Genotoxicity of Noscapine Nanosuspension Prepared by Microfluidic Reactors on HepG2 Cell Line

Azarian, Maryam [•]
Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, I.R. IRAN

Amani, Amir [••]
Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, I.R. IRAN

Faramarzi, Mohammad Ali
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, I.R. IRAN

Eidi, Akram
Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, I.R. IRAN

Divsalar, Adeleh* [+]
Department of Cell and Molecular Biology, Faculty of Biological Sciences, Kharazmi University Tehran, I.R. IRAN

ABSTRACT: *Noscapine is an antispasmodic alkaloid used as antitussive and anti-cough obtained from plants about Papaveraceae family which this benzylisoquinoline alkaloid and its synthetic subsidiaries (called noscapinoids) are being assessed for their anticancer potential. The present research aimed to investigate the induction of DNA destruction and viability of HepG2 tumor spheroid culture influenced by noscapine and nanosuspension of noscapine. Culture of HepG2 cells as spheroids was treated with different concentrations of noscapine for 24 h on Day 11. Afterward, viability assay and alkaline comet assay methods were applied to examine the viability and induced DNA destruction, respectively. Based on the results, no significant impact was observed from Tween 40 on the viability and DNA damage levels in comparison with the control ($p > 0.05$). Moreover, increasing noscapine concentration resulted in a dose-dependent reduction in viability of hepatic cancer cells and elevation of DNA damages, showing a correlation between rises of DNA damages and viability decline.*

KEYWORDS: *Genotoxicity; Noscapine; Nanosuspension; Microfluidic reactors; HepG2.*

***** *To whom correspondence should be addressed.*
+ E-mail: divsalar@khu.ac.ir
• Other Adrress : Department De Bioquimica I De Biologia Molecular, Institut De Biotecnologia Biomedicina (BB), Universitat Autonoma De Barcelona, Barcelona, SPAIN
•• Other Address: Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, I.R. IRAN
1021-9986/2020/5/145-155 14/$/6.04

INTRODUCTION

A number of in-use substances for cancer chemotherapy such as vinca alkaloids (vincristine, vinblastine, and vindesine) [1], paclitaxel [2], and estramustine [3], specifically inhibit development of cell cycle. This causes metaphasic arrest through interacting with tubulin as major protein of mitotic spindles. As a result, polymerization or depolymerization of tubulin is altered, mitosis in quickly dividing cells is arrested, and apoptosis is induced, hence, they are effectively used in cancer chemotherapy [1,3]. Nonetheless, a problem commonly associated with repeated and prolonged administration of chemotherapeutic agents is drug resistance, which may be caused by multiplication of a membrane glycoprotein which mediates drug outflow [4]. Anti-microtubule agents are reported to be highly toxic to normal tissues, thus may be used only for particular cancerous cells [5-7]. It is therefore necessary to find novel and more effective chemotherapeutic drugs. The chemical structures of several microtubule inhibitors were compared to determine potential new anti-microtubule drugs. It was also pointed out that lots of such chemicals have a hydrophobic trimethoxyphenyl group and various hydrophobic domains including lactone, tropolone, or other aromatic rings [8]. Noscapine, [L-a-methyl-8-methoxy-6, 7-methylenedioxy-1-1(6, 7-dimethoxy-3-phthalidyl)-1, 2, 3, 4 tetrahydroisoquinoline], a non-narcotic derivative of opium [9] has anti-microtubule activity [10]. Induced apoptosis and antitumor activity against examined murine and human solid tumors have also been noted for this drug [8,16]. It has no analgesic, sedative or respiratory depressant activities, nor produces euphoria or addiction [15]. Researchers have lately reported possible stoichiometric binding of noscapine to tubulin to improve microtubule polymerization, inhibiting growth of tumor cells throughout mitosis [8, 16].

In recent years, a prominent role for nanosuspensions has been highlighted in novel drug delivery systems [17-20]. As opposed to conventional drug delivery systems, nanosuspensions have shown benefits such as augmented solubility and dissolution rate, improved bioavailability [21] and enhanced biological activity [22]. Nanoemulsions are typically prepared via two common methods: use of an anti-solvent to precipitate drug molecules in a solution, and application of elevated shear forces (e.g. milling) to form finer particles from coarser ones [17].

Microfluidics can be considered both a science and a technology. It is defined as the study of fluid behavior at a sub-microliter level and the investigation into its application to cell biology, chemistry, genetics, molecular biology and medicine [25]. Liquids in such devices flow within channels with characteristic interior diameters of below 1 mm. Liquids flow linearly into microfluidic channels and develop a diffusion interface in the channel midpoint. Exposure of an anti-solvent to the solvent flow leads to diffusion of the drug molecules in the solvent through the interface (i.e. a diffusion layer), resulting in nucleation and size increment [26]. Using this technique, processes such as enzymatic reactions or extraction of active components could benefit from enlarged surface area to volume ratio. The striking function of such systems is also advantageous in handling solutions with micro- and nano-liter volumes. In comparison with other methods for nanosuspension formation, microfluidic reactors also present an inexpensive procedure with production of minimum residue [27] and commonly yield a monodispersed product [28, 29]. It is possible to control the growth/precipitation rate by surfactants or polymers in such apparatuses [30].

The present study aimed to produce a noscapine nanosuspension using microfluidic reactors and investigate factors that may influence size of nanoparticles using Artificial Neural Networks (ANNs). The nanosuspensions were then used to improve cellular drug delivery and examine cytotoxic and genotoxic impacts of encapsulation of noscapine against spheroid model of HepG2 hepatic carcinoma cells [31]. HepG2 is a well-known cell line with ability to self-assemble into large, stable spheroids aided by combi ned intercellular communication and diffusion [32].

EXPERIMENTAL SECTION

Noscapine (pharmaceutical grade) was kindly donated by Behansar Pharmaceutical, Iran. Acetonitrile (HPLC grade) and polysorbate 40 (Tween 40) were purchased from Sigma-Aldrich (Germany).

Nanoprecipitation in microfluidic reactor

To make noscapine nanosuspension by microfluidic reactor, saturated solutions of noscapine in acetonitrile at predetermined temperatures were injected into the reactor at certain flow rates of solvent/antisolvent. The antisolvent

system was water at controlled room temperature (i.e. 22 ± 2°C) and contained different concentrations of Tween 40. Hydrodynamic micropumps were employed to inject very small volumes of fluid. The microreactor was made of polylactide (polylacetic acid) with internal diameter of 1 mm and an inlet angle of 30°, 60°, 90°, 120° and 180°. Therefore, four different input variables were considered in this study: longitude output arm, angle between two entrances (inlet angle), antisolvent flow rate and Tween 40 concentration, which were assumed to potentially influence the sedimentation of prepared nanoparticles. To determine factors affecting stability of the prepared nanosuspension, time of sedimentation was taken as an indicator of physical stability of the nanosuspension. The prepared samples were sealed and kept at controlled room temperature (i.e. 22 ± 2°C) and observed daily to determine phase separation of prepared nanodispersions. The data were then employed to evaluate impact of the variables on the sedimentation time using a model obtained from ANNs.

Cell line

Human HepG2 hepatic carcinoma cells were obtained from Pasteur Institute, Iran, and incubated in RPMI 1640 (Gibco) containing 10% Fetal Bovine Serum (FBS) (Biosera), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Biosera).

Culture of spheroids

Liquid overlay technique was applied for culture of spheroids. The cells (n= 5×10^5) were placed into culture Petri dishes (100 mm) thinly layered with 1% agar (Bacto Agar) supplemented with RPMI (10 mL) plus 10% FBS. The Petri dishes were maintained in a dampened milieu with 5% CO_2 at 37 ºC. Fresh culture medium was used to replace half the culture medium two times in a week.

Treatment of spheroid culture with noscapine and noscapine nanosuspension

Multicellular spheroid formation was obtained by cell culture. After 10 days, various concentrations (0, 50, 75, and 100 µM) of free noscapine and noscapine nanoparticles were added to the spheroids (average diameter of 100 µm) for 24 h. Dimethyl sulfoxide (DMSO) was used to dissolve noscapine with a final concentration of 0.25% (v/v). The control cultures were treated with a similar volume of DMSO. Subsequently, 300 µl of 1mM EDTA/0.25% Trypsin (w/v) in PBS was added to the spheroid cells for 5 min at 37 ºC. After counting of single cells, their viability was tested through Trypan blue dye exclusion assay.

Alkaline comet assay

Alkaline comet assay was used to verify induction of DNA damages, which was a modification of the method previously developed by *Singh et al.* [33]. Agarose (1%) with a normal melting point was used to coat ordinary microscopic slides. The suspension contained nearly 10,000 cells in 100 µl of 0.5% agarose with a low melting point. Using a pipette, the cell suspension was quickly transferred to the agarose layer. The solidified slides were submerged in lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris-base with 1% Triton X-100, pH=10, prepared freshly) and incubated for an hour. Since this step onward, the whole procedures were performed at 4 ºC. After removal of the slides from the lysis buffer, they were transferred to a horizontal gel electrophoresis tank (Cleaver Scientific Ltd, CSLCOM20) filled with fresh cold denaturation buffer (300 mM NaOH, 1 mM EDTA, pH=13) and maintained for 30 min. The same denaturation buffer was used to conduct electrophoresis with a voltage of 1V/cm and a current of 300 mA for 30 min. To neutralize the excess alkali, the slides were subsequently washed in Tris buffer (0.4 M Tris HCl, pH=7.5), after which they were stained by ethidium bromide (20 µg/mL). A fluorescent microscope (Zeiss, Axioskop 2 plus) was employed to view and photograph individual cells or comets, which were analyzed by Comet Score® software. Evaluation of DNA damages was based on an increase in tail moment, the product of DNA level (fluorescence) in the tail, and the distance between the means of the head and tail fluorescence distributions.

Evaluation of DNA damage

Individual cells (n=100) on each slide (three slides per sample) were scored through visual assignment to one of the five above-mentioned classes depending on the tail length by appointing values of 0 (no tailing) to 1, 2, 3, or 4 (Max. tailing). The comets could be totally scored in a range of 0 (all no tailing) to 400 (all utmost tailing):

$$DD\ (au) = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / (\Sigma n /100)$$

The variables in the above equation are as follows: DD (au): arbitrary unit DNA damage score, n_0-n_4: number of Class 0-4 comets, and Σn: total number of scored comets. Each class of comet is weighted by factors of 0-4 coefficients [34, 35]. Such a visual classification may be doubted to be low-grade compared to computerized analyses, including CCD camera image analysis of tail moment. Evaluation of DNA damages was based on an increase in tail moment, the product of DNA density (fluorescence) in the tail, and the distance between the average distribution levels of the head and tail fluorescence.

Statistical analyses

Data were presented using mean values ± SEM (standard error of mean) and "n", indicating the number of experimental attempts. SPSS (Version 23.0) software was applied for statistical analyses, i.e. one-way analysis of variance (ANOVA), then, Turkey's test as the post-hoc analysis, considering significant values when $P < 0.05$.

RESULTS AND DISCUSSION

Validation of the model using statistical methods

The following attributes were recommended to assess performance of the ANN model [36]:

1) A model output of $|R| > 0.8$ represents significant correlations among the predicted and real values.

2) A model output of $0.2 < |R| < 0.8$ indicates a correlation among the predicted and real values.

3) A model output of $|R| < 0.2$ denotes an insignificant correlation among the predicted and real values.

MSE value should be minimal in all conditions [37]. According to the findings, very precise predictions were provided for both the training (MSE=0.0002) and testing (MSE=0.00005) datasets by the MLP model (Table 1).

External validation of the model was also examined in terms of newly suggested factors [38] using the testing dataset. A minimum of one slope of the regression lines (k or k′) through the origin is recommended to approximate to 1 [39]. k and k′ are slopes of the regression lines between the regressions of actual output (h_i) against predicted output (t_i) and t_i against h_i through the origin, i.e. $h_i = kt_i$ and $t_i = k'h_i$, respectively. Additionally, the functioning indices of m and n (as two factors for model performance assessment) should be below 0.1. A confirmed indicator (R_m) was recently presented for the external predictability of models [40]. The condition for $R_m > 0.5$ is acceptable. It is necessary that either the squared correlation coefficient (through the origin) between predicted and experimental values (R_0^2), or the squared correlation coefficient between experimental and predicted values ($R_{0'}^2$) to approximate to R^2 and 1 [37, 41, 42]. Table 2 represents the validation criteria studied and the pertinent findings gained from the model.

R should exceed 0.8 for the first item, which is 0.9979 in the established model. k should range from 0.85 to 1.15 for the second item. The k value is 1.05 in the created model indicating a satisfactory condition. k´ should be in the range of 0.85 and 1.15 for the third item. The k′ value (0.957) for the ANN model lies in the above limit. m and n values need to be below 0.1 based on items 4 and 5. The developed model yielded values of 0.098 and 0.046 for m and n, respectively. Eventually, R_m demands values above 0.5, for which a value of 0.61 was obtained by the established model. It was therefore observed that the model developed fulfills the whole conditions required. A strong suitability and applicability of the recommended model is endorsed by this validation phase.

Characterization of nanoparticles

Zeta sizer (company, country) was employed to measure the size and size distribution of the prepared nanosuspension using DLS (dynamic light scattering). As shown in Fig. 1, diameters of nanoparticles averaged 20 nm. Zeta potnetial of the particles was also measured as -1.44 mV.

Morphological examinations

Characterizing the morphology of nanoparticles was carried out by TEM measurements. The images of noscapine nanoparticles are depicted in Fig. 2. Accordingly, a regular spherical shape was noticed for the majority of nanoparticles at optimum conditions. Furthermore, the estimated particle size was lower than 30 nm, showing a proper consistency with the outcomes of DLS

Cell characteristics

The growth of HepG2 hepatic carcinoma cell line forms a monolayer on plastic culture vessels, showing an approximate population doubling time of 20 hrs. The cells are capable of surviving in low-density populations

Table 1. Statistical factors of the decision model for external validation

1	R	$0.8 < R$	0.9970
2	$k = \frac{\sum_{i=1}^{n}(h_i \times t_i)}{{h_i}^2}$	$0.85 < k < 1.15$	1.05
3	$k' = \frac{\sum_{i=1}^{n}(h_i \times t_i)}{{t_i}^2}$	$0.85 < k' < 1.15$	0.957
4	$m = \frac{R^2 - {R_o}^2}{R^2}$	$m < 0.1$	0.098
5	$n = \frac{R^2 - {R_o{}'}^2}{R^2}$	$n < 0.1$	0.046
6	$R_m = R^2 \times \left(1 - \sqrt{\left\|R^2 - {R_o}^2\right\|}\right)$	$0.5 < R_m$	0.610
where	${R_o}^2 = 1 - \frac{\sum_{i=1}^{n}(t_i - {h_i}^o)^2}{\sum_{i=1}^{n}(t_i - \bar{t_i})^2}$, ${h_i}^o$=k $\times t_i$		
	${R_o{}'}^2 = 1 - \frac{\sum_{i=1}^{n}(h_i - {t_i}^o)^2}{\sum_{i=1}^{n}(h_i - \bar{h_i})^2}$, ${t_i}^o = k' \times h_i$		

Table 2. The training parameters used during the modeling procedure

Parameters	Value
Hidden Layers	2
Number of neurons of the first hidden layer	4
Number of neurons of the second hidden layer	3
Function of the first hidden layer	Sig (X)
Function of the second hidden layer	Tanh (X)
Learning rule of the first hidden layer	Momentum
Learning rule of the second hidden layer	Momentum
Function of the output layer	Linear Sig (X)
Learning rule of the output layer	Delta Bar
Number of epochs	1000

developing colonies with a minimum of 50 cells during 11 days. Moreover, HepG2 cells can produce spheroids in cultures encumbered by liquid. Following 10 days, spheroids (mean diameter of 100 μm) were used to evaluate ability of HepG2 cells to develop colonies in spheroid model of culture.

Trypan blue exclusion assay (Viability assay)

Spheroid cells were scattered as single cells once the cells were treated with DMSO solution free noscapine, Tween 40, and noscapine nanoparticles. After counting the cells, their viability was specified via Trypan blue dye exclusion assay. Impact of DMSO solutionsolution free noscapine, and noscapine nanoparticles on the viability of HepG2 cells from spheroid cultures are represented in Fig. 3. It clearly shows that DMSO solutionsolution Tween 40 concentrations, and free noscapine nanoparticles exhibited no significant effects on the viability of cells in spheroid culture ($P > 0.05$).

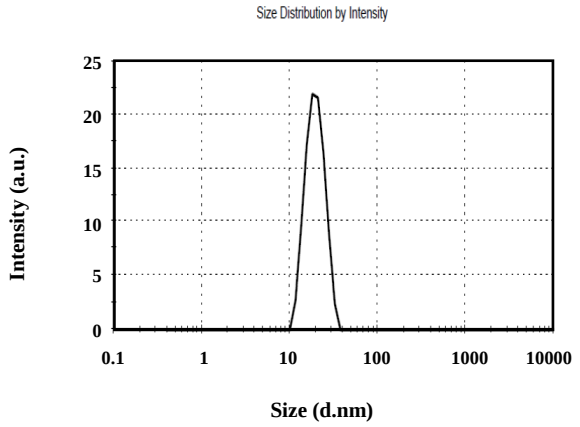

Fig. 1: Size distribution of noscapine nanoparticles, measured using DLS.

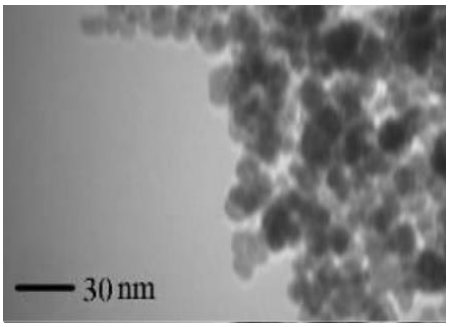

Fig. 2: TEM image of noscapine nanoparticles.

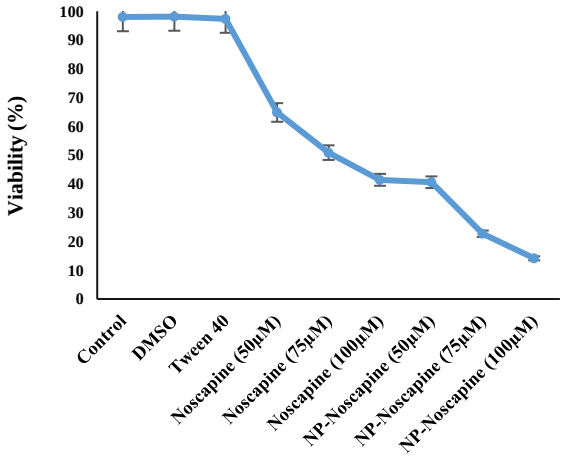

Fig. 4: Mean (SEM) effect of different concentrations of noscapine/noscapine nanoparticles for 24 hours on viability of HepG2 spheroid culture cells (n=3). NP-Noscapine represents nanoparticles containing noscapine).

Effects of noscapine/noscapine nanoparticles on induced DNA damages

DNA damages were evaluated by alkaline comet assays. Intercellular distribution of DNA migration (number of cells in the five visual comet classes) among control and treated cells with noscapine/noscapine nanoparticles for 24 h, is presented in Fig. 4A, B. Both groups of noscapine and noscapine nanoparticles displayed significant rises in the number of comets scored in visual Class 4 parallel to elevated doses of noscapine. The bulk of comets were increasingly disseminated into the next visual category of higher DNA damage resulting from exposure to rising doses of noscapine, with more sever effects on the group of noscapine nanoparticles. As an indication of DNA damage, the average tail moments was used in each category of cells.

The quantitative DNA damages assessed by comet score software are shown in Fig. 5 A, B. DNA damage (DD) and non-induced DNA damage ($DD-DD_0$) in two groups of noscapine (A) or noscapine nanoparticles (B) are obvious in both Figures. Both induced and non-induced DNA damages (Fig. 5 A) indicate no significant effect of DMSO solutionsolution on DNA damages as opposed to control ($P = 0.056$). Furthermore, Fig. 5 A shows increased induction of DNA damages as a result of rising noscapine concentrations in spheroid cultures.

Tween 40 concentrations resulted in no significant impacts on the induced DNA damages in comparison with the control (Fig. 5 B; $P = 0.085$). Similar to Fig. 5 A, this figure also reveals elevated induction of DNA damages as a result of rising concentrations of noscapine nanoparticles in spheroid cultures. A comparison of Figs. 5 A and 5 B represents increased DNA damages in the spheroid cultures, caused by various concentrations of free noscapine and nanoparticle-encapsulated noscapine. Noscapine nanoparticles, however, led to a significantly higher induction of DNA damages compared to noscapine. For instance, treatment with drug nanoparticles releasing 50 and 75µM noscapine for 24 h, caused genotoxic damages the same as those treated with 75µM and 100µM of noscapine, respectively.

Discussion

Noscapine is a phthalideisoquinoline alkaloid that comprises 1-10% of the alkaloids in opium, and is used as a cough suppressant in humans and animals [14, 43-47]

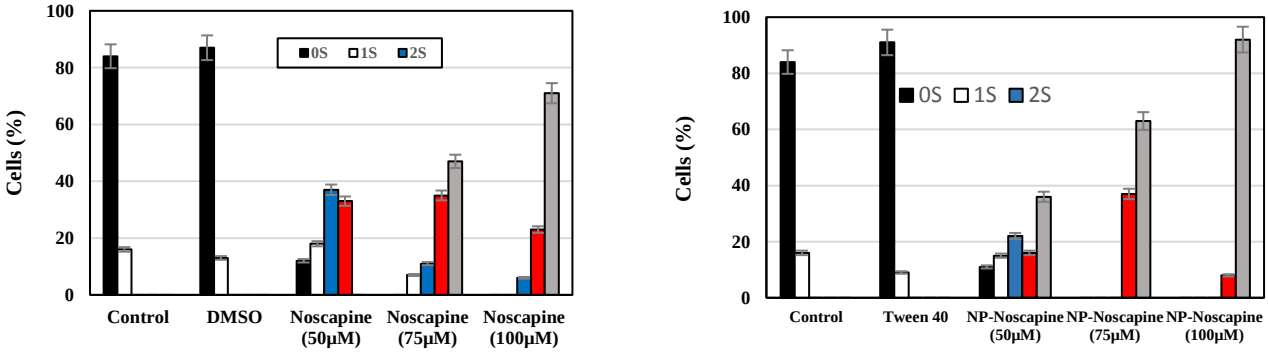

Fig. 4: Mean (SEM) distribution of DNA migrations (stages 0 to 4) among HepG2 cells of 50µm spheroids after treatment with noscapine (A)/or noscapine nanoparticles (B) for 24 hours. Data are based on analysis of 100 cells per slide, triplicate slides per samples (n=3).

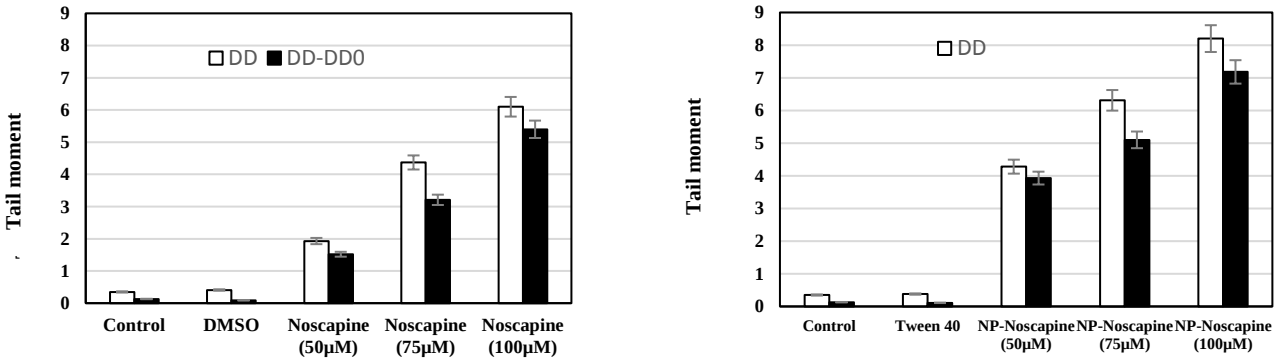

Fig. 5: Effects of different concentrations of noscapine (A) or noscapine nanoparticles (B) for 24 hours on induced DNA damages (DD) and net induced DNA damages (DD-DD_0) of HepG2 spheroid culture cells. Mean ± SEM of 3 experiments.

Anti-stroke and anti-cancer activities of noscapine are the basis for other clinical usages of this drug [48]. Anxiolytic impacts of noscapine in mice have been also reported in some studies [49]. Acute and chronic toxicity trials in animals reveal a large safety margin for noscapine, suggesting a low toxicity and good tolerance for the drug [50]. It was also shown in a study that 80% of 30 cancerous patients tolerated large doses of noscapine [51]. Similarly, Dahlsruom et al. [44] confirmed such an observation in five healthy volunteers (4 males and 1 female), who administrated the drug as oral (150 mg) and intravenous (66 mg) and exhibited no side effects.

This finding is supported by the present results. The tail moments increased as a result of higher noscapine concentrations in spheroid cultures. Changes in the viability of cells do not cause such a reduction. There is a general understanding that using drug delivery systems can considerably promote effectiveness of numerous conventional pharmaceutical therapies. Thus, pharmacokinetics and biodistribution of their associated drugs could be altered to function as drug reservoirs through designing such systems [52].

Nanosuspensions have presented various benefits compared with conventional drug delivery systems, including improved dissolution velocity and saturation solubility, diminished administered dose [21], enhanced biological functioning, scaling up capability, and promising improvements in stability and versatility [22]. A considerable ability to deal with poorly water soluble drugs have also been reported for nanosuspensions [21], which are composed of drug particles with colloidal suspensions of < 1 µm [23,24]. They are usually produced via two well-known approaches: use of an anti-solvent to precipitate drug molecules in a solution, and application of high shear forces to form finer particles from coarser ones (i.e. milling) [17]. It is well-known that microfluidic

instruments are shrunken forms of macroscale devices that reveal two striking features, namely elevated surface area to volume ratio enhancement and laminar flow occurrence [25]. Liquids in such devices flow within channels with characteristic interior diameters of below 1 mm. Linear liquids flow into microfluidic channels and develop a diffusion interface in the channel midpoint. Anti-solvent exposure of a drug solution in a solvent leads to diffusion of the drug molecules in the solvent through the interface (i.e. diffusion layer), resulting in nucleation and size increment [26].

Our results, using high concentration of Tween 40 revealed no significant impacts on HepG2 cells in terms of colony formability and plating efficiency. The compound had no effect on damage levels of HepG2 cells in spheroid culture model as well. On the other hand, colony numbers and plating efficiency of HepG2 cells, treated with nanoparticle scarring noscapine in spheroid cultures reduced due to rising concentration of noscapine expelled from nanoparticles. In comparison, treatment with free noscapine or noscapine loaded in nanoparticles with all various drug concentrations resulted in significantly lower colony numbers and plating efficiency of HepG2 cells than those treated with the same concentrations of free noscapine. As an example, treatment with noscapine nanoparticles (50 and 75 µM) for 24 h led to cytotoxic damages to the same extent as those resulted from treatment with free noscapine at concentrations of 75 and 100 µM, respectively.

According to the results, treatment with noscapine-loaded nanoparticle caused an increase in the level of DNA damage and tail moment of HepG2 cells because of increased concentration of released noscapine. However, the highest level of Tween 40 used led to no statistically considerable impacts on the DNA damage levels and tail moment score; the substance resulted in no damages in HepG2 cells in spheroid culture model as well. Significantly higher levels of DNA damage and tail moment score were observed as a result of treatment with different concentrations of noscapine loaded in nanoparticles than those solely treated with similar noscapine concentrations. For instance, treatment with drug nanoparticles releasing 50 and 75µM noscapine for 24 h caused genotoxic damages the same as those treated with 75µM and 100µM of noscapine, respectively.

CONCLUSIONSS

Our research lead us to the fact noscapine can be an interesting treatment for cancer. Actually, nanoparticle-based noscapine delivery seems to be precise, effective and safe method to treat the hepatic carcinoma cells line.

In addition, we clarified that nanoparticle synthesis procedure can make our nanosuspensions form of noscapine more applicable. However, it is arguable that application of incremental dose of noscapine nanosuspensions up to 100µM, effectively reduces cells viability and makes damages on their DNA that can cause cell death, the best result was obtained at the optimized dose of 75µM. Nonetheless, studies clarified that using nanosuspension form of noscapine has had beneficial aspects, but more experiments are needed on other cell lines and on animal models.

Acknowledgments

We would like to thank Dr. Nematollah Gheibi for providing his heartfelt support and guidance at all times. Also, I have the great pleasure to Sergi Montané Bel for her excellent contribution to assist me in this article.

Received : Mar. 10, 2019 ; Accepted :Jul. 1, 2019

REFERENCES

[1] Yadav N., Ganie SH.A., Singh B., Chhillar A.K., Yadav S.S., Phytochemical Constituents and Ethnopharmacological Properties of Ageratum Conyzoides L. *Phytotherapy Research.* **1**:16- (2019).

[2] Rowinsky E.K., Cazenave L.A., Donehower R.C., Taxol: a Novel Investigational Antimicrotubule Agent, *J. Nat. Can. Inst.*, **15**: 1247-1259 (1990).

[3] Bhatnagar A.S., The Discovery and Mechanism of Action of Letrozole, *Breast Cancer Res Treat.*, **105**: 7- (2007).

[4] Stark, G.R., Progress in Understanding Multidrug Resistance, *Nature*, **324**: 407- (1986).

[5] J. Francis Md, N. Coakley Mlis, L. Elit Md, H. Mackay Md, And The Gynecologic Cancer Disease Site Group., Systemic Therapy for Recurrent Epithelial Ovarian Cancer: A Clinical Practice Guideline, *Current Oncology*, **24(6)**: e540–e546 (2017).

[6] Prip, Anne, Møller, Kirsten Alling, Nielsen, Dorte Lisbet, Jarden, Mary, Olsen, Marie-Helene, Danielsen, Anne Kjaergaard, The Patient–Healthcare Professional Relationship and Communicationin the Oncology Outpatient Setting, *Cancer Nurs.*, **41(5)**: E11–E22 (2018).

[7] Rowinsky E.K., Donehower R.C., The Clinical Pharmacology and Use of Antimicrotubule Agents in Cancer Chemotherapeutics, *Pharmacol. Ther.*, **52**: 35-84 (1991).

[8] Ye K., Ke Y., Keshava N., Shanks J., Kapp J.A., Tekmal R.R., Petros J., Joshi H.C., Opium Alkaloid Noscapine is an Antitumor Agent that Arrests Metaphase and Induces Apoptosis in Dividing Cells, *Proc. Nat. Acad. Sci. USA*, **95**: 1601-1606 (1998).

[9] Altinoz M.A.,·Topcu G., Hacimuftuoglu, A., Ozpinar A., Ozpinar1 Hacker A.E., Elmaci I., Noscapine, A Non-Addictive Opioid and Microtubule-Inhibitor in Potential Treatment of Glioblastoma, *Neurochemical Research.*, **145**: 195-203 (2019).

[10] Karlsson M.O., Dahlstrom B., Serum Protein Binding of Noscapine: Influence ofa Reversible Hydrolysis, *J. Pharm. Pharmacol.*, **42**: 140 (1990).

[11] Quisbert-Valenzuela E.O., Gloria M. Ca., Apoptotic Effect of Noscapine in Breast Cancer Cell Lines, *International Journal of Oncology*, **48**: 2666-2674 (2016).

[12] Christe Sonia Mary, M., Sasikumar, S., Sodium Alginate/Starch Blends Loaded with Ciprofloxacin Hydrochloride as a Floating Drug Delivery System - *in Vitro* Evaluation, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **34**: 25-31 (2015).

[13] Schofield, J.R., Lawrence, B.A., Recognition and Management of Medication Excipient Reactivity in Patients with Mast Cell Activation Syndrome, *The American Journal of the Medical Sciences.* **357**: 507-511 (2019).

[14] Empey D.W., Laitinen L.A., Young G.A., Bye C.E., Hughes D.T.D., Comparison of the Antitussive Effects of Codeine Phosphate 20 mg Dextromethorphan 30 mg and Noscapine 30 Mg Using Citric Acid Induced Cough in Normal Subjects, *Eur. J. Clin. Pharmacol.*, **16**: 393-397 (1979).

[15] Taghavi S.M., Momenpour M., Azarian M., Ahmadian M., Souri F., Taghavi S.A., Sadeghain M., Karchani M., Effects of Nanoparticles on the Environment and Outdoor Workplaces, *Journal of Electronic Physician*, **5**:706-712 (2013).

[16] Yong, Ke. Á., Keqiang, Ye, Á., Hans, E. Grossniklaus, David, R., Archer, Harish Á., Joshi C., Judith Á., Kapp A., Noscapine Inhibits Tumor Growth With Little Toxicity to Normal Tissues or Inhibition of Immune Responses, *Cancer Immunol. Immunother*, **49**: 217-225 (2000)

[17] Müller, R.H., Jacobs, C., Kayser, O., Nanosuspensions as Particulate Drug Formulations In Therapy: Rationale for Development and What We Can Expect for the Future, *Adv. Drug Deliv. Rev.*, **47(1)**: 3–19 (2001).

[18] Nayak, S., Panda, D., Sahoo, J., Nanosuspension: a Novel Drug Delivery System, *J. Pharm. Res.*, **3**(2): 241-246 (2010).

[19] Aghajani, M., Shahverdi, A.M., Amani, A., The Use of Artificial Neural Networks for Optimizing Polydispersity Index (PDI) in Nanoprecipitation Process of Acetaminophen In Microfluidic Devices, *AAPS Pharmscitech*, **13(4)**: 1293–1301 (2012).

[20] Ali, S.M., York, P., Amani, A., Blagden, N., Evaluation of a Nanodispersion Formulation Prepared Through Microfluidic Reactors for Pulmonary Delivery of Budesonide Using Nebulizers, *Iran J. Pharmaceut. Res.*, **13(3)**: 785-795 (2014).

[21] Das, S., Suresh, P.K., Nanosuspension: a New Vehicle for The Improvement of The Delivery of Drugs to the Ocular Surface. Application to Amphotericin B, *Nanomedicine: NBM*, **7(2)**: 242–247 (2011).

[22] Patravale, V.B., Date, A.A., Kulkarni, R.M., Nanosuspensions: A Promising Drug Delivery Strategy, *J. Pharm. Pharmacol.*, **56(7)**: 827–840 (2004).

[23] Kocbek, P., Baumgartner, S., Kristl, J., Preparation and Evaluation of Nanosuspensions for Enhancing the Dissolution of Poorly Soluble Drugs, *Int. J. Pharm.*, **312(1–2)**: 179–186 (2006).

[24] Mokarram, A.R., Preparation and In-Vitro Evaluation of Indomethacin Nanoparticles, *DARU*, **18(3)**: 185–192 (2010).

[25] Weigl, B.H., Bardell, R.L., Cabrera, C.R., Lab-On-A-Chip for Drug Development, *Adv. Drug Deliv. Rev.*, **55(3)**: 349–377 (2003).

[26] Beebe, D.J., Mensing, G.A., Walker, G.M., Physics and Applications of Microfluidics in Biology, *Ann. Rev. Biomed. Eng.*, **4(1)**: 261–86 (2002).

[27] Voldman, J., Gray, M.L., Schmidt, M.A., Microfabrication in Biology and Medicine, *Ann. Rev. Biomed. Eng.*, **1(1)**: 401–425 (1999).

[28] Dollet B., Van Hoeve W., Raven J.P., Marmottant P., Versluis M., Role of the Channel Geometry on the Bubble Pinch-Off In Flowfocusing Devices, *Phys. Rev. Lett.*, **100(3)**: 34504 (2008).

[29] Rosenfeld C., Serra C., Brochon, C., Hadziioannou G., Influence of Micromixer Characteristics on Polydispersity Index of Block Copolymers Synthesized in Continuous Flow Microreactors, *Lab Chip.*, **8(10)**: 1682–1687 (2008).

[30] Peppas N.A., Hilt J.Z., Thomas J.B., "Nanotechnology in Therapeutics: Current Technology and Applications", Taylor & Francis, London (2007).

[31] Tomuleasa C., Soritau O., Rus-Ciuca D., Pop T., Todea D., Mosteanu O., Pintea B., Foris V., Susman S., Kacsó G., Irimie A., Isolation and Characterization of Hepatic Cancer Cells with Stem-Like Properties From Hepatocellular Carcinoma, *J. Gastrointes. Liver Dis.*, **19**: 61-67 (2010).

[32] Achilli T.M., Meyer J., Morgan J.R., Advances in the Formation, Use and Understanding of Multi-Cellular Spheroids, *Expert Opin. Biol. Ther.*, **12(10)**: 1347–1360 (2012).

[33] Singh N.P., Mccoy M.T., Tice R.R., Schneider E.L., A Simple Technique for Quantitation of Low Levels Of DNA Damage in Individual Cells, *Exp. Cell Res.*, **175(1)**: 184-191 (1988).

[34] Chen C.Y., Wang Y.F., Huang W.R., Huang Y.T., Nickel Induces Oxidative Stress and Genotoxicity in Human Lymphocytes, *Toxicol. Appl. Pharmacol*., **189(3)**: 153-159 (2003).

[35] Meybodi M.A., Mozdarani H., DNA Damage in Leukocytes from Fanconi Anemia (FA) Patients and Heterozygotes Induced by Mitomycin C and Ionizing Radiation as Assessed by the Comet and Comet-FISH Assay, *Iran Biomed. J.*, **13(1)**: 1-8 (2009).

[36] Azarian M., Amani A., Faramarzi M.A., Divsalar A., Eidi A., Design and Optimization of Noscapine Nanosuspensions and Study of its Cytotoxic Effect, *Journal of Biomolecular Structure and Dynamics*, **37**:147-155 (2017).

[37] Ghalehkhondabi, I., Ardjmand, E., Weckman, G. R., Young W.A., An Overview of Energy Demand Forecasting Methods Published in 2005–2015, Energy Syst. **8**: 411–447 (2017).

[38] Scholten, K., Collin, W. R., Fan, X., Zellers, E.T., Nanoparticle-Coated Micro-Optofluidic Ring Resonator as a Detector for Microscale Gas Chromatographic Vapor Analysis, *Nanoscale*, **7**: 9282–9289 (2015).

[39] Mollahasani, A., Alavi, A.H., Gandomi, A.H., Empirical Modeling of Plate Load Test Moduli of Soil *via* Gene Expression Programming, *Comput. Geotech.*, **38**: 281-286 (2011).

[40] Roy, P.P., Roy, K., On Some Aspects of Variable Selection for Partial Least Squares Regression Models, *QSAR Comb. Sci.*, **27**: 302-313 (2008).

[41] Segal, M.S., Goldstein, M.M., Attinger, E.O., The Use of Noscapine (Narcotine) as an Antitussive Agent, *Dis. Chest.*, **32**: 305-309 (1957).

[42] Mostafavi, E.S., Mousavi, S.M., Hosseinpour, F., Gene Expression Programming as a Basis for New Generation of Electricity Demand Prediction Models, *Comp Indus. Engin.*, **74**: 120–128 (2014).

[43] Alavi A.H., Aminian P., Gandomi A.H., Esmaeili M.A., Genetic-Based Modeling of Uplift Capacity of Suction Caissons, *Exp. Sys. Applic.*, **38**: 12608-12618 (2011).

[44] Dahlstrom B., Mellstrand T., Lofdahl C.G., Johansson M., Pharmakokinetic Properties of Noscapine, *Eur. J. Clin. Pharmacol.*, **22**: 535-539 (1982).

[45] Mooraki A., Jenabi A., Jabbari M., Zolfaghari, M, Javanmardi, S., Mahmoudian M., Bastani, B., Noscapine Suppresses Angiotensin Converting Enzyme Inhibitors- Induced Cough, *Nephrol.*, **10**: 348-350 (2005).

[46] Karlsson M.O., Dahlstrom B., Eckernas S.A., Johansson M., Tufvesson-Alm A., Pharmacokinetics of Oral Noscapine, *Eur. J. Clin. Pharmacol.*, **39**: 275-279 (1990).

[47] Ebrahimi S.A., Zareie Rostami P., Mahmoudian M., Interaction of Noscapine with the Bradykinin Mediation of the Cough Response, *Acta Physiol. Hun.*, **90**: 147-155 (2003).

[48] Mahmoudian, M., Recent Progress in Clinical Application of Noscapine: A Review, *Cur. Top. Pharmacol.*, **10**: 81-86 (2006).

[49] Khodarahmi P., Rostami P., Rashidi A., Khodarahmi Qahnavieh I., Anxiolytic Effect of Noscapine in Mice., *Pharmacological Reports*, **58**: 568-570 (2006).

[50] Harris E. A., The Effects of Noscapine and Codeine on the Ventilatory Responses to Excess of Carbon Dioxide and Lack of Oxygen, *Br J Pharmacol Chemother.*, **24(2)**: 532–537. (1965).

[51] Chougule M.B., Patel A.R., Jackson T., Singh M., Antitumor Activity of Noscapine in Combination with Doxorubicin in Triple Negative Breast Cancer, *Plos ONE,* **6(3)**: E17733 (2011). Doi:10.1371/Journal.Pone.0017733.

[52] Cukierman E., Khan D.R., The Benefits and Challenges Associated with the Use of Drug Delivery Systems in Cancer Therapy, *Biochem. Pharmacol.*, **80(5)**: 762-770 (2010).