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Tretinoin-loaded Polymeric Nanocapsules: Evaluation of the Potential to Improve the Antiproliferative Activities on *Allium cepa* root-tip Compared to the Free Drug

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SUMMARY. Tretinoin-loaded nanocapsules were prepared by the interfacial deposition of preformed polymer method. For the antiproliferative activity assay a tretinoin solution and tretinoin-loaded nanocapsules were tested on *Allium cepa* root-tip model. Tretinoin-loaded nanocapsules presented nanometric mean size, low polydispersity index, acidic pH value and encapsulation efficiency higher than 99%. Tretinoin-loaded nanocapsules presented a significant decrease in the mitotic index (1.36%) compared to the control-water (8.20%), as well as to the free drug (2.76%). This improvement in the antiproliferative activity did not lead to an increase in the frequency of chromosome aberrations. Encapsulation in polymeric nanocapsule suspensions improves the *in vivo* antiproliferative efficacy of tretinoin on the tested model.

RESUMEN. "Nanocápsulas Poliméricas conteniendo Tretinoína: Evaluación de su Potencial para Mejorar la Actividad Antiproliferativa en Raíz de *Allium cepa,* Comparado con el Fármaco Libre". Se prepararon nanopartículas poliméricas conteniendo tretinoína por el método de depósito interfacial de polímero preformado. Para el ensayo de actividad antiproliferativa se utilizaron: tretinoína en forma de solución y contenida en nanocápsulas poliméricas, que fueron ensayadas usando el modelo de raíz de *Allium cepa*. Las nanocápsulas mostraron un tamaño promedio en el rango de los nanómetros, con un bajo índice de polidispersidad, pH ácido, y una eficiencia de encapsulación mayor al 99%. Las nanocápsulas ensayadas mostraron una disminución significativa del índice mitótico (1,36%) comparado con el control-agua (8,20%), así como respecto al fármaco libre (2,76%). Esta mejora en la actividad antiproliferativa no conllevó a un aumento en la expresión de aberraciones cromosómicas. En conclusión, la encapsulación en nanocápsulas poliméricas mejora la eficacia antiproliferativa de la tretinoína en el modelo ensayado.

INTRODUCTION

Tretinoin (*all-trans*-retinoic acid) is the active form of a metabolic product of Vitamin A (Fig. 1). It belongs to the first generation of retinoids along with isotretinoin, a cis-isomer of retinoic acid [1].

The drug is effective in the topical treatment of different skin diseases such as acne vulgaris, ichtiosys, psoriasis and neoplasias such as Kaposi's sarcoma [2-5]. Retinoids have also demonstrated inhibition of cellular proliferation and the induction of differentiation and apoptosis in various cancers [6-11].

The systemic administration of tretinoin by oral or parenteral route presents some drawbacks like its poor aqueous solubility (8 x 10-3 mg/ml) [12], a wide interpatient variation in the bioavailability [13] and its high chemical instability [14-17]. Some drawbacks, such as poor solubility, high chemical instability and irritation of the treated area are also found in the development and dermal administration of topical systems containing tretinoin. Currently the administration of tretinoin is almost exclusively by the topical route [14,18], even so its isomer (isotretinoin) is widely administered by oral route.

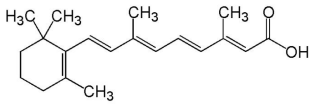

Figure 1. Chemical structure of tretinoin ($C_{20}H_{28}O_2$ - M_r = 300.435).

KEY WORDS: *Allium cepa,* Antiproliferative activity, Nanocapsules, Tretinoin.
PALABRAS CLAVE: Allium cepa, Actividad antiproliferativa, Nanocápsulas, Tretinoína.

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Many efforts have been made over the last years to overcome some of these drawbacks. The association/inclusion of tretinoin with different kinds of delivery systems (liposomes, lipid nanoparticles, cyclodextrins, niosomes) has improved its solubility [11,19], chemical stability [3,10,15-19], bioavailability [10,20] and/or efficacy [7,20].

Over the last 15 years, nanoparticulated systems like polymeric nanocapsules and nanospheres have been extensively studied as drug carriers including anticancers, peptides, antinflammatories and antibiotics [21-23]. Nanospheres are defined as a matricial polymeric structure, in which drugs can be entrapped or molecularly dispersed, while the nanocapsules are characterized by a lipophilic core surrounded by a polymeric layer, in which drugs can be dissolved in the oil, dispersed within the particle [22] or adsorbed at the interface particle/water [24]. These polymeric nanoparticulated systems have been widely studied as drug delivery systems due to their possibilities to increase drug efficacy, to reduce toxicity and/or to obtain modified drug release systems [22,23,25].

Recently we reported the improvement of tretinoin photostability by its loading on polymeric nanocapsules [19], which shows the potentiality of these suspensions to be incorporated in novel topical or systemic dosage forms containing tretinoin. So, in the present work, we evaluated the potential improvement on the in vivo antiproliferative activities and the genotoxicity potential of the encapsulated drug compared to its free form using a simple model. Nanocapsule suspensions were characterized by means of drug content, encapsulation efficiency, size, polydispersity index and pH. The *in vivo* antiproliferative activities were assessed by the in vivo *Allium cepa* root-tip cell test, which is known to give similar results to in vitro animal cytotoxicity tests [26-29].

MATERIAL AND METHODS

Materials

Tretinoin was obtained from Roche (Nutley, EUA) and poly-ε-caprolactone (PCL) was acquired from Sigma (São Paulo, Brazil). Caprylic/capric triglyceride mixture was delivered from Brasquim (Porto Alegre, Brazil), sorbitan monooleate (Span 80®) and polysorbate 80 (Tween 80®) were supplied by Delaware (Porto Alegre, Brazil). All other chemicals and solvents presented pharmaceutical grade and were used as received. *Allium cepa* bulbs were obtained from an organic farmer.

Preparation of polymeric nanocapsule suspensions

Nanocapsule (NC) suspensions were prepared by interfacial deposition of preformed polymer method as described by Fessi *et al.* [30]. Briefly, an organic solution composed of tretinoin (0.0125 g), the caprylic/capric triglyceride mixture (0.80 ml), Span 80® (0.194 g), the polymer (PCL) (0.25 g) and acetone (67.0 ml) was added under moderate magnetic stirring to an aqueous solution (134.0 ml) containing Tween 80® (0.194 g). The magnetic stirring was maintained for 10 min. Then, the acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure to a final volume of 25 ml (10 $mg.ml^{-1}$ of polymer and 0.50 $mg.ml^{-1}$ of drug). Blank NC suspensions were prepared as described above omitting the drug. All preparations were carried out protected from the light and the formulations were kept in the dark during all time [19].

Characterization of polymeric nanocapsule suspensions

Determination of drug content, encapsulation efficiency and pH

Drug content (mg/ml) was determined (n = 3) after the dissolution of nanocapsules in acetonitrile (1 ml of suspension to 10 ml of acetonitrile) and assayed by high performance liquid chromatography - HPLC (concentration range: 1-20 $\mu g.ml^{-1}$; r = 0.9998, interday and intraday precision: < 2.00%, n = 3). The chromatographic system consisted of a Gemini RP18 column (150 x 4.60 mm, 5 µm, Phenomenex, Torrance, USA) and a Shimadzu instrument: LC-10Avp Pump, UV-VIS SPD-10AvP Module, Class-Vp Software (Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 1.0 $ml.min^{-1}$ consisted of acetonitrile/water (85:15% v/v) containing 1% of acid acetic. The volume injected was 20 µl and tretinoin was detected at 342 nm [19].

For the evaluation of the encapsulation efficiency, the free drug was determined in the clear supernatant following the separation of the nanocapsules from the aqueous medium by a combined ultrafiltration-centrifugation technique (Ultrafree-MC® 10,000 MW, Millipore, Bedford, USA). The encapsulation efficiency (%) was calculated by the difference between the total and free drug concentrations determined in the nanocapsule suspension (drug content) and in the ultrafiltrate, respectively.

The pH values of the suspensions were determined by the immersion of the electrode di-

rectly in the suspension using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil).

Particle size analysis

Particle sizes and polydispersity indices (n = 3) were measured by photon correlation spectroscopy after adequate dilution of an aliquot of the suspension in water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK).

Evaluation of the antiproliferative effects and genotoxicity potential on Allium cepa root-tip cells

For the onion root-tip cell test, we used 24 *Allium cepa* bulbs divided into four groups of six onion bulbs for each treatment (distilled water, tretinoin aqueous solution, tretinoin-loaded nanocapsules and blank nanocapsules). Tretinoin aqueous solution and tretinoin-loaded nanocapsules were tested at a concentration of 1.6 x 10^{-3} M (0.50 mg.ml^{-1}). This concentration was choosing considering the stability of the systems (tretinoin crystallization occurs at higher concentration) and the impossibility to dilute this suspension due to the entrapped drug displacement. Tretinoin aqueous solution was prepared using ethanol as a cossolvent (ethanol: water, 10:90 v/v). For each treatment, all bulbs were rooted in distilled water for three days and after they were placed in their respective treatment for 24 h. The control group, which had not received treatment, remained in distilled water. After 24 h, control and experimental bulbs were collected and fixed in 3:1 (v:v) ethanol-acetic acid for 24 h before being placed in 70% (v/v) aqueous ethanol and refrigerated (4 ± 2 °C) until analyzed. For each bulb, five slides were made using five root-tips hydrolyzed in 1N hydrochloric acid for five minutes and washed in distilled water. The fragmented meristematic regions were stained with 2% (w/v) acetic orcein. Five fields of each slide were assessed by bright-field optical microscopy at 500X magnification and the number of interphase, prophase, metaphase, anaphase and telophase cells was recorded. At least 5000 cells for each treatment and the control were scored. Mean values for the different cell cycle phases, mitotic index (MI) and percentage (%) of division inhibition in relation to the control (distilled water) were calculated [31,32]. Chromosome aberrations were observed during cell division and counted as breaks (all mitotic phases), bridges and laggards (telophase and anaphase).

Statistical analysis

Results are expressed as mean ± SD (standard deviation). The t-test was performed to compare the formulations (tretinoin-loaded nanocapsules and blank nanocapsules). For the antiproliferative studies and chromosome aberrations, statistical analyses were performed using the Chi-squared (χ^2) test at $p \leq 0.05$. All analyses were run using the Bioestat statistical program (Version 3.0).

RESULTS AND DISCUSSION

Several studies have been carried out over the last years to improve the solubility, chemical stability and/or the efficacy of tretinoin either for topical or systemic treatment [3,12,17,18,20]. In the present study, we evaluated the possibility to encapsulate tretinoin in polymeric nanocapsules in order to evaluate the potential to increase its antiproliferative efficacy compared to the free drug. It is important to point out that this study is the first report about the evaluation of the increase of antiproliferative activities on *Allium cepa* root-tip cells by means of nanoencapsulation of a drug.

Tretinoin-loaded NC suspensions were prepared at a concentration of 0.50 mg.ml^{-1} of tretinoin. As a control, we also prepared blank NC omitting the presence of the drug. Both formulations presented particle size in the nanometric range (200-250 nm), polydispersity index below 0.25 and pH values of the formulations were in the acidic range [19]. The HPLC assay of tretinoin-loaded NC samples used in this study showed a drug content (0.529 ± 0.020 mg.ml^{-1}) close to the theoretical value (0.50 mg.ml^{-1}) and similar to our previous report [19] with a very high encapsulation efficiency (> 99.90%). Tretinoin-loaded NC suspension prepared in this study improved tretinoin water solubility, which is almost nil (8 x 10^{-5} mg.ml^{-1})[12] to 5 x 10^{-1} mg.ml^{-1} (more than 6000 times).

After the preparation, we evaluated the antiproliferative activities of free and encapsulated tretinoin on *Allium cepa* root-tip cells. The cytotoxicity tests, employing a vegetal *in vivo* test as the *Allium cepa* test, are validated by several researchers with a good correlation to the in vitro test using animal cells [27,28]. Although the vegetal metabolism is different from the animal metabolism, the *Allium cepa* test is an excellent tool of cytotoxic or antiproliferative analyses [27,33-35].

The number of interphase, prophase, metaphase, anaphase and telophase, as well as

Treatment	Number of cells in different phases of the cell cycle					Mitotic index (%)*
	Interphase	Prophase	Metaphase	Anaphase	Telophase	
Water (control)	4530	124	90	55	141	8.20 [a]
Tretinoin solution	4862	33	39	36	30	2.76 [b]
NC-tretinoin	4932	23	19	14	12	1.36 [c]
NC	4895	65	10	14	16	2.10 [b]

Table 1. Number of cells in different phases of the cell cycle and the mitotic index of onion root-tip cells treated for 24 h in water, tretinoin aqueous solution (0.5 mg/ml), tretinoin-loaded nanocapsules - NC-tretinoin (0.5 mg/ml) and blank nanocapsules - NC. The total number of cells analyzed for each treatment was 5000. *Means, in column, with the same letter are not significantly different (χ^2 test, p (0.05).

the mean mitotic index (%) for all tested groups are shown in Table 1. It can be observed that for all treatments no phase of the cellular cycle was suppressed. Tretinoin, in its free form in aqueous solution, presented a mitotic index of 2.76%, which demonstrated a significant difference ($p \leq 0.05$) in relation to the control group-water (8.20%) as well as to the nanoencapsulated tretinoin (1.36%). In the same way, tretinoin-loaded nanocapsules also presented a significant decrease ($p \leq 0.05$) in the mitotic index (1.36%) compared to the blank nanocapsules-NC (2.10%).

Tretinoin has a well-documented antiproliferative activity in cancer cells [10,36-38]. In this study, we used a simple *in vivo* method to study the potential to improve its antiproliferative activity by means of nanoencapsulation. The inhibition of the cellular division by tretinoin (as free drug) observed in our study is in accordance with its *in vitro* antiproliferative activity, previously reported in the literature using other cell lines [8,11,36,37]. There have been no studies evaluating the antiproliferative activity of tretinoin on onion root-tip cells. This evaluation was necessary in this study in order to compare the results of the free and encapsulated tretinoin and as a form to validate our experiment.

Considering that tretinoin-loaded nanocapsules presented a significant difference in the mitotic index compared to the tretinoin solution (2.76%) as well as to the blank nanocapsules (2.10%), we can suggest that the nanoencapsulation of tretinoin improves its antiproliferative efficacy. However, in order to verify if this decrease in the mitotic index (%) may be related to important chromosome aberrations, breaks, bridges and laggards were counted for each slide by microscopic observation. The total and the percentage number of chromosome aberrations observed in each treatment are shown in Table 2. The occurrence of these aberrations (Fig. 2) was low and not significantly different among the treatments and the negative control (χ^2 test, $p > 0.05$). These results showed that neither tretinoin nor tretinoin-loaded nanocapsules and blank nanocapsules induced to an increase in the frequency of chromosome alterations on onion root-tip cells.

Since the ability of retinoids to act as antiproliferative agents by modulation of the growth of both healthy and malignant cells is facilitated by the interaction with their respective receptors encoded by separate genes at distinct chromosomal loci [38], we can suggest the higher antiproliferative activity of tretinoin-loaded nanocapsules on onion root-tip cells by the higher penetration of the drug promoted by its

Treatment	Chromosome aberration			Chromosome aberration (%)
	Breaks	Laggards	Bridges	
Water	-	-	-	0.00
Tretinoin solution	1	1	10	0.24
NC-tretinoin	-	-	6	0.12
NC	-	-	8	0.16

Table 2. Number and percentage (%) of chromosome aberration observed on *Allium cepa* root-tip cells treated for 24 h in water, tretinoin aqueous solution (0.5 mg/ml), tretinoin-loaded nanocapsules - NC-tretinoin (0.5 mg/ml) and blank nanocapsules - NC. Total number of cells: 5000 (χ^2 test, $p \leq 0.05$).

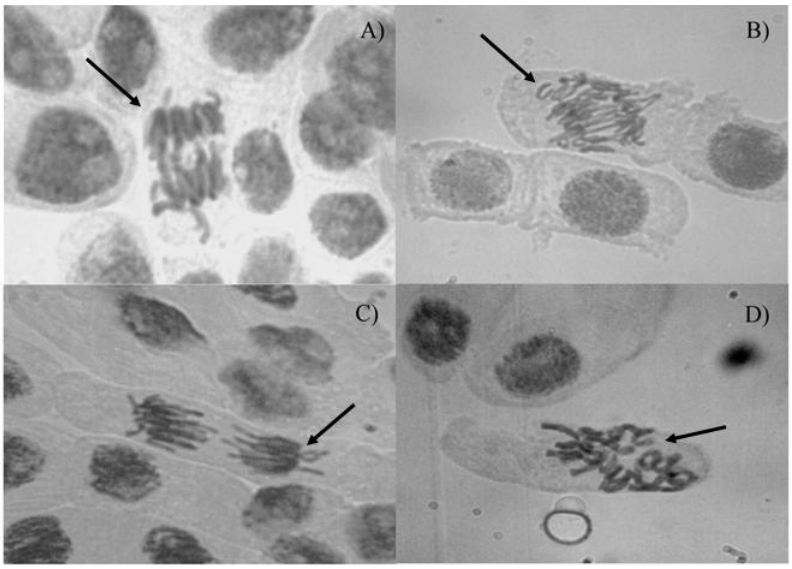

Figure 2. *Allium cepa* cells treated with tretinoin. **A**) cell with irregularity during cell division, arrow indicating anaphasic bridge; **B**) cell with chromosomal disorganization, arrow showing fragmented chromosome; **C**) cell with chromosomal aberration, arrow indicating chromosomal break; **D**) cell in mitotic division with chromosomal disorganization, arrow showing non-orientation (disturbed orientation). Scale bar = 4 µm.

nanoencapsulation. This higher penetration of drug encapsulated in nanoparticles has been reported by several authors to explain the improvement of the drug efficacy in intracellular infection and anti-cancer therapy [39-41].

CONCLUSIONS

The preparation of tretinoin-loaded nanocapsules was proposed in this study to evaluate the possibility to improve the antiproliferative activities of the free drug (tretinoin). Our results demonstrated that nanocapsule suspensions containing tretinoin improved the antiproliferative activities of the drug in comparison to its free form using the *in vivo* onion *(Allium cepa)* root-tip cell test, without an increase in the frequency of chromosome aberrations. Tretinoin-loaded nanocapsules represent a potential system to be further studied in animal models as an alternative for the treatment of cancer either by topical or system administration.

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