Genotoxicity of noscapine nanosuspension on DU145 human prostate cancer (spheroid cell model)

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Abstract

Objective: In Iranian folk medicine, the cytotoxic effects of some members of the papaveraceae family have been reported. Recent reports have shown that the fraction of opium alkaloids may be responsible for its cytotoxic effect, but this mechanism is not fully understood. This study was designed to investigate the selective cytotoxic and genotoxic effects on DU145 cancer cell lines of noscapine one non-addictable opium alkaloids.

Methods: Cells were treated with different noscapine concentrations and nanosuspension of DNA and the viability of DU145 tumor spheroid culture. The genotoxicity was tested with a comet test if the concentration of each of these drugs is lower than IC50. The DNA damage of the DU145 cells have also been examined and the results compared.

Results: The cytotoxicity and genotoxicity of noscapine specifically on cancer cell lines were noted in this study. In addition, at the IC50 concentration, noscapine showed genotoxicity in the studied cell line.

Conclusions: We found a decrease in the viability of prostate cancer cells depending on the dose and damage to the DNA by increasing the concentration of noscapine, which implies a relationship between damage to the DNA and decreased viability. This experiment suggests that, due to their specific cytotoxicity and genotoxicity, noscapine can be used in cancer treatment. However, to confirm its usefulness in cancer treatment, further in vivo studies are needed.

Keywords: Papaveraceae, Genotoxicity, Cancer, DU145, Noscapine.

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Introduction

Noscapine (Nos) is considered as a phthalideisoquinoline alkaloid extracted from *Papaver somniferum* plant, which is generally applied as a cough suppressant drug among human being and experimental animals [1]. Over the past years, Nos has shown remarkable anticancer effects despite low toxicity; thus, it has gained a considerable attention in the field of cancer researches. This antitussive and innate tubulin-binding compound is currently undergoing phase I/II in clinical trials of cancer treatment [2,3]. Also, this compound has the potential of mitosis inhibition and induces apoptosis in lung, breast, lymphoma, and prostate cancer cells [4]. Based on above-mentioned exclusive characteristics, it is currently taken as an alternative chemotherapeutic choice. Many authors published reports over the past few years, which suggest several positive outcomes of Nos and its derivatives in the field of oncology [5,6]. An investigation was carried out by Chougule et al. which was related to the xenograft inhibition of lung tumor cells. Results of the mentioned investigation indicated that a synergistic anticancer effect of Nos is in combination with Gemcitabine [7]. Also, another study was carried out by Joshi et al in which Nos was found to disrupt tubulin dynamics and induce mitotic arrest [8]. Considering various tumor cells including glioma, melanoma, nonsmall cell lung cancer, prostate, multiple myeloma, colon, ovarian, and breast cancer [9-11]. we can observe *in vitro* and *in vivo* antitumor activities of Nos in different researches. Nowadays, Nos and its derivatives are considered as potential anticancer drugs and have many advantages; however, there are many unsolved key-problems in the field of pharmacology and pharmacokinetics researches.

Recent studies focuse on prominent roles of nanosuspensions including the synthesis of medicinal compounds, and replenishment of aqueous insoluble or less soluble drugs' formulations [12]. However, results indicate that various advantages including the augmented dissolution rate and saturation solubility, decreased doses of administration [13]. enhanced biological functioning, scale up potentiality; moreover, probable improvements related to constancy and adaptability [14]. are shown by nanosuspensions despite conventional drug delivery systems. Also, results of studies indicate that

nanosuspensions have the considerable capacity of interacting with poorly water-soluble drugs. The preparations contain little colloidal suspensions with the sizes of less than 1 μm [15,16]. however, they are typically prepared by applying two normal approaches: 1) using an anti-solvent in order to precipitate drug molecules in a solution, and 2) applying elevated shear forces in order to form finer particles from coarser ones [17].

It is recognized that microfluidic instruments are considered as shrunken forms of macro-scale devices that mainly focuse on two significant features: 1) the enhancement of surface area to the volume ratio, and 2) the occurrence of laminar flow [18]. Liquid flows can be observed within those chanals of such devices that are with interior diameters of less than 1 mm. There are linear liquid flows inside microfluidic channels; however, they lead to the development of a diffusion interface within the channel midpoint. It was found that the drug molecules' diffusion, which is existed in the solvent, is occured by the anti-solvent exposure of a drug solution through an interface (i.e. a diffusion layer), and leads to the nucleation and size increment [19]. Factors inluding enlarged surface area and volume ratio have positive impacts on processes including enzymatic reactions or active components' extraction. Also, significant functions of these systems could be usefull in performing the process of handling solutions with micro- and nano-liter volumes. Considering other approaches of the nanosuspension formation, it could be recognized that a cost-effective procedure can be offered by microfluidic reactors, which is not with a significant residue production [20]. However, it normally yields a monodispersed product [21,22] in order to avoid the unrestrained development, and sedimentation of the drug nuclei which is formed in the solution. Also, the growth/precipitation rate can be controlled by surfactants or polymers existed in such apparatuses [23].

The purpose of this study is to examine impacts of various concentrations of Nos nanosuspension on the viability and induced DNA damages of DU145 tumor spheroids and finally, the correlation between these two biological effects.

Material and Methods

It should be noted that processes of preparation and purchase of Noscapine (Nos), acetonitrile (HPLC grade) and polysorbate 40 (Tween 40) were carried out in Sigma-Aldrich (Germany).

Nanoprecipitation in microfluidic reactor

Preparing Nos nanosuspension by microfluidic reactor is a process which is as the following: first, the injection of Nos saturatated solutions, which are existed in acetonitrile and are also at predetermined temperatures, into the reactor should be carried out at certain solvent/anti-solvent flow rates. Second, water at the controlled room temperature (i.e. 22°C ± 2°C), which contains various Tween 40 concentrations, should be considered as the anti-solvent system. Then, hydrodynamic micro-pumps should be used in order to carry out the process of injecting very small volumes of fluid. Finally, it should be noted that polylactide, which is with 1-mm internal diameter and 60° inlet angle, is the constitutive of micro-reactor [24].

Dynamic light scattering

Determination of the average particle size of the best formulation was carried out by using the ζ sizer (Malvern, UK), which is provided by the Malvern PCS software (Version 1.27). Samples were measured while they were at the fixed 22°C temperature; also, there was no dilution before the measurements. The confirmation of ζ sizer calibration was carried out by a 500 nm nanosphere™ size standard (Duke Scientific Corporation, USA).

Transmission electron microscopy

A transmission electron microscope (TEM) was applied in order to evaluate the size and morphology of QU nanoparticles existed in the optimum formulation (ZEISS EM 10C). It should be noted that there was a fixed preparation (three drops) on a copper grid that was coated with carbon, and was also exposed to the air-drying. To carry out the pocess of TEM observation with an accelerated voltage (80 kV), we located the sample grid on a frame.

Cell line

Human DU145 prostate carcinoma cells were obtained from Pasteur Institute, Iran; however, they were cultured in RPMI 1640 (Gibco) that contained 10% fetal bovine serum (FBS) (Biosera), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Biosera).

Culture of spheroids

The liquid overlay technique was applied for spheroids' culture. Cells were placed on culture Petri dishes while they were thinly layered with 1% agar (Bacto Agar), and supplemented with RPMI plus 10% FBS. Petri dishes were maintained in a dampened milieu with 5% CO_2 at 37°C. Also, the process of replacing half of the culture medium was carried out two times a week by the fresh culture medium.

Treatment of spheroid culture with Nos/Nos nanosuspensions

Results show that the process of cell culture leads to multicellular spheroid formation. It should be noted that the 24-hour process of adding various Nos and Nos nanosuspensions concentrations (100 μM, 300 μM and 500 μM) to spheroids (average diameter of 100 μm) was carried out after 10 days. However, dissolving Nos with the 0.25% (v/v) final DMSO concentration, which was supplemented to the medium, was performed by applying dimethyl sulfoxide (DMSO).

Alkaline comet assay

Ordinary microscopic slides were coated with agarose (1%), which was with a normal melting point. It was found that the suspension contained almost 10,000 cells in 100 μl of the low-melting-point 0.5% agarose. Using a pipette, the cell suspension was quickly transferred on the foremost agarose layer. Then, we submerged solidified slides in the freshly-prepared lysis buffer, and incubated them during an hour. The suitable temperature of performing the whole procedures of the next steps was considered to be 4°C. Slides were then transferred into a horizontal gel electrophoresis tank (Cleaver Scientific Ltd, CSLCOM20), which was filled with fresh cold denaturation buffer, and maintained there for 30 minutes after we removed them from the lysis buffer. The process of 30-minute conduction

of electrophoresis, with the 1V/cm voltage and 300 mA current, was carried out by using the same denaturation buffer. However, neutralization of the excess alkali was carried out based on the two following steps: first, slides were subsequently washed in Tris buffer and then, they were stained by ethidium bromide. Individual cells or comets were first viewed and photographed by a fluorescent microscope (Zeiss, Axioskop 2 plus) and then, they were analyzed by Comet Score® software. Evaluation of DNA damages was based on an increase in the tail moment, the product of DNA level (fluorescence) existed in the tail, and the distance between means of the head and tail fluorescence distributions.

Evaluation of DNA damage

Applying the process of visual assignment, we considered each slide's individual cells (n=100) in one of the five above-mentioned classes based on the tail length by appointing values of 0 (no tailing) to 1, 2, 3, or 4 (Max. tailing). Totally, comets could be scored in the range of 0 to 400:

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

The above equation's variables are as the following. DD (au) is the arbitrary unit DNA damage score, n_0-n_4 is the number of Class 0-4 comets, and Σn is considered as the total number of scored comets. However, factors of -4 coefficients are applied in order to weight each class of comets. It can be considered that such a visual classification is low-grade, compared to computerized analyses, including CCD camera image analysis of tail moment. It was found that the evaluation of DNA damages was based on an increase in the tail moment, the product of DNA density in the tail, and the distance between the average distribution levels of the head and tail fluorescence.

Statistical analyses

Mean values ± SEM (standard error of mean) imply on results, while "n" implies on experimental attempts. Statistical analyses were carried out by using SPSS (Version 23.0) software (i.e. one-way analysis of variance (ANOVA)). Turkey's test was then applied as the post-hoc analysis; however, it considers significant values when there is $P<0.05$.

Result and Discussion

Design and characterization of nanosuspensions

There are three steps involved in the nanosuspension formation process of a microfluidic reactor: a solution supersaturated with the active ingredient, drug nucleation, and subsequently drug nuclei growth. There are several approaches suggested in order to estimate prepared nanosuspension's stability that show effective applications related to this field. These approaches include differential scanning calorimetrvy combined with X-ray analysis [25] microscopic observation of nanoparticles [26] growth of the particle size and changes in ζ potential [27] change of crystalline state [28] observation of sedimentation or creaming and the use of HPLC in the process of nanoparticle contents' recovery existed in suspension [29]. It should also be noted that more significant outcomes, which are typically considered as the long-lasting ones, are yielded by the classical approach; however, this approach examines the preparation [29,30]. Moreover, this study physically investigates prepared samples. We can observe the development of a compact deposit was developed in this investigation; however, this could hardly be re-scattered by using the process of shaking and shows an un-aggregated suspension [31] in which particles deposit, individually. This leads to a slow precipitation rate with a compact sediment layer, which is easily visible by naked eyes [32].

Results of measurements showed that there was a ~20 nm particle size for the prepared sample; furthermore, it was assessed by DLS (Figure 1A) and verified by using TEM (Figure 1B). Moreover, nanosuspension's physical stability, which is obtained with the finest particle size, is determined by the visual observation of stable Nos precipitation. However, this is considered as a method of evaluating the produced nanosuspension stability [33]. Since the formation of a deflocculated nanosuspension is carried out because of the stable Nos and makes a compact deposit, we can visually observe the sedimentation. Finally, a 12-day physical stability is exhibited by the obtained sample.

Trypan blue exclusion assay

Studies indicate that Nos is considered as a phthalideisoquinoline alkaloid that comprises 1-10% of the alkaloid contained in

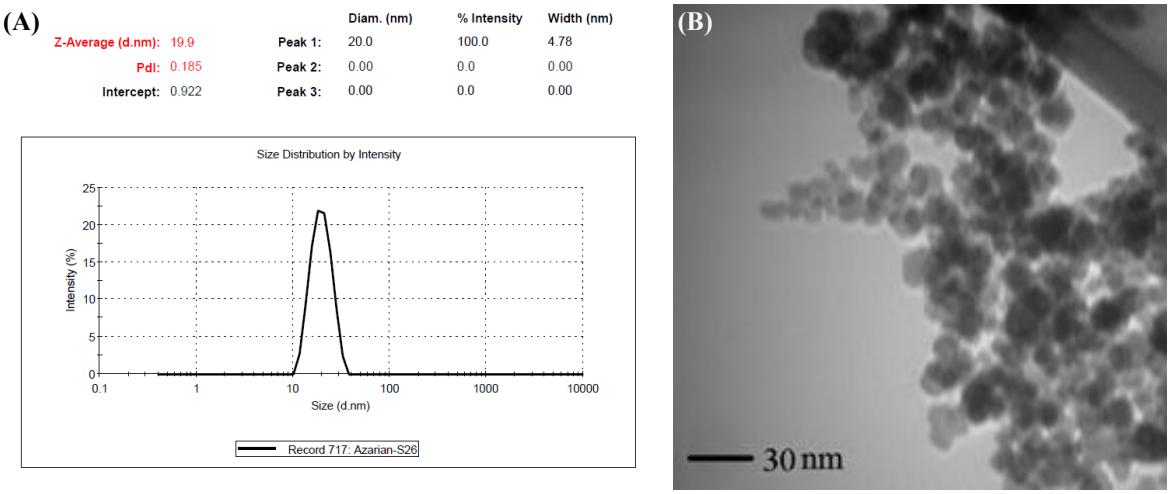


Figure 1. *Size distributions (A), and TEM image (B) of noscapine nanoparticles.*

opium; also, it is is used as a cough suppressant among humans and trial animals [34-38]. It should be noted that this drug contains two basic clinical advantages: anti-stroke and anti-cancer activities of Nos [39]. Results of some investigations that were carried out on mice reported anxiolytic effects of Nos [40]. There is a large safety margin for Nos because of the existence of acute and chronic toxicity trials among animals, which indicates that the drug contains a low level of toxicity and good tolerance [41]. Results of another investigation also showed that 80% of 30 cancerous patients could tolerate high doses of Nos [42]. Similarly, B. Dahlsruom et al., confirmed that there was such an observation among five healthy volunteers (4 males and 1 female) who administered the drug orally (150 mg) and intravenously (66 mg); however, they did not exhibit any side effects [43].

When DMSO, Nos, Tween 40, and Nos nanosuspension added to the cells, then spheroid cells were scattered as single cells. Applying the Trypan blue dye exclusion assay, we could specify cells' viability after counting them. Figure 2 shows impacts of DMSO, Nos, and Nos nanosuspension on the viability of DU145 cells resulted from spheroid cultures. It clearly shows that DMSO and Tween 40 exhibit no significant effects on the viability of those cells that are existed in spheroid culture (P>0.05).

Following results confirm the mentioned hypothesis. Such a reduction does not occur because of the existence of changes in cells' viability. It is generally well-known that the drug delivery system (DDS) can considerably promote the effectiveness of numerous conventional pharmaceutical therapies. It could be resulted that while designing DDS, pharmacokinetics and their associated drugs' bio-distribution could be altered in order to function as drug reservoirs [44].

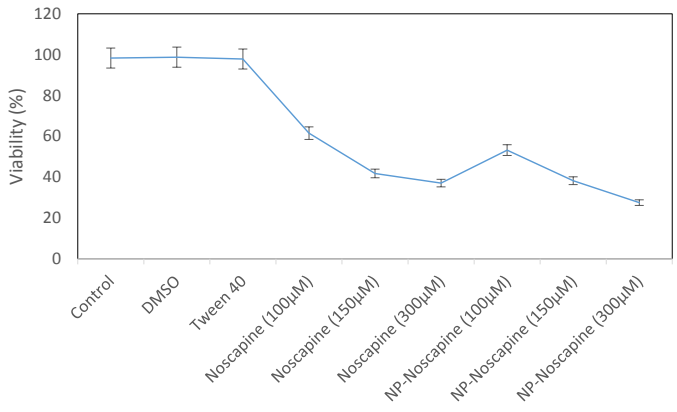


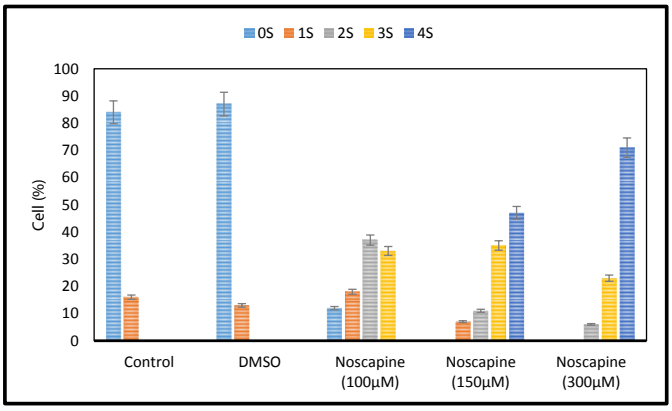
Figure 2. *Effects of various concentrations of Nos and its nanosuspension on the viability of DU145 spheroid culture cells during 24 hours. Mean ± SEM of 3 experiments.*

Alkaline comet assay

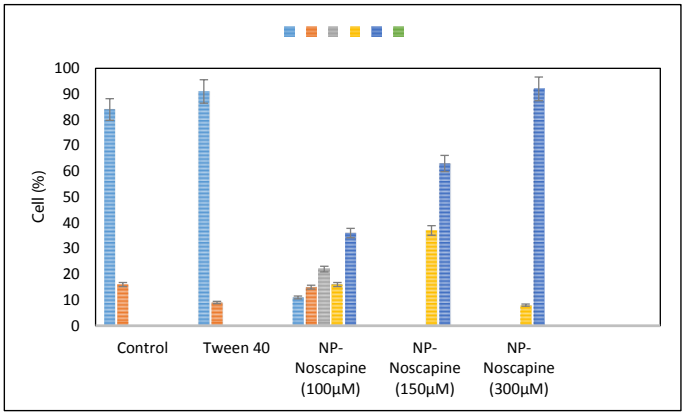
The process of DNA damages evaluation was carried out by alkaline comet assays. Figure 3A and 3B shows DNA migration's intercellular distribution (the number of cells in five visual comet classes) among those cells that were controlled and treated by Nos and its nanosuspension during 24 hours, respectively. Both Nos and its nanosuspension led to a considerable increase of comets' number scored in the visual Class 4 parallel to elevated Nos doses. Comets were increasingly disseminated into the next visual category of higher DNA damage, which was resulted from the exposure to the increase of Nos doses. This should also be noted that it was associated with more sever effects on Nos nanosuspension group. We can refer to the average of tail moments, which was applied in each cell's category, in order to imply on the DNA damage.

The quantitative DNA damages assessed by comet score software are shown in Figure 4A and 4B. However, both figures clearly show the DNA damage (DD) and non-induced DNA damage (DD-DD0) in two groups of Nos(A) or its Nos nanosuspension (B). Both induced and non-induced DNA damages (Figure 4A) indicated no significant effects of DMSO diluent on DNA damages as opposed to control (P=0.056). Figure 4A and 4B shows DNA damages' increased induction as a result of the increase of Nos or its nanosuspension concentrations, which are existed in spheroid cultures.

Tween 40 concentrations did not have considerable impacts on induced DNA damages compared to the control (Figure 4B; P=0.085). This figure is similar to Figure 4A; however, it could be found that an elevated induction of DNA damages is resulted from the increase of concentrations of Nos or its nanosuspension in spheroid cultures. Finally, we carried out a comparison of (Figure 4A and 4B) which represent increased DNA damages in the spheroid cultures; however, these damages are caused by various concentrations of Nos or its nanosuspension. Moreover, results showed that Nos nanosuspension, leads to a significant

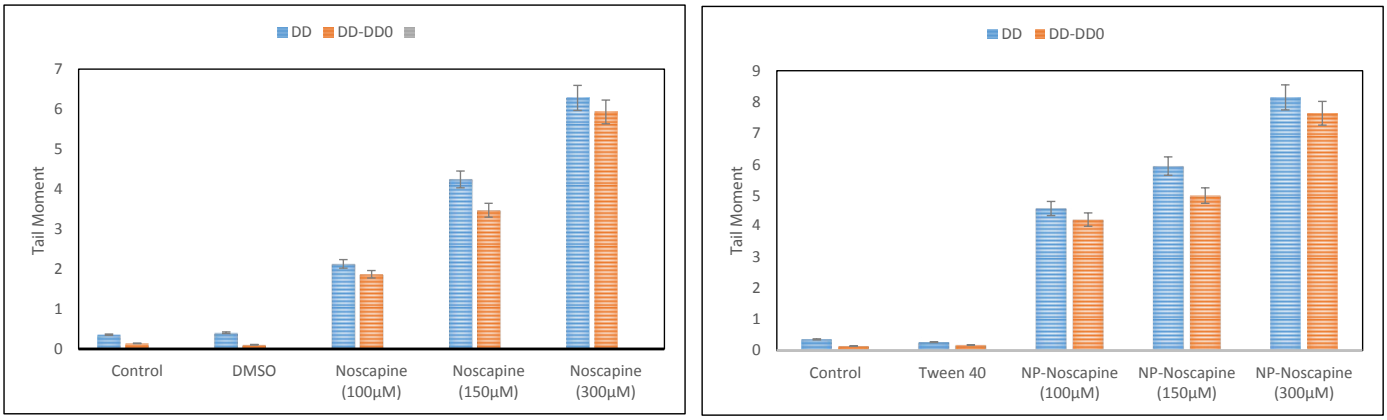


(A)



(B)

Figure 3. *Distribution of DNA migrations (stages 0 to 4) among DU145 cells spheroids after performimg the 24-hour treatment process with Nos (A)/Nos nanosuspension (B). Data is based on the analysis of 100 cells per slide, and triplicate slides per samples. Mean ± SEM of 3 experiments.*



(A) (B)

Figure 4. *Effects of Nos' (A) or its nanosuspension's (B) various concentrations on induced DNA damages (DD) and net induced DNA damages (DD-DD0) of DU145 spheroid culture cells during 24 hours. Mean ± SEM of 3 experiments.*

higher induction of DNA damages compared to Nos.

Conclusions

The current study implies on the fact that Nos can be considered as a usefull treatment of cancer. Actually, nanoparticle-based Nos delivery seems to be a precise, effective, and safe method in order to treat the prostate carcinoma cells' line.

Furthermore, it is clarified that the nanoparticle synthesis procedure can lead to the more applicability of nanosuspensions. Hence, it could be argued that applying the incremental dose of Nos nanosuspensions, which is up to 500 µM, effectively reduces cells' viability and leads to damages on their DNA. This can cause the cell death; however, the best result was obtained at the optimized dose of 300 µM. Finally, studies clarified that using the nanosuspension form of Nos has many beneficial aspects; however, it is required to carry out more experiments on other cell lines and animal models.

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