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In-Vitro Screening of Etoposide and Its Thermosensitive Hydrogel in Lung Cancer (L132) Cell Lines

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KEYWORDS

Lung Cancer, Thermosensitive Hydrogel, Etoposide, L-132 Cell line, MTT Assay.

ABSTRACT:

Introduction: Cancer causes genetic abnormality (mutation) in healthy cells which leads overgrowth of abnormal cells leads to the development of tumors. In lung cancer, abnormal cell overgrowth occurs, which are vital organ for breathing. Sustained release drug delivery system is comparatively better than the conventional drug therapy because the conventional methods for delivery fail to achieve desire therapeutic concentrations and effectiveness of drugs, despite reaching toxicity, hydrogel overcomes the shortcomings of the conventional delivery. Thermosensitive hydrogel (Poloxamer 407) of etoposide are excellent candidates for long term depot formations in our body. Etoposide (DNA Topoisomerase-II inhibitor) is one of the most commonly used drugs in the chemotherapy of cancer.

Objective: To carried out in-vitro screening of etoposide and its thermosensitive hydrogel in lung cancer (L132) cell lines.

Methods: This study was carried out to evaluate cell viability & proliferation activity on Lung cancer cell line (L132) by etoposide hydrogels & other chemotherapeutic drugs. Fresh L132 cell lines were observed under microscope for any contamination. Cell lines were incubated for 24 hours in a CO2 incubator (Heraeus Hera cell) at 37 °C and 5% CO2. In vitro cell viability assays with cell lines are mainly used for drug screening. For MTT Assay, 100 µl of DMEM media was placed in 96-well plates. 98 µl L132 cell line was then added into the DMEM media and incubated for 24 hours at 37°C and 5% CO2 in a CO2 incubator. The average value were determined from reading & proliferation indices & percentage proliferation were calculated.

Results: Anticancer activity of hydrogels were compared with different established chemotherapeutic drugs by MTT method and etoposide hydrogel 2 showed maximum anticancer activity when compared to all the established chemotherapeutic drug including standard etoposide with a percentage of inhibition of cancerous cells of 147.09% (0.63±0.007). The standard etoposide showed 125% percent inhibition of cancerous cells (0.536±0.011). Etoposide hydrogel 1 showed 117.8% inhibition of lung cancer cells (0.502±0.014). Anticancer activity of etoposide hydrogel 1 was found to be statistically very significant when compared to etoposide hydrogel 2, Dacarbazine, Ifosfamide with a p value of <0.001 and significant when compared to normal control with a p value of <0.05.

Conclusion: Etoposide loaded Thermosensitive hydrogel could be effectively used for continuous release of drug. The present study will not only approach the cancerous cells but could be able to

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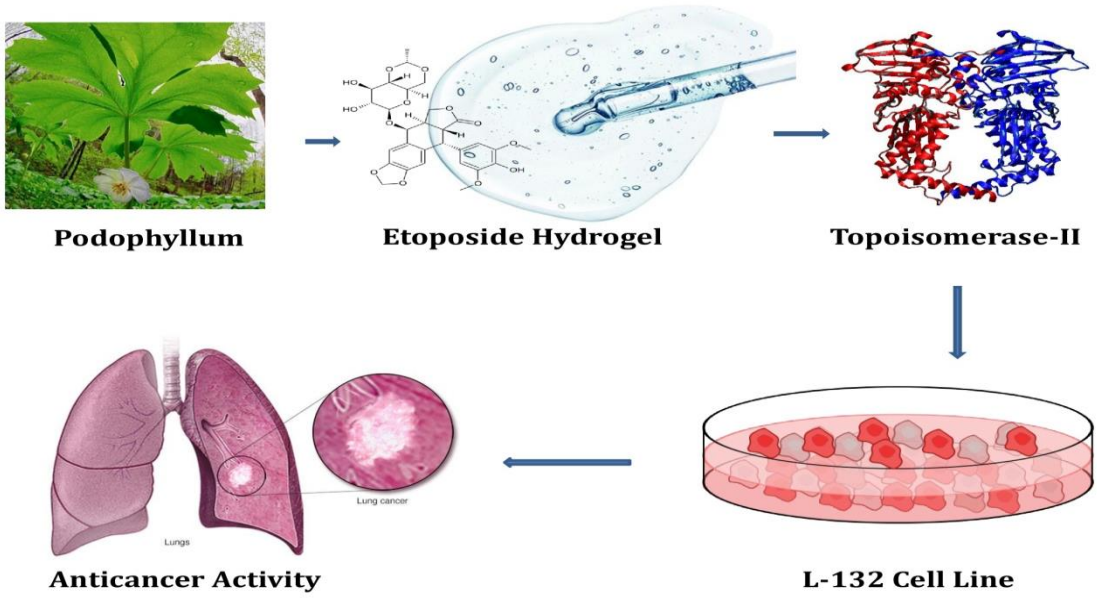
protect normal cell cellularity & morphology.

Graphical Abstract



INTRODUCTION-

Cancer is a group of diseases that can start in any tissue or organ of the body when abnormal cells spread uncontrollably to invade adjoining parts of the body and spread to other organs or tissues.[1] According to WHO, Cancer is the 2nd leading cause of mortality. Globally, approx 9.6 million deaths in 2018 & 1 in 6 deaths is because of cancer. Cancer starts from the mutation of a normal gene called oncogenes.[2]

Lung cancer is the most deadly cancers for males and females. 2 basic types of lung cancer are small-cell lung carcinoma (15%) & non-small-cell lung carcinoma (85%).[3] Lung cancer primarily caused by smoking, tobacco and its products. Some other factors like exposure of air pollution, asbestos, radon gas, chronic infections can grow lung cancer. Multiple mechanisms like inherited & acquired of susceptibility to lung cancer.[4]

American Mayapple toxin podophyllotoxin derivative Etoposide synthesized firstly in 1966 and USFDA approval for cancer therapy in 1983.[5] Etoposide targets Topoisomerase-II which stabilizes a covalent topoisomerase II-cleaved DNA intermediate complex wand inhibit DNA synthesis leading to instability of genome as well as abnormal cell death.[6]

Hydrophilic gel called Hydrogel constitute a group of cross linked polymeric materials, which is capable of holding large amounts of water in their 3D configurations. Hydrogels ability to absorb water starts from its hydrophilic functional groups which is attach to the polymeric backbone.[7]

Thermo-sensitive hydrogels contain hydrophobic and hydrophilic component and have been developed widely due to the controllable release of drug is sensitive to changes in temperature and have many advantages, like site-specific, increased solubility of lipophilic drugs, simple drug formulation & its administration, sustained release behavior and delivery of various types of drugs. When injected,

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thermosensitive hydrogel work as an implantable depot device for sustaining the release of the entrapped drug.[8]

Poloxamer based hydrogels are non-ionic, water soluble triblock copolymer made up of lipophilic residue of polyoxypropylene between two hydrophilic units of polyoxyethylene (POE). These are excellent candidates for long term depot formulations because of excellent water permeability, biocompatibility & also exhibit an interesting reversible thermal character. They are liquid at room temperature but convert into gel form when administered at body temperature, which makes them valuable candidates for pharmaceutical drug delivery system.[9] Poloxamer 407 is block copolymers of poly (ethylene oxide-b-propylene oxide-b-ethylene oxide) (PEO-PPO-PEO, Pluronic 127) is used for low toxicity, solubilizing capacity, drug-release characteristics and its compatibility with other biomolecules & chemical excipients. It is liquid when refrigerated in an aqueous solution (3-5°C) but convert into gel around 30-32°C which is ideal candidate for thermosensitive delivery.[10]

Material and Method:

(A) Sample collection

(i) Etoposide hydrogels were obtained from Rajiv Gandhi College of Pharmacy, Bhopal

(ii) Lung cancer cell lines (L132) were obtained from NCCS, Pune, Maharashtra.

(b) Culture of cell lines:

Fresh L132 cell lines were observed under microscope for any contamination. Cell lines were incubated for 24 hours in a CO_2 incubator (Heraeus Hera cell) at 37 °C and 5% CO_2. After 24 hours, medium from the cell lines were removed & rinsed with 5ml of trypsin solution (0.5%). Flasks were allowed to sit at room temperature until the cells detached from it. The disaggregated cells were centrifuged at 2100 rpm for 20 min. for removal of trypsin. The pellets were suspended in fresh complete DMEM medium and dispensed into new sterile culture flask.[11]

(c) *In-vitro* cell viability assay:

In vitro cell viability assays with cell lines are mainly used for drug screening.

Trypan Blue Assay

Preliminary cell viability test was performed using the trypan blue dye assay. 10µl of Cancerous cell line (L132) was placed in laminar air flow and 10µl of 0.4% trypan blue stain was added thoroughly. It was allow standing for 5 min at 20^0C-30^0C. Neubaurs chamber were filled with the help of micropipette for cell counting. The chamber was observed under a microscope for stained non-viable cells while viable cells excluded the stain.[12]

Calculated the number of cells/cubic millimeter of sample as follows,

Number of cells = Cells counted × 20 × 10 (depth factor) / 4

Number of cells = cells counted × 50

MTT Assay

100 µl of DMEM media was placed in 96-well plates. 98 µl L132 cell line was then added into the DMEM media and incubated for 24 hours at 37°C and 5% CO_2 in a CO_2 incubator. After 24 hours, 30 µl of different drugs were placed in well and 96 well plate was incubated for 24 hrs in CO_2 incubator. After completion of 24 hrs, 5mg/ml concentration of MTT dye in PBS (pH 7.2-7.4) buffer was prepared in UV treated dark room. 30µl of MTT dye solution was placed in each wells & placed on shaking table for 5 min in order to mix properly. 96 well plates were incubated at 37^0C & 5% CO_2 for 3-4 hrs. After incubation, 150µl of the sample was discarded and 150 µl of 5% DMSO was added to each well & placed on shaking table for 5 min so that formazan is thoroughly mixed with solvent. The plated cover was removed & absorbance was measured in each well including blank, vehicle, control & standard at 490 nm at test & reference respectively in ELISA (Lisaquant).[13] The average value were determined from reading & proliferation indices & percentage proliferation were calculated by following;

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a) Proliferation indices (Ratio) = Abs (Test) × 100 / Abs (Control).
b) Percentage of proliferation = Abs (Test) × 100 / Abs (Control).

Result and Discussion:

This study was carried out to evaluate cell viability & proliferation activity on Lung cancer cell line (L132) by etoposide hydrogels & other chemotherapeutic drugs.

Trypan Blue Assay

Cell viability is counted by trypan blue assay &
Calculated the number of cells / cubic millimeter
Number of cells in 10 µl = 343 × 50
Number of cells in 10 µl = 17150
Number of cells in 3000 µl = 51,45,000 cells

MTT Assay:

Anticancer activity of hydrogels were compared with different established chemotherapeutic drugs by MTT method and etoposide hydrogel 2 showed maximum anticancer activity when compared to all the established chemotherapeutic drug including standard etoposide with a percentage of inhibition of cancerous cells of 147.09% (0.63±0.007). The standard etoposide showed 125% percent inhibition of cancerous cells (0.536±0.011). Etoposide hydrogel 1 showed 117.8% inhibition of lung cancer cells (0.502±0.014). Anticancer activity of etoposide hydrogel 1 was found to be statistically very significant when compared to etoposide hydrogel 2, Dacarbazine, Ifosfamide with a p value of <0.001 and significant when compared to normal control with a p value of <0.05. Anticancer activity of etoposide hydrogel 2 was found to be significant when compared to normal control, Dacarbazine Cyclophosphamide, Ifosfamide, Cisplatin, Paclitaxel with a p value of <0.001 and significant when compared to standard etoposide with a p value of <0.01.

Table 1: Absorbance of different chemotherapeutic drugs and etoposide hydrogel on Lung cancer cell line (L132)

	1	2	3	4	5	6	7	8	9	10
A	0.408	0.511	0.478	0.651	0.444	0.473	0.474	0.617	0.497	0.157
B	0.419	0.583	0.518	0.602	0.467	0.472	0.443	0.601	0.445	0.208
C	0.454	0.506	0.474	0.658	0.344	0.458	0.472	0.537	0.499	0.180
D	0.399	0.592	0.508	0.653	0.451	0.464	0.405	0.551	0.325	0.180
E	0.415	0.551	0.587	0.609	0.420	0.528	0.501	0.524	0.288	0.183
F	0.478	0.510	0.551	0.642	0.440	0.482	0.435	0.560	0.302	0.193
G	0.462	0.526	0.470	0.626	0.459	0.486	0.400	0.567	0.284	0.201
H	0.415	0.533	0.479	0.636	0.447	0.472	0.442	0.524	0.321	0.192

Where,
A1-H1 = Normal control A2-H2 = Standard etoposide A3-H3 = Etoposide hydrogels 1
A4-H4 = Etoposide hydrogels 2 A5-H5 = Cyclophosphamide A6-H6 = Cisplatin
A7-H7 = Paclitaxel A8-H8 = Distilled water A9-H9 = Dacarbazine
A10-H10 = Ifosfamide

Table 2: Average & percentage of absorbance of different chemotherapeutic drugs in comparison to etoposide hydrogel on Lung cancer cell line (L132)

S.NO.	GROUP	SAMPLE	ABS. (Average)	Percentage	Mean ± SEM
1.	A1-H1	Normal control	0.431	100%	0.425±0.010
2.	A2-H2	Standard etoposide	0.539	125.05%	0.535±0.011
3.	A3-H3	Etoposide hydrogel 1	0.508	117.86%	0.502±0.014

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4.	A4-H4	Etoposide hydrogel 2	0.634	147.09%	0.630±0.007
5.	A5-H5	Cyclophosphamide	0.434	100.69%	0.430±0.135
6.	A6-H6	Cisplatin	0.479	111.13%	0.475±0.007
7.	A7-H7	Paclitaxel	0.446	103.48%	0.443±0.012
8.	A8-H8	Distilled water	0.560	129.93%	0.556±0.012
9.	A9-H9	Dacarbazine	0.370	85.84%	0.365±0.032
10.	A10-H10	Ifosfamide	0.186	43.15%	0.183±0.005

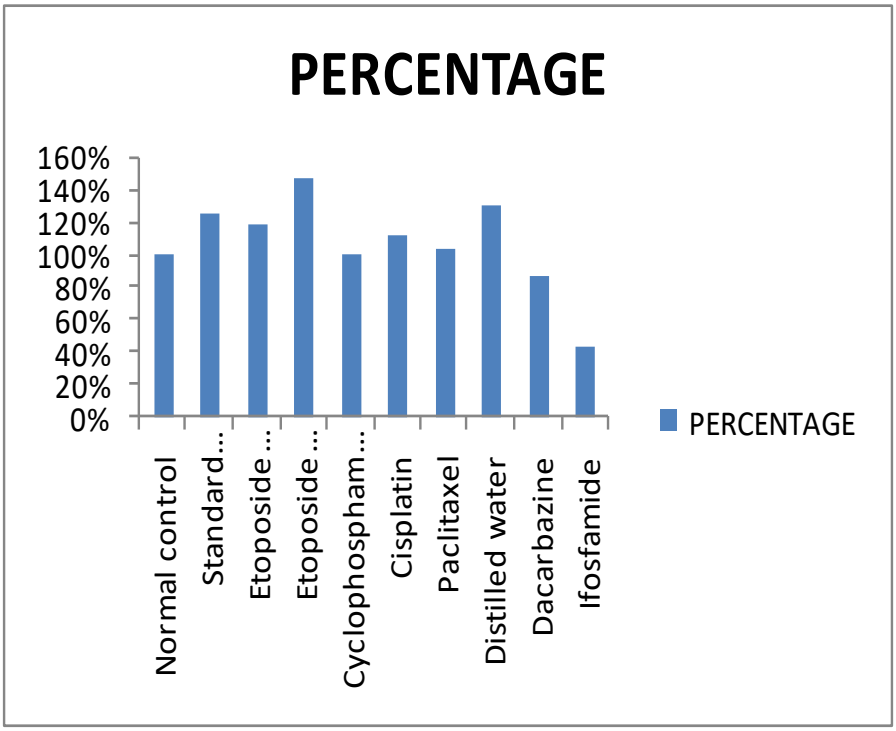

Fig 1: Percentage of absorbance of different chemotherapeutic drugs in comparison to etoposide hydrogel on Lung cancer cell line (L132)

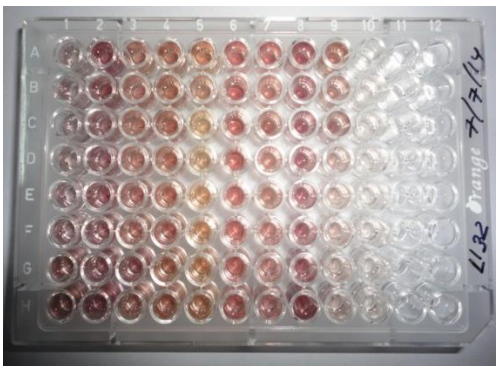

Fig 2: MTT assay of lung cancer cell line (L132) day 1

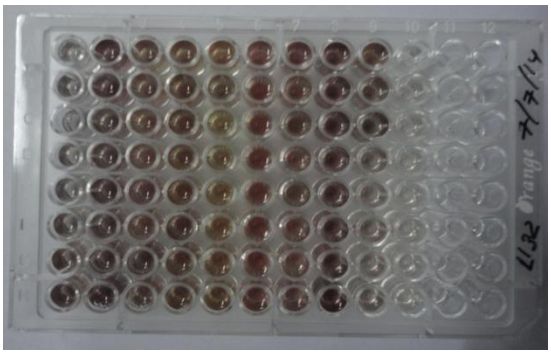

Fig 3: MTT assay of lung cancer cell line (L132) day 2

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Conclusion:

The present study is a comparative study between old conventional therapeutic regimen and modern approach of sustain release of drug i.e. hydrogels. It demonstrates that the hydrogel of etoposide have effectively approach the cancerous cells to inhibit its potentiality. Such drug delivery is in demand in the field of oncomedicine where the conventional drug causes huge number of cellular as well as organ toxicity. The present study will not only approach the cancerous cells but could be able to protect normal cell cellularity & morphology.

Conflict of interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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