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Drug Formulations for Localized Treatment of Human Papillomavirus-Induced Lesions

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ABSTRACT

Background: The human papillomavirus (HPV) is responsible for over 90% of all cervical cancer cases. The use of vaginal gels is often indicated for local vaginal drug delivery. Previous studies have shown that Thymus vulgaris essential oil (TEO) exhibits anticancer properties besides antifungal and antibacterial properties. Its activity derives from a specific increase in free radicals and oxidative stress caused in cancer cells. Furthermore, mitoxantrone (MTX), an anthracenedione, and C_8, an acridine orange derivative, were shown to inhibit the growth of the cervical cancer cell line HeLa.
Results: The results showed that TEO + C_8 is the most promising formulation in terms of viscosity and osmolality properties in vaginal fluid simulant (VFS). The combined action of TEO with the compounds MTX and C_8 resulted in HeLa cell viability reduction compared with the effect obtained with the individual formulations containing each one of the compounds.
Conclusions: The formulation TEO + C_8 holds promise in terms of cost-benefit and topical application of the active compound for the HeLa cells.

Introduction

Cervical cancer is the fourth most common cancer among women worldwide, with estimated 604,127 new cases and 341,831 deaths in 2020 (GLOBOCAN),[1] and high-risk HPVs are the leading cause of this cancer. [2]

In the recent years, the combination of screening programs for cervical cancer and vaccination against HPV has greatly reduced cervical cancer incidence and mortality.[3]

The majority of infections are solved spontaneously within two years; however high-risk HPVs are capable of inducing oncogenesis by the expression of oncogenes E6 and E7 which deregulate tumour suppressor genes, leading to the development of cervical lesions with increasing severity.[2,3] Currently, prophylactic HPV vaccines are available and efficient but ineffective for women already infected. In many HPV infections are only detected when cancer has already developed. Treatment options for precancerous states are usually excision or ablation of the lesion tissue and in case of advanced cancer the therapeutic options available are surgery, radio and chemotherapy. These methods are invasive, with limited effectiveness and

Abbreviations: HPV, Human papillomavirus; VFS, Vaginal Fluid Simulant; HPLC, High-performance Liquid Chromatography; MTX, Mitoxantrone; C_8, 10- (8- (4-iodobenzamide) octyl)) - 3, 6-bis (dimethylamine) acridinium iodide; HeLa, Human cervical cancer cells line; NHDF, Normal human dermal fibroblasts; TEO, *Thymus vulgaris* essential oil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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undesirable side-effects, and regrettably, about 50% of the cancer patients will die. [4,5] New compounds and anticancer therapy approach must be developed to induce cancer cell death and reducing their sustained proliferative signalling and growth. Certain essential oils have been reported as promising anticancer agents and are currently being investigated for their cytotoxic and antiproliferative activities in cancer cell lines.[6] Moreover, they can increase the cytotoxic activity of chemotherapeutic drugs open the possibility of reducing their dose while providing the same effect.[7] One example is the *Thymus vulgaris* essential oil (TEO), which is composed mainly by two phenolic compounds, thymol (2-isopropyl-5-methylphenol) and its phenol isomer carvacrol (5-isopropyl-2-methylphenol); however other compounds such as cineol, cimene, α-pinene and borneol, are present in low concentrations.[8] It is assumed that its pharmacological activities are mainly related to thymol, one of the main constituents (10 to 64%) followed by carvacrol (0.4 to 20.6%).[9] Although the mechanism of action of thymol and its derivatives is not well understood and it may include suppression of cell growth, induction of apoptosis, production of intracellular reactive oxygen species (ROS), depolarization of the mitochondrial membrane potential with activation of the protein Bax (pro-apoptotic mitochondrial protein) and interaction with caspases.[10]

The understanding and application of techniques to evaluate cell viability of drugs and their permeation through the vaginal epithelium, as well as their characterization in terms of pH and buffering capacity, viscosity, osmolality and bioadhesion, contributes to a well-prepared selection of drugs and vaginal formulations in the initial stage of development. Furthermore, *in vitro* and *ex vivo* approaches are extremely important, since they define the effectiveness and performance of vaginal products in the treatment of precancerous lesions.[11]

This study focuses on the development and characterization of intravaginal formulations based on TEO for the delivery of potential chemotherapeutic drugs with known cytotoxic activity on cervical cancer cell line, in order to prevent the development of the disease and evaluate their behaviour when in contact with the vaginal tract. The tested compounds are the commonly used antineoplastic agent mitoxantrone (MTX) and an acridine orange derivative (C_8) which has shown promising anticancer effect in cervical cancer cell lines.[6,7]

The cell viability of drug-loaded formulations was evaluated in the cervical cancer cell line HeLa, as well as their potential for drug delivery and retention in the target tissue by Ussing chambers. Altogether, our studies intend to show the potential of developing simple cost-effective gel formulations based on TEO and anticancer compounds for topical vaginal application.

Materials and Methods

Preparation of Vaginal Formulations

The excipients[12] selected for the development and preparation of the gel formulation are Generally Recognized as Safe (GRAS, FDA), pharmaceutical grade materials (European Pharmacopoeia 9.0) and have evidence of previous use in vaginal products marketed in Europe.

Preparation of the Base Formulation

The method used to prepare the gel formulations was based on the following steps:

0.1% (w/w) potassium sorbate (Merck, Germany) was used as preservative and dissolved in 10 g of water. The polymer hydroxypropylmethyl cellulose (Methocel K100 – viscosity 100000 cP, 2% aqueous solution, Dow, USA) was dispersed in the remaining water using a helical stirrer (Heidolph RZR 2041, Heidolph Instruments GmbH & Co., Germany). Propylene glycol (Acros, USA) was added under agitation. The resulting gel was centrifuged at 3000 rpm for 5 min, at 4 °C to allow for the removal of entrapped air.

The pH of the mixture was then adjusted to 5.5 using NaOH 10 M.

The TEO formulation was prepared using similar procedure by dispersing 1% (w/v) of TEO previously mixed with Tween 80 (1%) for emulsification purposes. Then, MTX and C_8 were added to the formulations at concentrations of 50 μM.

The same concentrations of MTX and C_8, and 1% Tween 80 were also added to the base formulation, thus resulting in 7 final formulations: Placebo/Base formulation (BF), 1% (w/v) TEO formulation (TEO), base formulation + MTX (BF + MTX), Base formulation + C_8 (BF + C_8), TEO + MTX, TEO + C_8 and Base formulation + Tween (BF+ Tween).

The composition of formulations and placebo are presented as % w/w in Table 1.

Preparation of Vaginal Fluid Simulants

The vaginal fluid simulant (VFS) was prepared as described by Owen and Katz in 1999.[11] Briefly, 3.51 g sodium chloride, 1.40 g potassium hydroxide, 0.22 g hydroxide calcium, 0.018 g of bovine serum albumin, 0.40 g of urea, 5.00 g of glucose, 2.00 g of lactic acid, 1.00 g of glacial acetic acid, 0.16 g of glycerol were added to less than 1 L of ultra-pure water and the solution was stirred vigorously until complete dissolution and complete homogenization. The pH of the mixture was then adjusted to 4.46 using HCl, and the final volume was adjusted to 1 L. For the preparation of the modified VFS, swine

Table 1
Composition of formulations and placebo presented as % w/w.

	Formulation name						
	Composition % (w/w)						
	BF	TEO	BF + C_8	BF + MTX	TEO + C_8	TEO + MTX	Tween 80
HPMC (Methocel K100M)	1.5	1.5					
Propylene glycol	8	8					
Potassium sorbate	0.1	0.1					
Water	90.4	90.4					
Thymus vulgaris oil	-	1					
Polysorbate 80	-	1					1
NaOH 10 M	Enough to adjust the pH to 5.5	Enough to adjust the pH to 5.5					
BF			99.6	99		-	99
TEO			-	-	99.6	99	-
C_8			0.4	-	0.4	-	-
MTX			-	1	-	1	-

Note: Formulations BF + C_8, BF + MTX, TEO + C_8, TEO + MTX and Tween are composed by formulation BF and TEO.

gastric mucin type II (Sigma, Germany) was added to the VFS as described by Katz in a concentration of 1.5% (w/w).[3]

Technological Characterization of Formulations

pH and Buffering capacity

The pH measurements were performed in triplicate (Thermo scientific Orion Star A211 pH meter, ThermoFisher) and the absolute buffering capacity was calculated and defined as the amount of NaOH and HCl needed to change by one unit the initial pH value.[12] The relevant pH-buffering capacity was calculated as the amount of NaOH required to reach a pH value of 5. For formulations with initial pH higher than 5, the reverse buffering capacity was performed by adding 20 μL of HCl 1 M until pH lower than 3.[13]

Viscosity

Viscosity determination was performed using a conical plate rheometer (Brookfield DV-3T, Brookfield, USA). The measurements were made directly in formulations and after dilution in VFS, at the physiological vaginal temperature (37 °C). The cones used were CPA-52Z and CPA-40Z, 3° and 8°, and 1.2 cm and 2.4 cm, cone angle and radius, respectively (Brookfield, USA), and 500 μL of sample was placed on the plate. The tests were performed in triplicate, and left to equilibrate for 1 min between measurements, with rotation speeds between 5 and 250 rpm in order to obtain an acceptable torque (10-100%).

Osmolality

The osmolality was determined using a freezing point osmometer (Osmomat 3000, Gonotec, Germany), as previously described.[12,13] Before the experiments, three reference standards were used to calibrate the equipment, which were distilled water (zero point), NaCl 300 mOsm/Kg and NaCl 850 mOsm/Kg commercially available from the equipment manufacturer. Each experiment used 50 μL aliquots of the simple and diluted VFS formulations and the measurements were performed in triplicate. The procedure in VFS was established to estimate the osmolality of the product when put in contact with vaginal fluid, since 0.75 mL is the estimated mean volume of fluid present in the vagina at any moment.

Bioadhesion

Porcine vaginal tissue (obtained from a local slaughterhouse) was isolated from the other genital organs using surgical scissors. Then, the tissues were washed in 0.9% NaCl and stored at -80 °C until used.

The bioadhesion of the formulations to the porcine vaginal tissue was assessed using a texturometer (TAXT Plus, Stable Micro Systems, United Kingdom), in which the method consisted of assessing the tensile strength of the interfacial layer formed between the formulation and the vaginal epithelium. To determine the bioadhesive strength, a cylindrical probe with a diameter of 10 mm (P10) was used.[14]

The measurements were performed at 37 ± 1 °C in an oven to mimic body temperature. The epithelial tissue samples were fixed with the mucoadhesion device and placed on the base of the equipment. The tissue was hydrated with 50 μL of VFSm, assuming that mucin is the protein responsible for bioadhesion. A double-sided adhesive tape was used to attach a small piece of cellulose acetate membrane to the probe, where 30 mg of formulation was directly weighed. The cellulose membrane attached to the probe without formulation was used as a control. The software was used in adhesive mode according to the conditions described previously[12], connected to the texturometer. The detachment force was recorded, as well as the negative graphic area, representative of the adhesion work (N. mm) required to detach the two surfaces.

Evaluation of Formulations in the Culture Medium

The formulations containing MTX and C_8 were diluted to concentrations of 30%, 20%, 10%, 5%, 1%, 0.4%, 0.2%, 0.1% (w/v) in DMEM medium supplemented with 10% fetal bovine serum, and 1% penicillium/streptomycin antibiotic containing 0.5% DMSO to ensure adequate solubility of the formulations. The formulations were left in contact with the culture medium for 48 h and then they were visually inspected for precipitation identification.

Cell Viability Assay

Normal human dermal fibroblasts (NHDF) were grown in RPMI-1640 medium (Sigma-Aldrich, Missouri, USA) supplemented with 0.01 M HEPES, 0.02 M L-glutamine, 0.001 M sodium pyruvate, 10% fetal bovine serum, and 1% penicillium/streptomycin antibiotic (Sigma-Aldrich, Missouri, USA). Human cervical cancer cells (HeLa) were grown in DMEM medium supplemented with 10% fetal bovine serum (Life Technologies, California, USA) and 1% penicillium/streptomycin antibiotic. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 96-well plates (1 × 10 cells/mL) and incubated overnight for cell adhesion. Then, cells were incubated for 24 h with 100 μL of each concentration of formulations (10%, 5%, 1%, 0.4%, 0.2% and 0.1%).

Wells containing untreated cells were used as control. At the end of incubation, the media was replaced with fresh media containing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide salt (MTT, Sigma-Aldrich, Missouri, USA) and HeLa cells were further incubated at 37 °C for 45 min and NHDF cell for 1 h 15 min. The resultant formazan crystals were then dissolved in 100 μL DMSO and the absorbance recorded in a BioRad xMark™ microplate reader (BioRad, USA) at 570 nm. Cell viability was obtained through the following calculation: mean absorbance of treated / mean of controls x 100 and expressed as mean ± SEM from at least three different plates with each condition tested in triplicate wells. All data treatment was performed using GraphPad Prism 6. The percentage of cell viability is calculated with the following equation (where A is the absorbance, and the blank contains only medium).

$$\%\ Cell\ Viability = \frac{(A\ treament\ -\ A\ blank)}{(A\ control - A\ blank)} \text{x}100$$

Permeation of formulations in vaginal tissue (ex vivo assay)

Vaginal epithelial tissue was excised from freshly porcine tissue in the nearest slaughterhouse (Oviger, Alcains, Castelo Branco, Portugal).

Square pieces of vaginal tissue were cut using a dermatome with around 300 μm thickness, and mounted on NaviCyte horizontal 9 mm circular Ussing chambers with an exposed tissue surface area of 0.64 cm2 (Harvard Apparatus, USA) with the mucosa facing the donor chamber. 1.8 mL of VFS was added to the receiving chamber. In the donor chambers, 200 μL of VFS was added to all cells, in order to balance the membrane. It was allowed to settle for 20 min and the VFS of all Ussing chambers, except the control, was replaced with 300 mg of the following formulations: TEO, TEO + C8 (50 μM), TEO + MTX (50 μM) and the base formulation. The samples (100 μL) from the receptor chamber of each formulation were collected at the following times: 0 (zero moment), 5 h, 20 h 30 min, 25 h 30 min and 56 h after the formulations were placed. At the end of 56 h, samples from the donor chambers and epithelial tissue were also collected and reserved for further analysis by the high-performance liquid chromatography (HPLC) method.

HPLC Analysis

A 1290 HPLC coupled to a 1260 fluorescence detector (FLD) from Agilent Technologies (Soquimica, Lisbon, Portugal) was used to determine the compounds concentration. The separation of the analytes was achieved using a YMC–Triart PFP (5 μm, 4.6 i.d. × 150 mm) analytical column coupled to a Guard–c holder (4 ×10 mm) and a Triart PFP (5 μm, 3 × 10 mm) pre–column, supplied by Solítica, Lisbon, Portugal). Methanol with 0.1% trifluoroacetic acid in water (9:1; v/v) was used as mobile phase at a flow rate of 0.5 mL/min in isocratic mode. Total chromatographic run was 20 min. The injection volume was 100 μL. The column and autosampler temperatures were set at 25 and 4 °C, respectively, and the analytes were detected at 498 and 530 nm as excitation and emission wavelengths, respectively. The retention times were 2.4 min for MTX and 9.3 min for C_8. The method was validated according to the guiding principles of the Food and Drug Administration (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics) for the analysis of drugs and biologics. The calibration curves were established between 0.2 and 6.50 μg/mL for MTX and 0.02 and 100 μg/mL for C_8. The limits of detection and quantification were considered the same as the limit's quantification, 0.20 and 0.02 μg/mL for MTX and C_8, respectively. Figure S1A describes a chromatogram of MTX at 0.20 μg/mL and Figure S1B describes a chromatogram of C_8 at 0.02 μg/mL.

ELISA Assay

The expression of interleukin (IL)-6 by NHDF cell line was evaluated with ELISA. Cells were seeded in 96-well plates (2×10^4 cells/mL) and incubated overnight for cell adhesion. Then, cells were incubated for 24 h with 100 μL of each concentration of formulations (1% and 0.2%). After 24 h, culture supernatants were collected. Three independent experiments were performed (n = 3). Then, the IL-6 levels present in the collected supernatant was measured by a commercially available ELISA assay kit (RayBio® Human IL-6 ELISA Kit; ref ELH-IL6). The ELISAs were performed according to the manufacturer's instructions. The sensitivity of the assay for IL-6 was 3 pg/mL. Samples and standards were tested in duplicate. After the enzyme-substrate reaction terminated, the color change was measured spectrophotometrically at a wavelength of 450 nm in a BioRad xMark™ microplate reader (BioRad, USA). The concentration of IL-6 in the samples was determined by comparing the optical density of the samples to the standard curve. Average the duplicate readings for each standard and samples was determined and the average zero standard optical density was subtracted. A standard curve was obtained, with IL-6 concentration on the x-axis and absorbance on the y-axis and the points were fitted and determined by regression analysis.

Statistical analysis

The statistical analysis was performed by using Student's unpaired t-test or ANOVA followed by Dunnet's and Sidak's test for multiple comparisons. A p value < 0.005, 0.0001 and 0.00001 was considered statistically significant. Data analysis was performed in GraphPad Prism 6 (San Diego, USA).

Results and discussion

Technological characterization of formulations

pH and Buffering capacity

The pH of the formulations and pH buffering capacity are important parameters since they must be compatible with the vaginal physiological acidic pH which is normally 3.5–4.5 in non-pregnant women in their reproductive age.[12] The determination of the pH buffering capacity, when performed in VFS dispersion intends to simulate the real conditions *in vivo* after the administration of the formulation. Although the formulations were developed with an initial pH > 5, when in contact with the VFS the pH decreased to approximately 4.5 (Table 2) being in accordance with physiological vaginal pH, possibly decreasing any undesirable effects. The pH variation of the formulations in VFS was also evaluated over a period of 72 h (Figure S2).

As previously described, the increase in vaginal pH (pH> 5) is associated with vaginal infections and those with lower pH (pH <3) conditions are still poorly understood.[2,12] However, experimental results in animals suggested that vaginal products with pH values <3 are less acceptable for human use.[10] Changes in vaginal pH due to HPV infections or cervical intraepithelial neoplasias have not been described in the literature. As shown in Fig. 1 all formulations showed higher buffering capacity after being diluted in VFS than in normal saline conditions, in accordance with results previously published, and explained by the influence of the dissolving medium.[13] For the pH buffering capacity of formulations, two parameters were considered: the relevant and the absolute buffering capacity. Although the formulations have a pH above the physiological limit, they present low capacity to maintain pH when in contact with VFS, revealing that they can easily reach the normal range of pH vaginal physiological. According to the formulations in VFS, the TEO + MTX showed the highest buffering capacity, suggesting that its application will not modify the vaginal pH, as showed in Figure S2.

Viscosity

The rheological properties of vaginal formulations influence the drug release and the passive displacement between epithelial surfaces, determining the dispersion in the vaginal cavity therefore and local time residence in the genitourinary tract providing efficacy.[15]

The viscosity of formulations was determined in mPa.s after dilution in mVFS at 37 °C and results are presented in Fig. 2.

The viscosity of all formulations through the experimental conditions revealed non-Newtonian and shear thinning behaviour properties.

The viscosity of all formulations was slightly lower after physiological dilution in VFS at room temperature. The same was observed by Machado et al., when evaluating the viscosimetric properties of

Table 2
Studies of pH and acid-base titration, carried out in dispersion with normal saline solution (NaCl 0.9%) and in the vaginal fluid simulant (VFS). Means and standard deviations are represented, n = 2.

Formulations	pH	Diluted pH in NaCl 0.9% ± SD	Volume of HCl pH < 3 (μL)	Diluted pH in VFS ± SD	Volume of NaOH pH > 5 (μL)
BF	5.5	7.54 ± 0.03	40	4.52 ± 0.01	180
TEO	5.46	6.89 ± 0.01	60	4.49 ± 0.02	180
BF + C_8	5.5	7.62 ± 0.01	40	4.51 ± 0.01	180
BF + MTX	5.56	7.48 ± 0.02	40	4.52 ± 0.01	180
TEO + C_8	5.49	6.31 ± 0.02	60	4.41 ± 0.01	160
TEO + MTX	5.4	6.64 ± 0.01	60	4.48 ± 0.00	160
Tween 80	5.5	7.03 ± 0.02	40	4.52 ± 0.01	180

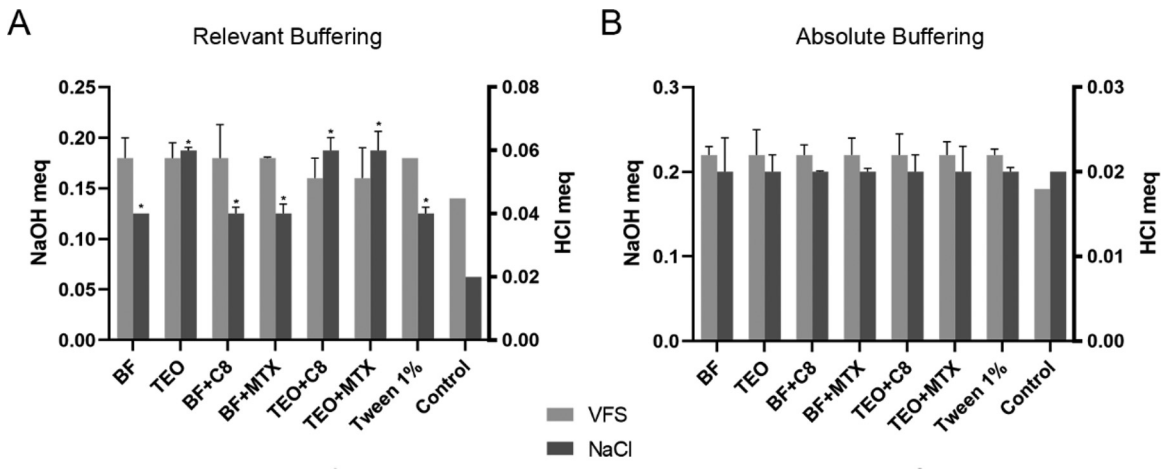

Figure 1. (A) Relevant and (B) Absolute pH-buffering capacity of formulations with dispersion in 0.9% NaCl (NaCl columns) and in vaginal fluid simulant (VFS columns). In the dispersion experiments performed in NaCl, titration was done using HCl (1M) since all formulations had pH > 5 and the experiments performed in VFS, NaOH (1M) was used since the pH changed to less than 5, until a pH >9. Thus, VFS columns are related with the left axe (NaOH meq used in the experiment) and NaCl columns with the right axe (HCl meq used in the experiment). Results are represented as mean ± standard deviation (n = 3). * Represents statistically different from the NaCl control (one way-ANOVA, $p < 0.05$).

commercial vaginal formulations, such as Gino-Canesten® and Sertopic®. According to this study, after diluted in the VFS, viscosities were noticeably lower when compared to simple formulations, not being proportional or similar to the variation between formulations, as each one presented a unique behavior due to its composition.[16]

TEO-based formulations were more viscous, and consequently lower shear stresses were needed to obtain an acceptable torque. These properties possibly circumvent the problems reported by women regarding the use of vaginal products, increasing retention and consequently decreasing the oozing and discomfort caused. More viscous formulations are less likely to leak through the vaginal canal, presenting longer retention time in the vagina; however they can promote greater resistance to dilutions, limiting the intravaginal application and dissemination of the product.[12] The TEO formulation, although showing higher viscosity, after dilution in VFS, the viscosity decrease, which could increase the probability of discomfort given the low viscosity. Although vaginal formulations with low viscosity flow better through the applicator, spread more easily and better coat the epithelium, the inability of the formulation to resist dilution in VFS would result in a short vaginal retention period, increasing runoff and consequent decrease in lining integrity.[12,15,17] Therefore, an optimal clinical performance may only be achieved when the elastic-viscous balance is carefully controlled.

Concluding, the most promising formulation given to the viscosity is the one containing TEO + C_8 since it was able to resist dilution in VFS, only slightly decreasing its viscosity, what would be favourable for the dispersion and coating of the formulation in the vaginal epithelium, and reduced runoff. The TEO + MTX formulation also showed identical viscosity, but lower than the one with TEO + C_8.

Osmolality

Products showing osmolality consistent with the normal osmolality of female secretions (260 to 290 mOsm/kg) and semen (250 to 380 mOsm/Kg) are considered iso-osmotic for the vaginal environment. However, wider intervals of osmolality rates for vaginal administration are well tolerated, reaching values of up to 1200 mOsm/Kg, the upper limit recommended, for vaginals lubricants, by the World Health Organization (WHO).[12,13]

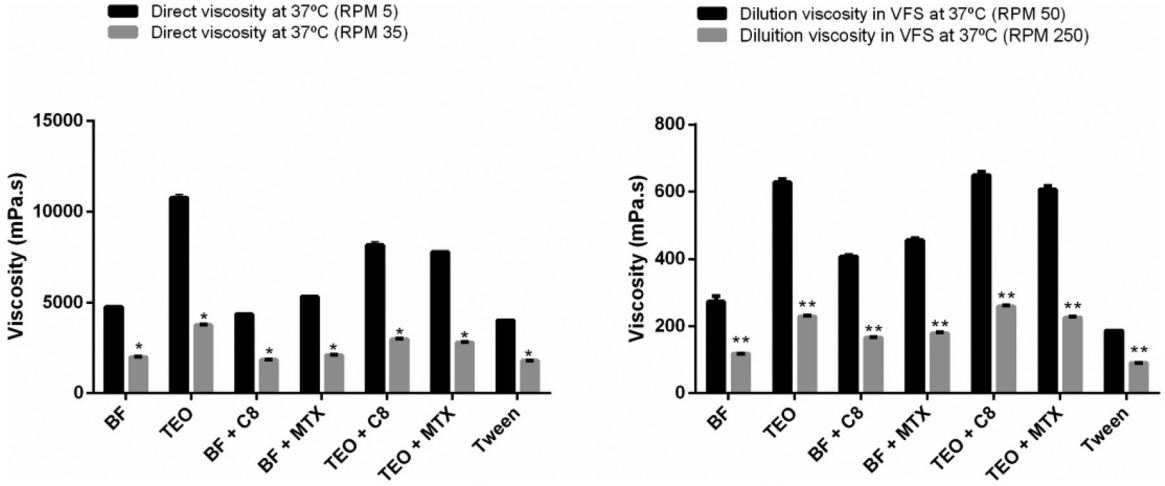

Figure 2. Viscosity comparisons for direct and diluted measurements at 37 °C. The dilutions were done with VFS. The viscosity results are expressed as mPa.s, corresponding to the mean and standard deviation of three determinations (n = 3). * Represents statistically different from direct viscosity at 35 rpm, and ** represent statistically different from dilution viscosity at 250 rpm (two-way ANOVA, $p < 0.0001$, Sidak's multiple comparison test). After dilution and at 50 and 250 rpm, the viscosity is lower compared to direct formulations at different speeds.

Table 3
Osmolality results of direct formulations and after dilution in VFS. At physiological dilution, all formulations were affected, with statistical differences (two-way ANOVA, $p < 0.001$), $n = 3$. * Represents statistically different from direct osmolality.

Formulations	Osmolality ± SD (mOsmol / kg)	
	Direct osmolality	Physiological dilution
BF	1562 ± 47.7	838 ± 10.1 *
T1%	1515 ± 19.0	876 ± 6.5 *
BF + C_8	1638 ± 7.1	894 ± 9.6 *
BF + MTX	1636 ± 12.1	856 ± 11.0 *
T1% + C_8	1500 ± 21.4	847 ± 13.5 *
T1% + MTX	1545 ± 7.6	877 ± 22.5 *

BF – Base Formulation; TEO – Base Formulation with essential oil of *Thymus vulgaris* (1%, w/v); BF + C_8 – Base Formulation with C_8; BF + MTX – Base Formulation with mitoxantrone; TEO + C_8 - Formulation with essential oil of *Thymus vulgaris* and C_8; TEO + MTX - Formulation with essential oil of *Thymus vulgaris* and MTX; Tween – Formulation with polysorbate 80.

According to the results obtained (Table 3), the 7 formulations were slightly above the upper limit recommended by WHO for lubrificants. Indeed, since high osmolality may be due to high concentrations of humectants, the WHO recommends that glycerine and propylene glycol concentrations should not exceed 9.9% (w/w) and 8.3% (w/w), respectively.[14] Higher levels of glycerine and propylene glycol were correlated with the irritation potential of vaginal products.[14] In this work the content of propylene glycol was set to 8% (w/w). Other ingredients of the formulations may also contribute for osmolality.

Cunha et al., carried out a study in which they evaluated 12 commercial vaginal gels, and of these, 7 had osmolalities up to 3 times higher than the recommended maximum limit.[13]

Adiraens et Remon[18] determined whether musosal irritation potency of personal lubricants is related to varying product osmolalities. Formulations with osmolalities, markedly lower than the normal physiologic range for fluids from the female reproductive tract (280 −290 mOsm/kg), resulted in negative mucus production (and a drier membrane surface). Whereas the hyperosmotic lubricants in study all contained glycerin (i.e., glycerol) and all induced some mucosal irritation in the slug mucosal irritation assay.[18]

After dilution in VFS, all formulations were affected, considerably decreasing their osmolality and with statistical differences (two-way ANOVA, $p < 0.001$) between the two measurements, with osmolality values in accordance with the maximum limit recommended by WHO. This attenuation in osmolality was already expected, since VFS is a hypotonic solution (185 mOsm/Kg) and the formulations have higher osmolalities, causing a progressive decrease in osmolalities compared to measurement without adding VFS.

Bioadhesion

The analysis of the bioadhesion of vaginal formulations allows to know the capacity of a formulation to adhere to a biological surface. Bioadhesion is characterized by the interaction between a pharmaceutical dosage form and the mucous membrane or mucus secreted present in the vagina. One of the main components of vaginal fluid mucus is composed by soluble mucins, highly glycosylated glycoproteins.

The bioadhesive properties allow the contact of the formulation with the vaginal surface, and consequently longer retention times in the genital tract. As shown in Table 4 the base formulation with C_8 (BF + C_8) showed greater bioadhesion to the vaginal tissue, followed by the formulation of TEO + MTX and the BF. The 1% Tween formulation showed less adhesion work, and consequently low detachment force. Given that the base formulation containing C_8 (BF + C_8) showed a greater bioadhesion to the vaginal tissue, presenting a higher adhesion work. The same did not occur for TEO + C_8 formulation since the adhesion work was lower for this formulation.

Bioadhesive dosage forms can contribute to prolonged *in situ* residence, resulting in advantageous characteristics, such as decreasing vaginal discharge, fewer daily applications and increase contact of the active compounds with the epithelial tissue.[19] However, the adhesion work (here described bioadhesion) and maximum detachment strength must be carefully analyzed due to the high standard deviation presented. Indeed, study design considers variability between vaginas and different portions of the same vagina, that also occur *in vivo*, but inevitable this impacts the standard deviation value.

The bioadhesive potential of pharmaceutical forms and polymers also depends on the specificities of the vaginal epithelium environment and its evaluation should take this into account.[15]

The self-cleaning mechanisms of the vagina, mechanical stress (for example, during penetration of the penis) and the slight inclination of the vaginal canal contribute to the expulsion of products for vaginal application. The variability of vaginal fluid (menstrual cycle, hygiene practices, etc.) can affect the phenomenon of bioadhesion of formulations to vaginal tissue, which is important to develop vaginal formulations with promising bioadhesive properties.[12,15]

Evaluation of Formulations in the Culture Medium

All formulations (BF, TEO, BF + MTX, BF + C_8, TEO + MTX, TEO + C_8 and BF + Tween) were dissolved in the medium, at concentrations of 10, 5, 1, 0.4 and 0.1%; however, for concentrations of 20 and 30%, the same result was not observed. BF, BF + C_8, BF + MTX and BF + Tween were quite viscous and the formulations containing TEO (TEO, TEO + C_8 and TEO + MTX) were not completely dissolved in the culture medium. As described in the literature, the use of TEO in formulations is limited, due to its low solubility.[4] This solubility issue was minimized by adding Tween 80 to 1% (w/v), a surfactant and emulsifier often used in formulations for therapeutic purposes, in order to increase its solubility, and avoid TEO precipitation when mixed with the other excipients of the formulations.[5]

However, according to the results obtained, at concentrations of 20 and 30% even with the incorporation of Tween 80, TEO in

Table 4
Bioadhesive parameters of formulations. Maximum detachment force (N), detachment distance (mm) and bioadhesion work (N.mm), represented by mean, $n = 5$. * Represent statistically different from the control (Two-way ANOVA, $p < 0.02$ Dunnett's multiple comparisons test).

Formulations	Peak-force Adhesiveness (N) ± SD	Debounding distance (mm) ± SD	Debounding time (sec) ± SD	Work of adhesion (N.mm) ± SD
Control	0.03 ± 0.02	1.89 ± 0.18	211.0567 ± 7.47	0.01 ± 0.01
BF	0.06 ± 0.02	2.33 ± 0.90	226.892 ± 3.46	0.07 ± 0.05
TEO	0.04 ± 0.02	2.20 ± 0.64	233.351 ± 7.13	0.05 ± 0.03
BF+ C_8	0.07 ± 0.03	3.54 ± 1.51	262.648 ± 8.68	0.11 ± 0.05 *
BF + MTX	0.07 ± 0.03	2.25 ± 0.39	230.566 ± 2.36	0.05 ± 0.02
TEO + C_8	0.05 ±0.03	2.74 ± 0.75	244.285 ± 12.31	0.06 ± 0.02
TEO + MTX	0.06 ± 0.05	2.67 ± 1.09	233.974 ± 16.49	0.07 ± 0.01 *
BF + Tween 1%	0.02 ± 0.01	1.92 ± 0.49	221.548 ± 14.01	0.03 ± 0.01

formulations was not homogeneous, observing precipitation of the formulations in the culture medium. Therefore, the highest concentrations (20 and 30%) were not considered for *in vitro* tests since they could hinder the exchange of nutrients between cells and present themselves as cytotoxic, thus interfering in the subsequent results.

Cell Viability Assay

The results of the MTT assay of the formulations are presented in Fig. 3.

All formulations (TEO, BF + C_8, BF + MTX, TEO + C_8 and TEO + MTX) inhibit the growth of both cell lines at concentrations of 5% and 10%, except the base formulations and Tween 1% (controls). The IC_{50} values of C_8 (alone) were already determined for HeLa and NHDF cells and was 0.15 and 0.48 μM, respectively.[7] For MTX, the IC50 for HeLa cells was 4.4 μM[20] and for TEO was reported to be > 200 μg/mL, having a lowest toxicity towards the HeLa cell line.[21] The formulation TEO + C_8 have a significantly higher effect on the viability of HeLa cancer cells in almost concentrations, except for 10% and 5%, where viability was practically 0% in both cell lines. The effect on cell viability in the presence of C_8 was greater in HeLa at concentrations of 5% and 10%, and moderate at concentrations of 0.2% and 0.4%; however, at 0.1% a slight decrease was observed on cell viability in NHDF.

Carvalho et al. studied the effect of the C_8 ligand conjugated with delivery systems (namely DNA aptamers) and in its free form in HeLa cells and in NHDF. In this study, they observed that free C_8 had the same effect in both lines, but nevertheless its cytotoxic effect was reduced in NHDF when conjugated to the carriers, demonstrating that these could increase the selectivity of C_8 for cancer cells.[7]

TEO, as already described in the literature, has a strong effect on cancer cell viability, namely in HeLa.[8] According to the results, there was a reduction in cell viability dependent on the concentration in HeLa cells, possibly induced by thymol, the main constituent of the TEO, as described by De La Chapa *et. al.*[22] Also according to the study by De La Chapa et al., thymol significantly reduced the growth of tumors *in vivo*, showing no adverse effect throughout the studies.[22]

The cytotoxic effect of thymol may have occurred through the dysfunction that it causes in the mitochondria and thereby inducing apoptosis.[10]

The synergistic action of combining TEO with potential drugs (C_8 and MTX) resulted in decrease of the growth of cancer cells, since both C_8 and MTX are described in the literature as inhibitors of HeLa cells.[6,7]

The high levels of pro-inflammatory cytokine IL-6 are correlated with inflammation severity, and they are widely used as markers of inflammation.[23] Thus, to study if the proposed formulations can cause an inflammatory response, the IL-6 levels were measured in the supernatants after exposing the NHDF cells to the concentrations 0.2% and 1% of the formulations. As showed in Figure S3, the formulations do not upregulate the IL-6 secretion to the cell medium, when

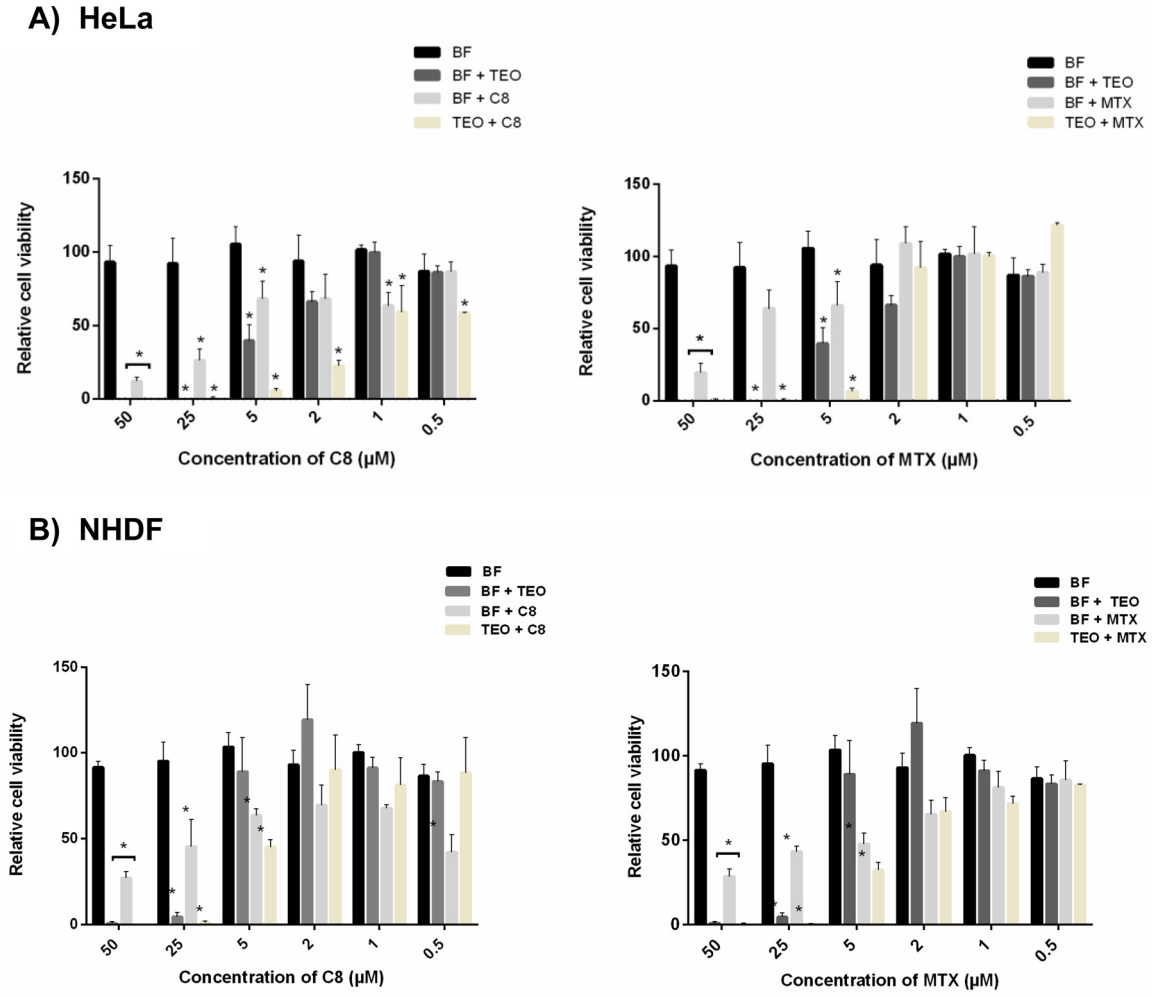

Figure 3. Relative cell viability of formulations with C_8, on the left, and MTX, on the right, tested, in variable dilutions of 0.1% to 10% (w/v) in HeLa (A) and NHDF (B). The results correspond to the mean and standard error of three determinations. * Represents statistically different from BF (control) (two-way ANOVA, $p < 0.0001$, Dunnett's multiple comparisons test).

Table 5
Quantification of C_8 and MTX through high-performance liquid chromatography, over 56 h. The permeation was carried out in Ussing chambers. To quantify the compounds C_8 and MTX, the formulations TEO + C_8 and TEO + MTX were tested in the porcine's epithelial tissue. Samples (100 μL) from the receiving chamber of each formulation were collected at the following times: 0, 5, 20, 30, 25, 30 and 56 h.

	Sample	Concentration (μg/mL)	Sampling period
	1	0	0 h
	2	n.d.	5 h
T1% + C_8	3	n.d	20 h 30
	4	0.02	25 h 30
	5	0.03	56 h
	DC C_8	1.81	56 h
	Tissue C_8	0.12	56 h
	RC C_8	0.02	56 h
T1% + MTX	1	n.d.	0 h
	2	0.56	5 h
	3	0.51	20 h 30
	4	0.47	25 h 30
	5	0.49	56 h
	DC MTX	0.50	56 h
	Tissue MTX	n.d.	56 h
	RC MTX	0.46	56 h

n.d. not detected

compared with the control condition (only medium), suggesting that the formulations do not cause an inflammatory response.

Permeation assay of the compounds in the porcine vaginal tissue (ex vivo)

The evaluation of the release and permeation of drugs is an important and mandatory factor in the development and quality control in pharmaceutical industry. This is particularly important when the objective is to limit the activity to the surface of the vaginal wall. To assess this possibility, it is crucial to select a correct method that mimics the environment *in vivo*, in order to test properly the performance of a formulation in promoting or avoiding permeation through vaginal tissue.

According to the results (Table 5), the active compound C_8 on the formulation TEO + C_8 only started to permeate the tissue after 24 h (25 h 30 min), and at very low concentrations. At the end of the test, the C_8 concentration quantified in the recipient chamber was considerably lower than that quantified in the donor chamber, revealing that very low amounts of C_8 permeated the tissue throughout the test. C_8 showed to have a low capacity to cross the vaginal epithelial tissue and reach blood circulation, thus reducing the possibility of causing systemic side effects.

In fact, the quantification of C_8 in the tissue demonstrates that it has been retained, which can be used as topical application in order to increase the bioadhesion to the vaginal epithelium. The balance between the amount of active ingredients that permeate the vaginal tissue and the amount that is retained in the tissue is important for safety and efficacy *in vivo*.

Interestingly, both propylene glycol and essential oil components (particularly limonene, terpinene, cineole and p-cymene, which are constituents of *Thymus vulgaris* essential oil) are well known for their permeation enhancing properties. Previous works have shown that combinations of these terpenes with propylene glycol significantly increased the skin penetration of drugs such as caffeine and hydrocortisone (for mice skin) while for other drugs such as triamcinolone no significant changes were observed.[24]

While using porcine epidermis S. Gao and J. Singh, observed that the terpenes thymol, carvone and 1.8 cineol, enhanced the absorption of the drug 5-fluorouracil.[25]

While referring to a different tissue, these studies highlight the importance of considering the interactions between excipients and the targeted site of action during product development. In the present study, C_8 extensive permeation through the vaginal tissue was not observed throughout 56 h despite the presence of both propylene glycol and terpenes.

In the case of the TEO + MTX formulation, an opposite effect was observed when compared to the formulation containing C_8. In the former formulation, the MTX was not detected in the first sampling, and in the second (5 h) it was possible to detect the compound in the receptor chamber. The concentration quantified in the 5 h period was practically the same as that quantified in the donor chamber, maintaining approximately the same concentration throughout the sampling points. This suggests that MTX started to permeate the issue minutes after the formulation was added, having reached an equilibrium between donor and receptor chambers, demonstrating that the drug extensively permeated the tissue and that experimental conditions are not ideal for carrying out the permeation test, since the sink conditions were not maintained. Sink conditions are one of the important principles to be maintained in a permeation study, where the solubility of the assets in the receiving solution must be known and if in fact they can dissolve or ensure that the volume is sufficient so that it can flow freely to the receiving chamber.[26]

Conclusion

In this work, we have evaluated the potential of drug-loaded formulations for the delivery of two antineoplastic agents to the female genital tract.

According to the permeation test of the compounds in the porcine vaginal tissue and the characterization of the compounds, the formulation of TEO + C_8 proved to be quite promising. Compound C_8 showed a lower capacity to permeate the porcine vaginal tissue, thus increasing its retention in the tissue, revealing itself promising since the objective of the study is to develop a formulation for topical application aiming at increasing the retention of the compounds to the target tissue. All the formulations had an initial pH > 5, but when diluted with the VFS, the pH decreased to approximately 4.5, revealing that they can easily reach the normal range of vaginal physiological pH (3.5 − 4.5). TEO + C_8 formulation showed promising viscosity and osmolality properties in VFS dilutions. Indeed, the viscosity results suggested that this formulation could be efficient at avoiding leakage and could facilitate its dispersion reaching the vaginal epithelium.

Relatively to bioadhesion experiments, the formulation BF + C_8 showed to be the most promising in vaginal tissues. The higher bioadhesive properties contribute to prolonged *in situ* residence, resulting in advantageous characteristics, such as decreasing vaginal discharge, fewer daily applications and increasing the contact of the active compounds with the epithelial tissue. The MTT experiments with the formulations showed decrease of cell viability in both cell lines at concentrations of 5% and 10%, except for the base formulation and Tween 1%.

According to the permeation assay, the formulation of TEO + C_8 showed that C_8 has a low capacity to cross the vaginal epithelial tissue, reducing the possibility of causing systemic side effects. On the other hand, MTX formulation permeated easier the epithelial tissue.

Overall, although further studies like pharmacokinetics and efficacy are clearly needed; however, the formulations developed provided some evidence for the use of TEO and C_8 for topical vaginal application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.02.004.

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