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CAMPTOTHECIN: SOLUBILITY, IN-VITRO DRUG RELEASE, AND EFFECT ON HUMAN RED BLOOD CELLS AND SPERM COLD PRESERVATION

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ABSTRACT

BACKGROUND: Camptothecin (CPT) is an anticancer drug, and is not employed in the clinic because of its high hydrophobicity and low active form stability. CPT may also have potential for use in cold preservation. **OBJECTIVE**: To overcome these drawbacks, CPT solubility variations in the presence of cyclodextrins (CDs) and polyethylene glycol (PEG) were evaluated by Higuchi solubility experiments. **MATERIALS AND METHODS:** CPT was encapsulated in different cyclodextrins and polyethylene glycol using a co-evaporation method. The CPT interactions with CDs and PEG 6000 were investigated by Fourier-transformed infrared spectroscopy (FT-IR), and X-ray powder diffraction (XRPD). Then, CPT complexes were evaluated for in-vitro drug release. To evaluate the potential anticancer efficacy of the CPT complexes system, in-vitro cytotoxicity studies on human red blood cells were carried out using UV assay. The impact of the CPT complex systems on sperm motility protection during cold preservation at 4°C was studied using CASA. **RESULTS:** The dissolution profile of these preparations shows the improvement of the dissolution of the CPT following a fickien diffusion. The CPT solubility and stability improvement were the cause of the cytotoxicity on the red blood cells test. However, CPT alone, encapsulated, dispersed, and chemically modified protected spermatozoids during cold preservation. **CONCLUSION:** We confirm the interest in CPT encapsulated and dispersed in anticancer treatments. We also found that CPT encapsulated or dispersed could protect sperm against oxidative damage and improve the membrane integrity of human sperm. Consequently, CPT encapsulated our dispersed could eventually be beneficial for infertility therapy.

Keywords: camptothecin; cold storage; cyclodextrins; human semen; PEG 6000; red globule.

INTRODUCTION

Natural chemicals are a valuable resource in the development of new drugs, particularly those used to treat cancer (1, 2, 3), and they may also serve as a safe replacement for a variety of synthetic drugs currently utilized in clinical treatments (4). Alkaloids are significant hotspots for creating plant-based antitumor medications. Alkaloids are tracked down

commonly in plants and are for the most part present in flowering plants (5). Specialists have brought about the identification of novel alkaloids that demonstrated powerful apoptotic and antineoplastic capacities in different malignant growth cells (6). Antitumor medicines have already been produced using alkaloids such as vinblastine and camptothecin (7, 8). Camptothecin (CPT), a broad-spectrum anticancer drug, was first isolated from *Camptotheca acuminate* (family Nyssaceae), a tree endemic to Tibet and China that has long been used in Chinese medicine (9). CPT analogs are used to treat cancers of the colon, ovary, and small-cell lung (5, 10). The CPT action mechanism is to inhibit the enzyme topoisomerase I. (Topo I). Human Topo I is a key enzyme that causes strand breaks and apoptosis induction by generating nonreversible and covalent Topo I-DNA complexes during DNA replication (11). Moreover, some synthetic analogs and semisynthetic compounds of CPT have been reported to serve as topoisomerase inhibitors by altering their catalytic activity by stabilizing covalent DNA-protein complexes (12, 13, 14, 15, 16). Irinotecan (CPT-11), is a soluble camptothecin-derived drug that was licensed for cancer treatment in 1994 and is still used as a key anti-cancer agent (17). CPT's therapeutic potential is limited by characteristics such as high toxicity, lactone ring instability, and water insolubility, limiting the drug's oral solubility and bioavailability in blood plasma (18). As a result, encapsulating CPT in various nano-sized delivery vehicles is an effective technique for overcoming these obstacles (19). Nanotechnology targets conveying drugs more viable to their objective for treating malignancies (20). These days, nanotechnology-based conveyance frameworks have acquired enormous consideration as a methodology for conquering the difficulties identified with bioavailability, dissolvability, dissemination, and poisonousness, and focusing on traditional chemotherapeutic specialists just as antitumor regular items (21, 22, 23, 24). Therefore, novel methodologies including pharmacological and low portions of CPT, either alone or joined with nanoparticles, could have powerful anticancer action in vitro and in vivo. One potential use CPT nanoformulations is on sperm protection, although little is known in this area. Consequently, the point of this work is to investigate, on one hand, the solubility effect of a potential enemy of malignant growth, camptothecin (alone, encapsulated with numerous cyclodextrins, cyclodextrin polymer, and in solid dispersion), and assess CPTs effect on human cells, such as cytotoxicity and apoptosis in the red globule. Moreover, we aimed to study the effect of the different systems on human semen cold preservation at 4°C.

MATERIALS AND METHODS

Material

Camptothecin (M.W. 348.11 g/mol) and irinotecan (M.W. 586.678 g/mol) were purchased from Shenzhen Boda Natural Product laboratory (P. R. China); beta Cyclodextrins(β-CD), 2-hydroxypropyl-β-cyclodextrin(HP β- CD), permethyl beta cyclodextrin (PM-β-CD) and poly β-CD polymer were purchased from SIGMA ALDRICH, Germany. Polyethylene glycol 6000, ethanol, methanol, dimethylsulfoxide (DMSO), di-sodium phosphate, mono-potassium phosphate, phosphoric acid, hydrochloric acid HCL andsodium chloride (NaCl) were purchased from (BIOCHEM Pharma). Fructose and TRIS (hydroxymethyl) aminomethane were purchased from SIGMA-ALDRICH.

Solubility studies

CPT solubility studies were carried out according to the Higuchi and Connors method (25). An excess of CPT was added to a deionized aqueous solution in vials containing increasing amounts of the oligosaccharide poly-β-CD and PEG 6000. These flasks were under magnetic stirring at room temperature for 7 d. Then, samples were filtered (0.45 µm) and the concentration of CPT was determined by UV. The presence of trace amounts of cyclodextrins did not interfere with the assay. The assays were performed in triplicate. The apparent stability constants (Kc) and the stoichiometry of camptothecin cyclodextrin complexes (CPT-CD) and camptothecin PEG solid dispersion (CPT-PEG6000) were estimated from phase solubility diagrams. For AL diagrams, apparent stability constant (Kc) of drug complex or solid dispersion can be calculated as follows (26):

$$KC = \frac{Slope}{S0\ (1-Slope)} \quad \text{[Eq.1]}$$

where S0 is the camptothecin molar solubility in the absence of poly-β-CD or PEG6000 and the slope is obtained from the initial straight-line portion of the plot of camptothecin concentration against the cyclodextrin or PEG concentration.

Moreover, complexation efficiency (CE) or solid dispersion efficiency (SDE) was calculated from the slope of the linear phase of the phase-solubility diagram (26):

$$CE = S0\ Kc = \frac{Slope}{(1-Slope)} \text{.....[Eq.2]}$$

Inclusion complexes and solid dispersion preparation

Co-evaporation. β-CD, HP-β-CD, PM-β-CD or poly-β-CD, and CPT (in a 1:1 molar ratio) were dissolved in 50 mL ethanol, the mixture was left under agitation for 2 h, and protected from light. After drying at 45°C for 1 h, the powder was preserved in a desiccator (27).

PEG 6000/ CPT dispersed. Polyethylene glycol 6000 and CPT were dissolved in ethanol by agitation. The solvent was then evaporated under vacuum at 40°C and the residue was kept in a desiccator until used (27).

Physical mixtures: CPT with different amounts of β-CD, HP β-CD, M β-CD, poly β-CD, and PEG 6000 were mixed in a mortar until an apparent homogeneous powder was obtained. The latter was preserved in a desiccator.

Infrared spectroscopy (IR). The successful encapsulation of CPT in CD and PEG 6000 was confirmed by infrared spectroscopy (IR) using a Frontier IR (IRAffinity-1 SHIMADZU). Pellets containing a mixture of samples and KBr were prepared and subsequently analyzed by infrared spectroscopy between the wavelengths at 500 to 4000 cm -1.

X-ray diffraction spectrophotometer. The samples were investigated by X- ray diffraction spectrophotometry (EXPERT Pro PANalytical theta II theta, vertical framework, programmed mode), based on scanning 2θ points somewhere in the range of 0 and 60°.

In-vitro drug release

In-vitro drug release was carried out according to a rotating basket method using a dissolution test system (Erweka, Germany). Briefly, 10 mg of CPT or its equivalent on complexes or solid dispersion were added to 900 mL of phosphate buffer (pH 7.2) at 37 ± 0.5°C. The rotation speed of the paddle was fixed at 50 rpm. Samples of 5 mL were collected at 0, 5, 10, 15, 20, 25, 30, 40, 60, and 120 min. CPT concentration was determined at 286 nm on Beckman DU 640 B spectrophotometer. Drug release data were analyzed using various models as follows: Zero order model, Higuchi model, first order model and Korsmeyer-Peppas model (28, 29, 30), in order to establish the mechanism of CPT release from different complexes and solid dispersion.

Cells test

Red blood cells and cellular hemoglobin test. All investigations were performed with new blood. The blood was centrifuged at 2000 rpm for 10 min at room temperature to isolate plasma and buffy coats. The supernatant was supplanted by an isotonic arrangement (NaCl 0.9%). Then, the blood was treated with various mixtures (v/v) (CPT, CPT11, β CD, HP β CD, M β CD, poly β CD, PEG, DMSO-CPT, and β CD-CPT, HP β CD-CPT, M β CD-CPT, poly β CD-CPT/NaCl 0.9%). The samples were incubated for 30 min at room temperature. All treatments were diluted: 100 µL / 3.9 mL of 0.9% NaCl. The cell fixations were evaluated by UV-visible spectroscopy at a frequency λ = 620 nm. Additionally, the measurement of hemoglobin was completed by UV-visible spectroscopy at a frequency λ equivalent to 412 nm and by recuperating hemoglobin by centrifugation of blood with the treatment chemicals for 10 mins at 2000 rpm.

Sperm motility analysis. Men with normal gamete characteristics that matched WHO criteria provided the sperm samples used in the current investigation (31). A Computer-Assisted Sperm Analyzer (CASA) (Sperm class analyzer, SCA Microptic, S.L., Version 3.2.0, Barcelona, Spain) was utilized to assess sperm motility parameters. To enable image capgture for the CASA analysis, sperm were diluted in order to have a good field of view (40×10^6 spermatozoa/mL. An aliquot of 5 µL of every sperm test was analyzed with a stage contrast magnifying lens (Nikon E200®-LED magnifying lens, Japan). Pictures were caught utilizing a camcorder (Caméra Digital Basler A312fc, Germany) at amplification x10 (negative stage contrast). Following a 24-h period of cold conservation at 4°C, the sperm

motility was assessed using the CASA method (full motility (%), moderate motility (%), straight direct speed (VSL m/s), and normal way speed (VAP m/s)).

RESULTS AND DISCUSSION

Solubility test

Solubility concentrates on stage solvency charts for the intricate development among camptothecin and the distinctive cyclodextrin and PEG are shown in Figure 1. This plot shows that the dissolvability of the medication in water increased linearly as a function of the oligosaccharide fixation. For the cyclodextrin polymer and PEG tried, the solvency graph of camptothecin can be named an AL type (25).

These outcomes would hypothetically propose the arrangement of a CPT cyclodextrin complex with a plausible 1:1 stoichiometry. Moreover, the same phenomena can be observed using dispersion in PEG. The evident dependability constants, Kc, calculated from the inclines of the straight stage dissolvability graphs were found to be around 23,9851 M^{-1} (for poly β-CD) and 4.7959 M^{-1} (for PEG6000). The complexation and solid dispersion efficiency was determined to be 0.0002 and 4×10^{-5} for poly β-CD, and PEG 6000, respectively. This indicates that the water-dissolvable framework shaped by the oligosaccharide and camptothecin expanded the dissolvability of CPT better than, at that point, PEG 6000 (Figure 1 and Figure 2).

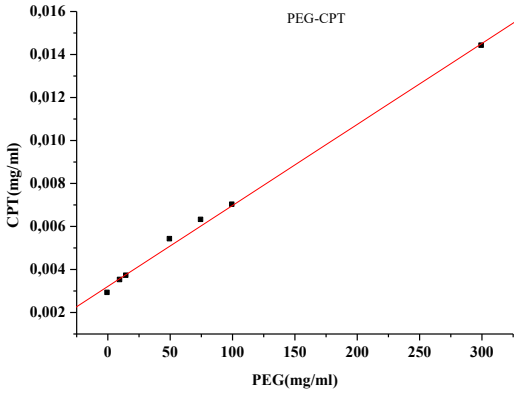

Figure 1. CPT/PEG6000 solubility diagram.

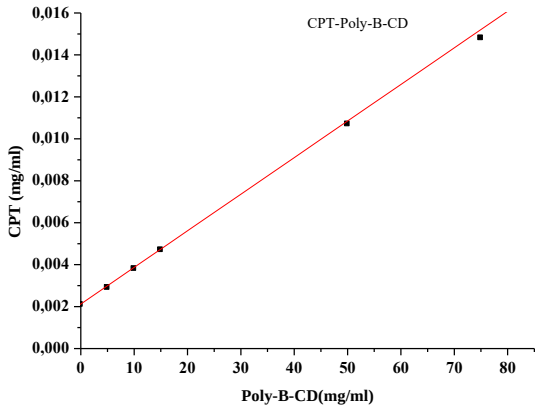

Figure 2. CPT/POLY β CD solubility diagram.

Infrared spectroscopy (IR)

The IR range of CPT shows trademark peaks that can be assigned to various elements, the most significant being (Figure 3):

(i) 2974.10 cm^{-1} corresponding to the vibrations of the fragrant rings;

(ii) 1741.30 cm^{-1} for the C=O lengthening vibration of the lactone ring;

(iii) 1655 cm^{-1} for the C = O lengthening vibration of the ketone gatherings;

(iv) 1490 cm^{-1} for the vibrations of the phenyl rings (32).

The actual blends of chemicals had peaks normal for CPT. These spectra are essentially comparable to the expansion of those of CDs and CPT. While on the spectra of the prepared complexes, we note the loss of some peaks characteristic for CPT related to the C=O bonds. The spectral expansion of CDs and the CPT shows that there is no evidence for the development of incorporation. This implies that there is no cooperation between the CPT and the CDs in a basic actual combination. The loss of at least one trademark peak of CPT on account of edifices demonstrates that there is a connection among CPT and CDs without the association of covalent holding. It seems that the entire particle or possibly a portion of its moieties (like C=O) are consolidated into depressions of CDs (32).

X-ray diffraction spectrophotometer

X-ray diffraction analysis of the CPT shows sharp, intense peaks, and characteristic peaks at 2θ between 0 and 60°. The diffractograms of the physical mixtures show a superimposed spectrum of the CPT and the added compounds with almost no disappearance of the characteristic peaks. While on the spectra there is evidence of inclusion

complexes, there is the disappearance or a clear decrease in the intensity of the peaks, as well as the appearance of the few new peaks. The PEG 6000 spectrum shows sharp and arranged peaks, but after solid dispersion of the CPT we note the disappearance of this arrangement. The intensity and shape of the characteristic peaks of CPT suggest that it is present in crystalline form (arranged state). The disappearance of its characteristic peaks or the decrease in their intensity, in the cases of complexes, indicates a marked reduction in crystallinity. This suggests the formation of amorphous inclusion complexes, which according to the literature confer better solubility to the active molecules (33). The disappearance of the peak arrangement in the case of dispersion in PEG 6000 indicates a transition from the crystalline to the amorphous state, which can be explained by the co-evaporation dispersion of CPT in PEG 6000 (Figure 4).

In-vitro drug release

The amount of untreated CPT dissolved increases until it reached 7%, then it stabilized at 120 min, reaching 14% (Figs 5, 6). CPT 11 dissolves almost instantly, reaching 97% within the first few minutes, and then reaching 100% as shown in Figures 5 and 6.

All other curves showed an increase in the amount of CPT dissolved, such that:

(i) The complexes (CDs-CPT) and the solid

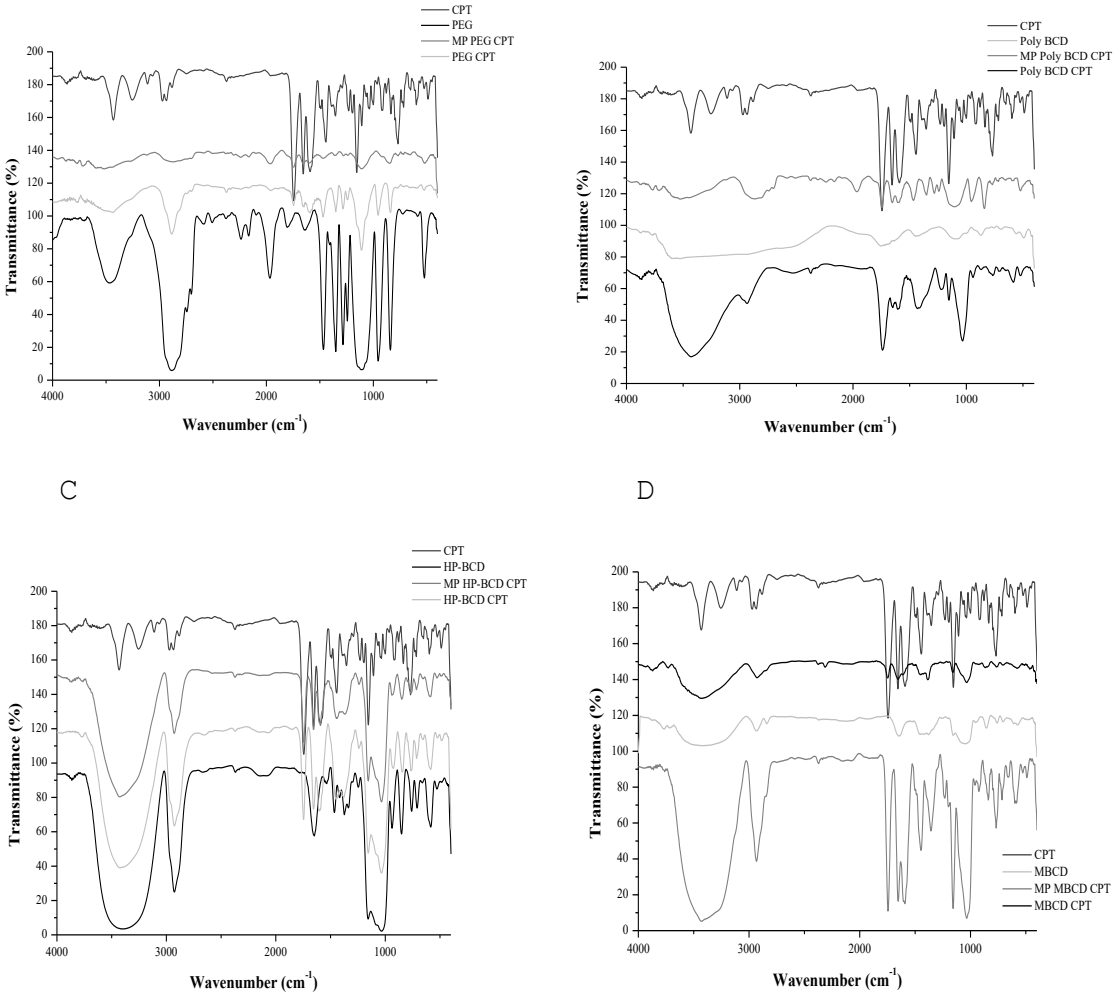

Figure3. FT-IR spectra: (A) CPT, HP-BCD, MP HP-BCD CPT, HP-BCD CPT, (B) CPT, M BCD, MP MBCD CPT, MBCD CPT, (C)CPT, Poly B CD, MP Poly B CD CPT, Poly B CD CPT, (D)CPT, PEG, MP PEG CPT, PEG CPT.

dispersion (PEG 6000-CPT) formed to offer a better percentage of CPT dissolution than the physical mixtures. Overall, PEG releases CPT faster and at a higher dose.

(ii) Among the β CD-CPT, HP β CD-CPT and M β CD-CPT complexes, the latter achieved a better percentage of dissolution, such that it reaches a dissolution rate four times higher than that of CPT alone (Fig. 5).

(iii) As for the two polymers, they reach after about 120 min approximately the same percentage of dissolution (41% for PEG 600 and 39% for poly β CD) (Fig. 6).

(iv) The solubility problem of CPT explains its low dissolution rate (14%).

The spontaneous and complete dissolution of CPT 11 is due to the fact that it is a water-soluble derivative of CPT, which is why it was chosen in our study. The increase in solubility of CPT in the complex cases is directly related to the formation of inclusion complexes which subsequently increase the solubility of the PA. The β CD-CPT complex offers lower solubility compared to the other complexes because its native form β CD has limited water solubility. For poly β CD, the increase in dissolution is due to inclusion and lattice binding. In the case of PEG 6000 this increase is due to the hydrophilizing ability and wettability of PEG (32, 34), as well as a state change as observed during XRD. The low Kc calculated also explains the fact that the CPT release of PEG 6000 is higher than that of Poly β CD. The best percentage of dissolution was obtained when using PM β CD-CPT. This can be explained by the fact that the methyl groups are more polar than those of HP β CD, and PM β CD is more soluble than β CD and maybe even poly β CD.

The Higuchi model represents the best fit, as it gives the best correlation coefficient values compared to the other models, indicating that the dissolution (or relegation) of CPT follows a Fickien diffusion mechanism.

The release exponent n of the Korsmeyer-Peppas model is lower than 0.43 (Table 1), reflecting a dissolution phenomenon due to a chemical potential gradient, which is in agreement with the Fickien diffusion mechanism of the Higuchi model (35).

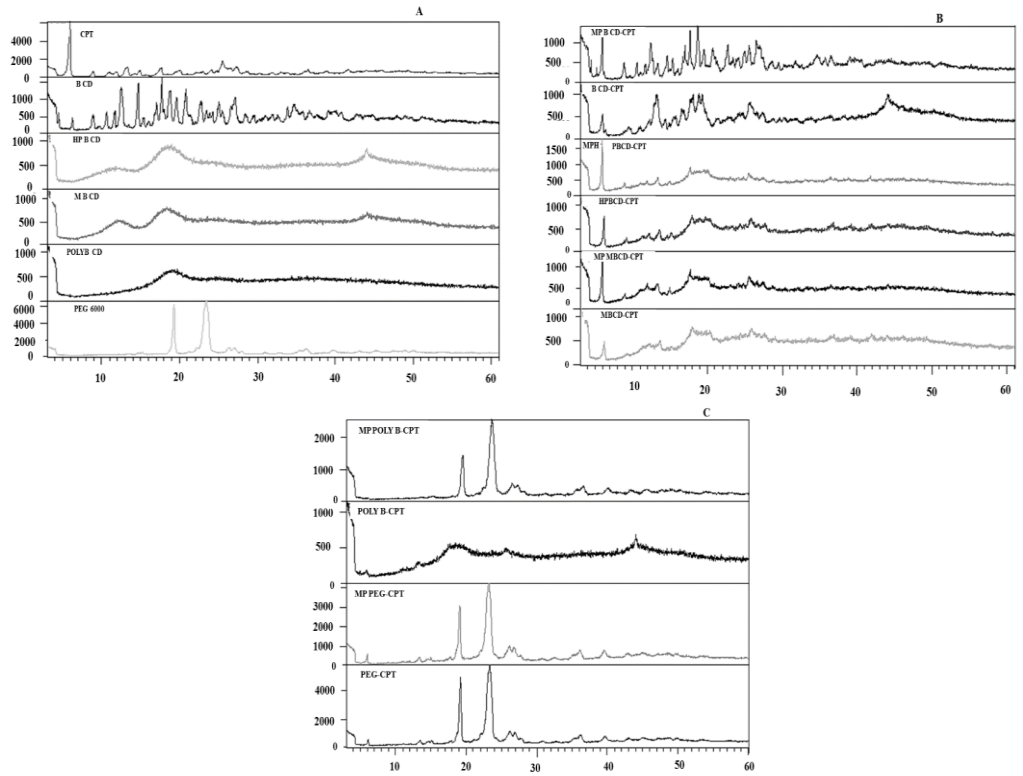

Figure 4. XRPD diffraction: (A) Diffractogram of CPT, β-CD, HPβ-CD, M β-CD, Poly β-CD and PEG, (B) Diffractogram of physical mixtures and complex of β CD- CPT, HP β CD- CPT and M β CD- CPT, (C) Diffractogram of physical and complex mixtures Poly β CD- CPT, et du PEG 6000- CPT

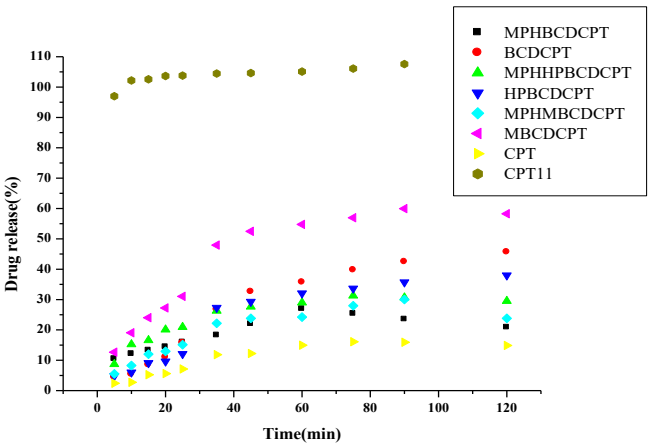

Figure 5. Release profiles of CPT 11, CPT, βCD/CPT, HPβCD/CPT, MβCD/CPT and their physical mixture.

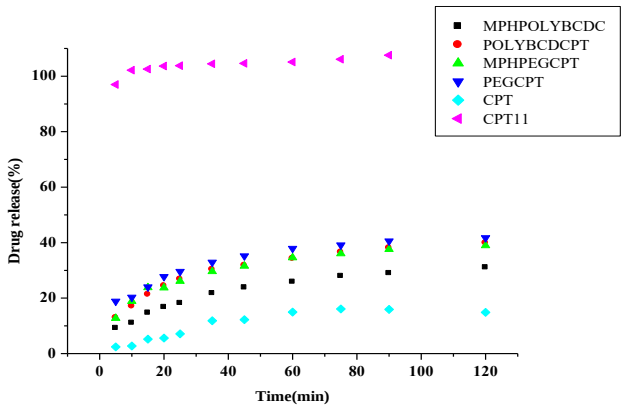

Figure 6. Release profiles of CPT 11, CPT, poly βCD-CPT, PEG 6000-CPT and their physical mixture.

Cells test

Test on red blood cell and hemoglobin. It can be seen that there is an inverse relationship between cell concentrations and hemoglobin for the majority of samples. It was also observed that values of cellular concentrations in all samples were close to the control with physiological medium (0.9% NaCl solution), except for the sample treated with DMSO. It is also apparent that globally cellular concentration values of complexes were low compared to their respective controls. Also in the case of the DMSO-CPT treatment, a decrease in cellular concentration values was obtained that remains close to that observed using DMSO alone. The above observations are more accentuated and clearer in Figure 7, representing a comparison by category (control and treatments).

The inversely proportional relationship between the amounts of hemoglobin and cell concentration can be explained by the hemolysis of red blood cells releasing hemoglobin.

The cell concentrations in all the controls used were high except for DMSO. This indicates that cyclodextrins and PEG controls do not influence red blood cells. Therefore, these molecules do not present a toxic effect at the concentrations used. This explanation can be supported by the fact that the Food and Drug Administration (FDA) has accepted these two molecules for pharmaceutical use (36, 37). DMSO presents low toxicity even at 1% (v/v). The same observations are noted for the CPT and CPT11 controls, which may be due to the

Table 1. Drug release kinetic data obtained from fitting drug release experimental data by various mathematical models.

Mathematics Models		β CD-CPT	HP β CD-CPT	M β CD-CPT	POLYβ CD-CPT	PEG-CPT
Zero order	K_0	0.0040	0.0032	0.0420	0.0022	0.0020
	R^2	0.8828	0.8244	0.7655	0.8315	0.8337
First order	K_{1st}	0.0055	0.0042	0.0072	0.0031	0.0030
	R^2	0.9168	0.8496	0.8050	0.8669	0.8619
Higuchi	K_H	0.0554	0.0451	0.0602	0.0309	0.0284
	R^2	0.9572	0.9070	0.8875	0.9438	0.9423
Korsmeyer-Peppas	K_p	0.1612	0.2504	0.9262	0.9467	1.0475
	R^2	0.8871	0.8682	0.8540	0.8865	0.9072
	n	0.2832	0.2529	0.1725	0.1172	0.0928

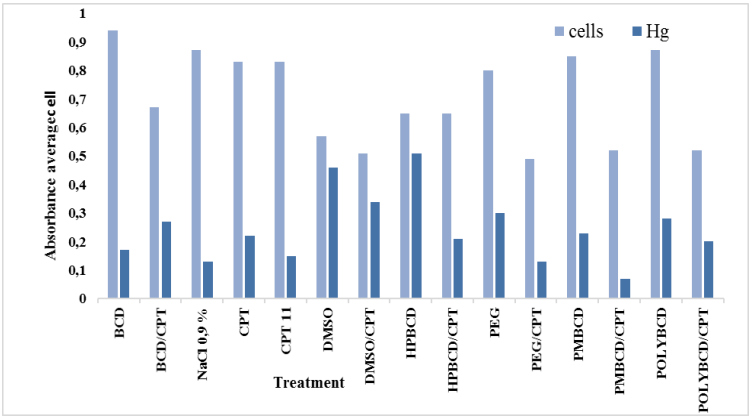

Figure 7. The average cell and hemoglobin absorbances.

low solubility of CPT, and to the instability of the lactam ring of these two molecules, essential to their anticancer effect (38). The DMSO-CPT treatment showed a decrease in cell concentration although it remained close to that of DMSO alone. This indicates that this result is probably due to the toxicity of DMSO and not to that of CPT even if the latter is soluble. The cell concentration decrease is due to the increase in the solubility and stability of CPT and thus its toxic effect due to the complexation with the different CDs and the solid dispersion in PEG 6000 (39, 40). Figure 7 confirms the observations as well as the hypotheses made because the tendency proves that the complexes formed and the solid dispersion carried out, have an effect more important than the controls. Free CPT showed cytotoxicity in a concentration-dependent manner (Figure 7). An almost similar trend of cell cytotoxicity was observed with CD-CPT and PEG-CPT. Importantly, the cell viability of CD-CPT and PEG-CPT-treated cells was slightly higher than that of free CPT. This result demonstrates a potential cancer-cell impact (39).

Human sperm motility analysis. The results show a maximum velocity (VSL and VAP) when using CPT 11 and the PM β CD-CPT, poly β CD-CPT, and PEG 6000-CPT solid dispersion complexes. Overall, CPT alone and when encapsulated showed a positive effect on sperm motility after 24 h of cold preservation at 4 °C (Figs 8, 9). It can be explained by the fact that camptothecin is an alkaloid, and it is known that alkaloids have protective effects on sperm cells (41). Based on our findings, CPT encapsulated or dispersed

could protect sperm against oxidative damage and improve the membrane integrity of human sperm. However, an optimization study of the concentration of CPT alone and/or encapsulated is mandatory to control the concentration of the treatment as well as to improve the protection of the spermatozoa as long as possible.

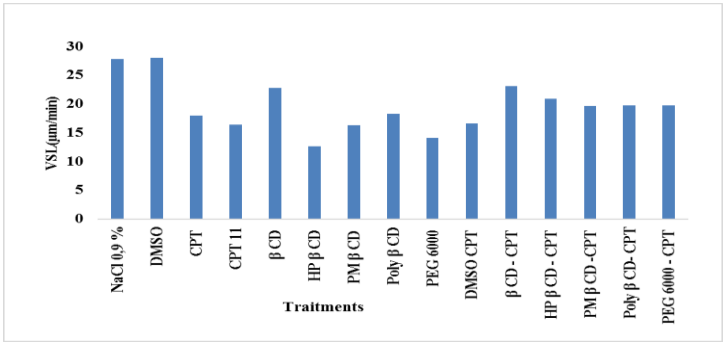

Figure 8. Sperm VSL according to the treatments after 24 h of cold preservation.

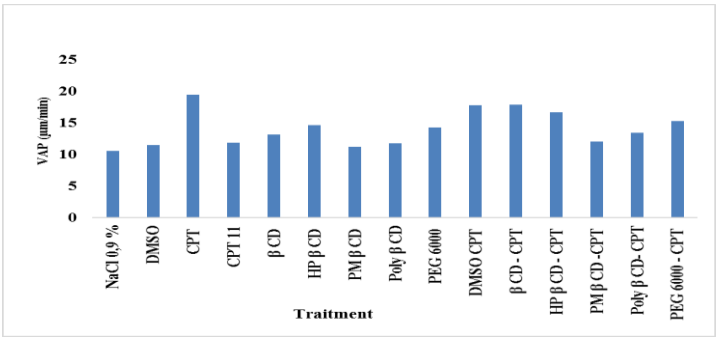

Figure 9. Sperm VAP according to the treatments after 24 h of cold preservation.

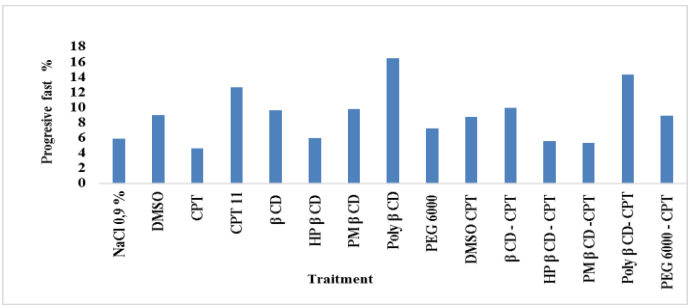

Figure 10. Fast progressive percentage of motility after 24 h of 4°C storage of human spermatozoa in the control group (NaCl 0.9 %) and groups treated with CPT and PM β CD-CPT, poly β CD-CPT, and PEG 6000-CPT.

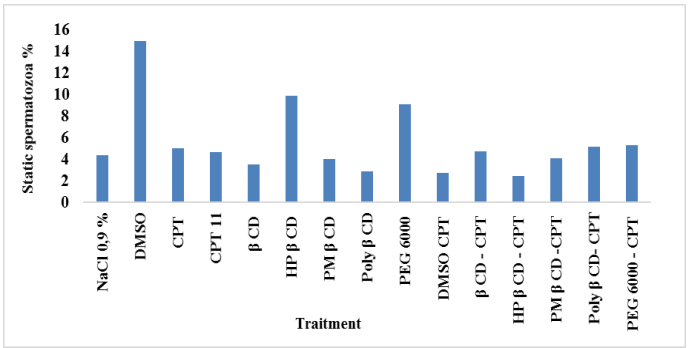

Figure 11. Static progressive percentage of motility after 24 h of 4°C storage of human spermatozoa in the control group (NaCl 0.9 %) and groups treated with CPT and PM β CD-CPT, poly β CD-CPT, and PEG 6000-CPT.

Percentage of fast progressive and static spermatozoa. The proportion of rapid and static progressive spermatozoa in each sample has been determined and the results presented in Figures 10 and 11. Fast progressive spermatozoa mobility showed that the CPT alone and encapsulated exhibited a significant percentage. Also, static progressive percentage showed that the treatments based on CPT encapsulated or dispersed induced fewer static spermatozoa than their respective controls (NaCl and DMSO). This indicates a positive effect of CPT on the progressive percentage of human spermatozoa. This outcome can be related to CPT's alkaloid nature, which is renowned for protecting spermatozoa (41, 42), and solubility enhancement by encapsulation in various systems.

Also, sugars and cyclodextrins are known to function as cryoprotectants. Consequently, their inclusion in the formulations aids in spermatozoa protection. Finally, we can state that this novel anti-cancer preparation (encapsulated CPT) has no harm to human sperm.

CONCLUSION

Our study aimed to improve the solubility of the anti-cancer agent camptothecin (CPT) using a novel combination of beta-cyclodextrin polymer (poly β CD) and CPT. It was demonstrated by a solubility test that there is an interaction between CPT and poly β CD. Inclusion complexes between this polymer, natural and modified cyclodextrins, and CPT were prepared, as well as a dispersion of the latter in polyethylene glycol 6000 (PEG 6000). These preparations were characterized by infrared and XRD, confirming the existence of interaction between the studied polymers and the active molecule. The dissolution profile of these same preparations shows the improvement of the dissolution of the CPT following a Fickien diffusion. The cytotoxicity discovered during testing on red blood cells is due to this improved stability and solubility. The current investigation demonstrated that sperm motility considerably increases using CPT encapsulated and dispersed. However, in future studies, an optimization of the concentration of CPT alone, encapsulated, and dispersed is mandatory. Then it might be possible to control the concentration of the treatment as well as improve the protection of the spermatozoa as long as possible.

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