.6	98.8	87.8
5	6.25	100	93.9	96.9	88.3	95.2
6	3.12	100	93.5	93.1	83.1	94.3
7	1.56	100	84.8	91.4	90.3	92.5

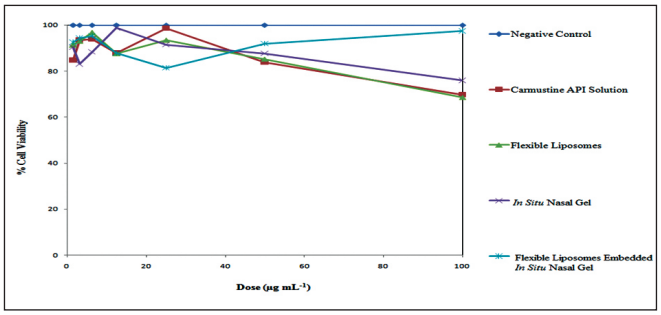

Fig. 1(a): Comparative *in vitro* cytotoxicity study of flexible liposomes, *in situ* thermoreversible intranasal gel, flexible liposomes embedded *in situ* thermoreversible intranasal gel, and carmustine API solution, tested on human L-929 fibroblasts cell line. The results were expressed as Mean ± SEM for n = 3

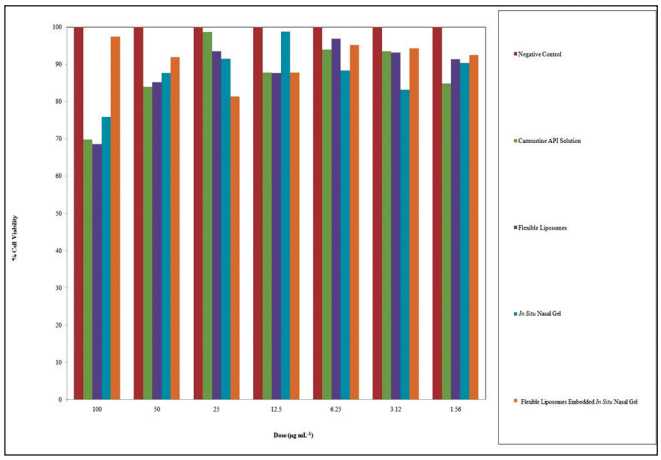

Fig. 1(b): Comparative *in vitro* cytotoxicity study of flexible liposomes, *in situ* thermoreversible intranasal gel, flexible liposomes embedded *in situ* thermoreversible intranasal gel, and carmustine API solution, tested on human L-929 fibroblasts cell line. The results were expressed as Mean ± SEM for n = 3

human L-929 fibroblast cell line. It could be due to carmustine being encapsulated by polymers during formulation of gel. Hence, these *in situ* thermoreversible intranasal gel and flexible liposomes embedded *in situ* thermoreversible intranasal gel were biocompatible.

MTT assay was performed to find out the cytotoxicity of flexible liposomes, *in situ* thermoreversible intranasal gel, flexible liposomes embedded *in situ* thermoreversible intranasal gel, and carmustine API solution against human glioblastoma U-87 MG cell line and its performance was compared. Then, the preparations were incubated with the cancerous cell lines for 24 h and the cell viability % was estimated [Fig. 2(a) and Fig. 2(b)].

A negative control group was incubated with human glioblastoma U-87 MG cell line to verify the normal performance of cell line. The percentage cell viability of the control group was found to be almost 100 %. The % cell viability of *in situ* thermoreversible intranasal gel on human glioblastoma U-87 MG cell line was observed to be 66.9 % at 100 µg mL^{-1} and % cell viability of flexible liposomes embedded *in situ* thermoreversible nasal gel on human glioblastoma U-87 MG cell line was observed to be 44 % at 100 µg mL^{-1}. It may be because of carmustine in flexible liposomes embedded *in situ* thermoreversible nasal gel increasing the ability of carmustine to enter in U-87 MG cell line. These observed results are a noticeable sign of better antitumor action of final optimized flexible liposomes embedded *in situ* thermoreversible intranasal gel of carmustine drug delivery system compared to the carmustine API solution, flexible liposomes, and *in situ* thermoreversible intranasal gel (Table II).

Table II: Effects of carmustine formulations on human glioblastoma U-87 MG cell line

Sr. No.	Dose (µg mL^{-1})	% Cell viability of negative control (n=3)	% Cell viability of carmustine API solution (n=3)	% Cell viability of flexible liposomes (n=3)	% Cell viability of *in situ* nasal gel (n=3)	% Cell viability of flexible liposomes embedded *in situ* nasal gel (n=3)
1	100	100	59.4	55.6	66.9	44
2	50	100	64.6	55.8	70.3	49.5
3	25	100	74.7	58.7	73.6	52.6
4	12.5	100	78.6	59.7	88.6	61.5
5	6.25	100	80.8	60.8	83.6	68.4
6	3.12	100	86.6	61.4	80.4	69.8
7	1.56	100	87	68.7	88	78.6

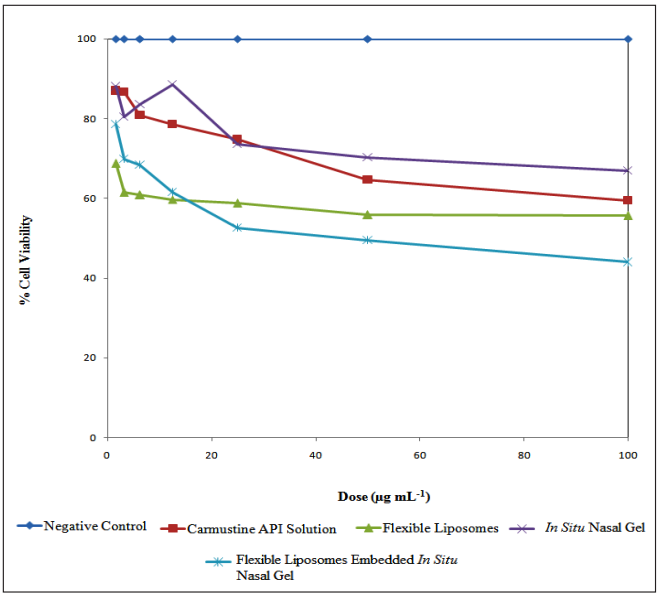

Fig. 2(a): Comparative *in vitro* cytotoxicity study of flexible liposomes, *in situ* thermoreversible intranasal gel, flexible liposomes embedded *in situ* thermoreversible intranasal gel, and carmustine API solution, tested on human glioblastoma U-87 MG cell line. The results were expressed as Mean ± SEM for n = 3

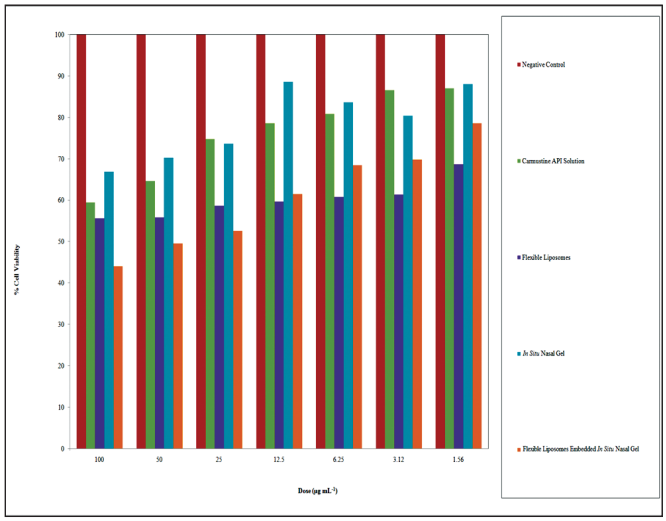

Fig. 2(b): Comparative *in vitro* cytotoxicity study of carmustine API solution, flexible liposomes, *in situ* thermoreversible intranasal gel, and flexible liposomes embedded *in situ* thermoreversible intranasal gel, tested on human glioblastoma U-87 MG cell line. The results were expressed as Mean ± SEM for n = 3

For the evaluation of cytotoxicity of carmustine formulations, the median inhibitory concentration (IC_{50}) value was determined. The IC_{50} data (Table III) shows that flexible liposomes embedded *in situ* thermoreversible intranasal gel promoted a 2.65-fold rise in cytotoxicity of carmustine in the human glioblastoma U-87 MG cell line as compared to only *in situ* thermoreversible intranasal gel, while a 1.90-fold rise in cytotoxicity of carmustine in the human glioblastoma U-87 MG cell line as compared to carmustine API solution, and 2.23-fold rise in cytotoxicity of carmustine in the human glioblastoma U-87 MG cell line as compared flexible liposomes (Fig. 3) was observed. It

Table III: IC_{50} values for human glioblastoma U-87 MG cell line exposed to carmustine API solution, flexible liposomes, *in situ* thermoreversible intranasal gel, and flexible liposomes embedded *in situ* thermoreversible intranasal gel

Sr. No.	Formulation	IC_{50} (µg mL^{-1})
1	Carmustine API solution	122
2	Flexible liposomes	143
3	*In situ* thermoreversible intranasal gel	170
4	Flexible liposomes embedded *in situ* thermoreversible intranasal gel	64

The data are shown as Mean ± SEM for n = 3

means that the optimized carmustine flexible liposomes embedded *in situ* thermoreversible intranasal gel is effective to act as a preparation, with marked cytotoxic impact in glioblastoma cells.

Fluorescence microscopy was employed to qualitatively estimate the uptake of the carmustine API solution, flexible liposomes, *in situ* thermoreversible intranasal gel, and flexible liposomes embedded *in situ* thermoreversible intranasal gel by human glioblastoma U-87 MG cell line (Fig. 4). To study the ability of carmustine formulations to be internalized in human glioblastoma U-87 MG cell line, the preparation containing a fluorescent dye (Rhodamine B) was incubated with human glioblastoma U-87 MG cell line for 1, 3 and 6 h. The cellular uptake of carmustine-containing formulations occurred in a time-dependent manner. Human glioblastoma U-87 MG cell line treated with flexible liposomes embedded

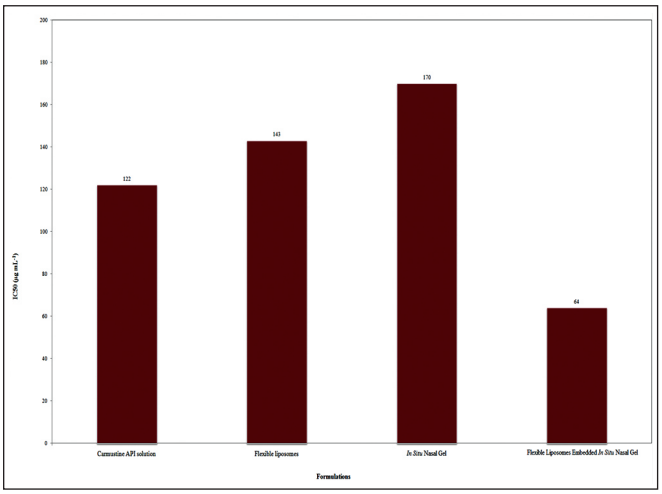

Fig. 3: Comparative IC_{50} values for human glioblastoma U-87 MG cell line exposed to carmustine API solution, flexible liposomes, *in situ* thermoreversible intranasal gel, and flexible liposomes embedded *in situ* thermoreversible intranasal gel. The data are shown as Mean ± SEM for n = 3

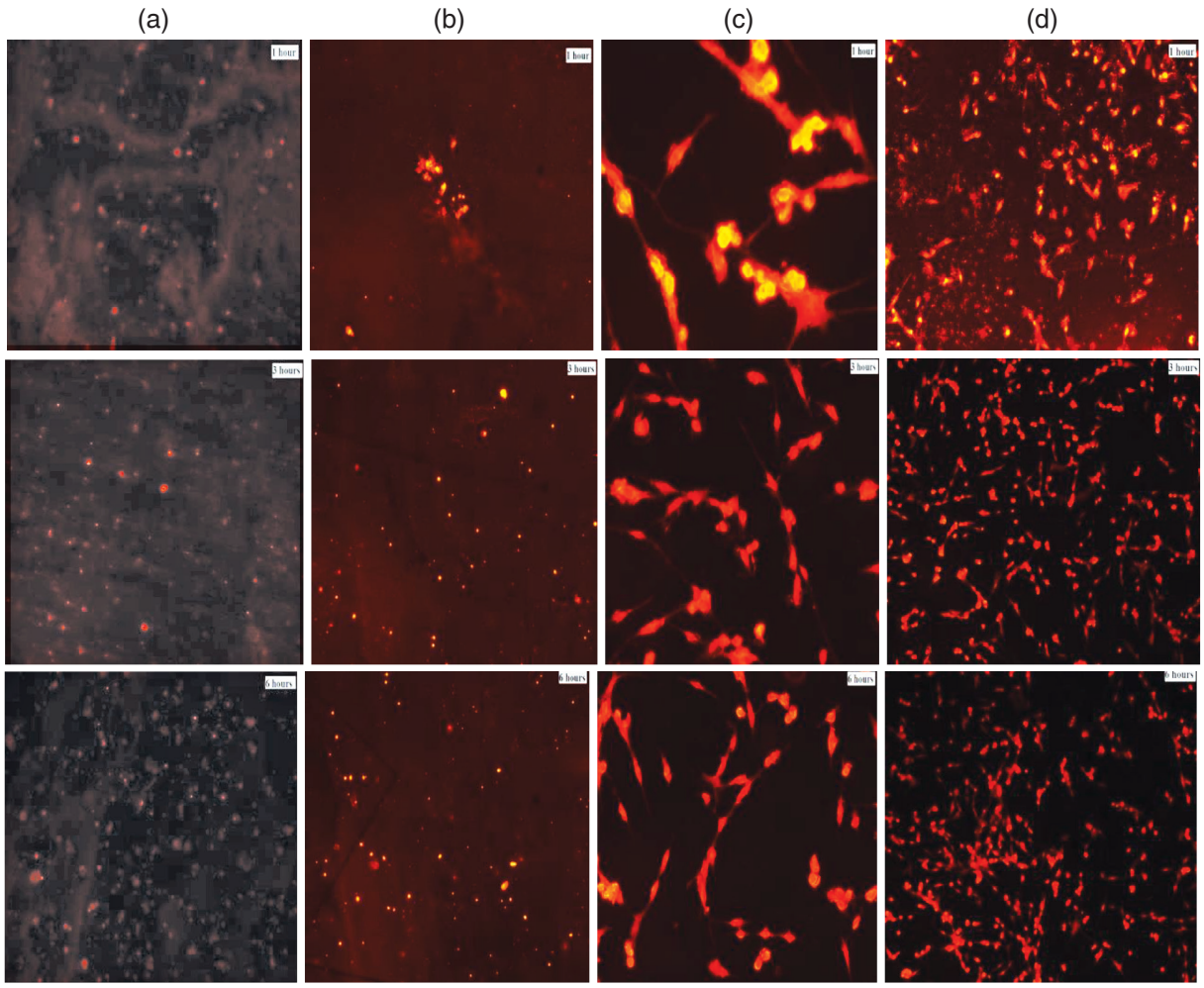

Fig. 4: Cellular uptake study of (a) carmustine API solution, (b) flexible liposomes, (c) *In situ* thermoreversible intranasal gel, (d) flexible liposomes embedded *in situ* thermoreversible intranasal gel. The fluorescence intensity measuring rhodamine B-labeled human glioblastoma U-87 MG cell line uptake after 1, 3, and 6 h is shown

in situ thermoreversible intranasal gel emitted stronger and higher intensity fluorescence as compared to those treated with carmustine API solution, flexible liposomes, and *in situ* thermoreversible intranasal gel. It indicates the presence of flexible liposomes embedded *in situ* thermoreversible nasal gel inside the cells and an effective carmustine delivery approach for the management of GBM. It could be due to more lipophilic property of flexible liposomes embedded *in situ* thermoreversible nasal gel sufficiently permeating through the U-87 MG cell line line, which is again a good finding towards effective use of the formulation for the management of glioma. It also proved that the optimized flexible liposomes embedded *in situ* thermoreversible nasal gel formulations were internalized in human glioblastoma U-87 MG cell line in a high amount, indicating the ability of the carmustine-containing formulation to convey into glioma cells to perform subsequently its antitumor action. The therapeutic impact against GBM depends on this substantial cellular absorption.

CONCLUSION

Cytotoxic evaluations of different formulations of carmustine were carried out in this research work. Amongst all carmustine formulations, flexible liposomes embedded *in situ* thermoreversible intranasal gel was found to be more cytotoxic to human glioblastoma U-87 MG cell line as compared to other formulations. This is also supported by cellular uptake study which shows increased penetration of carmustine in human glioblastoma U-87 MG cell line. The *in situ* thermoreversible intranasal gel and flexible liposomes embedded *in situ* nasal gel were observed to be safe, and biocompatible on normal human L-929 fibroblast cell line. Thus, flexible liposomes embedded *in situ* nasal gel of carmustine found to be safer and effective drug delivery system in the treatment of GBM.

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