Decreased inflammatory response in rat bladder after intravesical administration of capsaicin-loaded liposomes

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ABSTRACT

The objective of this work was to study the reduction in the capsaicin toxicity by encapsulation in liposomes. Capsaicin was extracted from peppers and characterized with high performance liquid chromatography (HPLC). We determined the zeta potential, polydispersivity index (Pdi) and vesicle size of liposomes. Wistar rats were submitted to intravesical instillation of liposomes (LIP), capsaicin (CAP) or liposomes with capsaicin (CAPLIP). After 24 hours, bladders were removed for histological analysis. Vesicle size ranged from 68 to 105 nm with Pdi smaller than 0.2 and zeta potential around -30 mV. The vesicles maintained stability over the 14-day study. The histological analysis of the CAP group showed intense inflammation in almost all bladder layers, as well as ulcer formation. Conversely, the CAPLIP group showed a smooth inflammatory reaction and hyperemia. In conclusion, the liposomes effectively protected the bladder against the irritative action of capsaicin.

Key words: capsaicin, liposomes, urinary incontinence, intravesical administration.

INTRODUCTION

Capsaicin is the chemical component responsible for the spiciness of peppers belonging to genus Capsicum. This substance is primarily found in the seeds of these fruits, and in mammals it leads to irritative symptoms caused by vanilloid receptor activation, subsequent activation of peripheral terminations and the liberation of substance P and other cytokines (Nirmal et al. 2012, Barbero et al. 2008, Petrovszki et al. 2014, Sharma et al. 2013).

This compound has been widely employed to treat urinary incontinence, a pathological condition characterized by the involuntary loss of urine that usually affects postmenopausal women (Tu et al. 2011, Thüroff et al. 2011). Capsaicin is neurotoxic and disrupts conduction in type C unmyelinated sensory afferent fibers that coordinate urine excretion control. Although this sensory neuron blockage is long lasting, it is also reversible. The length and onset of action depend on the dosage and the length of exposure (Kaufman 2011, Meel and Wyndaele 2012, Sharma et al. 2013).
Intravesical capsaicin administration is an effective treatment for urinary incontinence; it minimizes systemic side effects and is not subject to first-pass metabolism. Bladder anatomy allows the drug solution to be directly instilled via a catheter (Barthelmes et al. 2011, Lee et al. 2011). However, this treatment often causes local irritation and edema due to its effects on unmyelinated sensory afferent fibers, which are believed to signal pain and initiate inflammatory responses (Meel and Wyndaele 2012, Wyndaele et al. 2010, Cipullo et al. 2014). Alternative formulations have been proposed in order to minimize these side effects.

Capsaicin has nonpolar features; it has low solubility in biologically compatible solvents, such as buffers and saline solution. Capsaicin is usually dissolved in ethanol, vehicle that can also be irritating. Therefore, it is necessary to develop formulations that reduce adverse effects (Tyagi et al. 2004).

The development of alternative capsaicin delivery systems has received interest in recent years. One such option is the use of liposomes, which are spherical vesicles with a double lipid layer that are a popular vehicle because of their ability to encapsulate substances with varying polarity, biodegradability and biocompatibility (Peters et al. 2012). Several studies have demonstrated the successful use of liposomes to deliver substances, including capsaicin (Rollyson et al. 2014, GuhaSarkar and Banerjee 2010). Therefore, the aim of this work was to assess if delivering capsaicin via liposomes protected rodent bladder tissue against capsaicin-induced inflammation.

**MATERIALS AND METHODS**

**Preparation of Capsicum frutescens Extract**

Fresh samples from *Capsicum frutescens* pepper were sectioned to collect seeds and fruit skin. These products were dried at 55 °C for 24-30 h in a stove, ground and passed through a 60-mesh sieve. The obtained pepper powder was weighed and placed in separate tubes, each containing 3 g pepper powder and 10 mL acetonitrile, which were sonicated for 1 h. Finally, the material was filtered, and the solvent evaporated.

Capsaicinoids were quantified according to the method published by Karnka et al. (2002). A C-8 precolumn was conditioned with 0.5 mL MilliQ water, then with 0.5 mL methanol followed by 0.5 mL acetonitrile. Next, 0.5 mL aliquots were diluted in the conditioned precolumn and washed three consecutive times with 0.5 mL acetonitrile. The material obtained in this process was filtered through a membrane with 0.45 µm porosity (Millipore®) and placed in vials. The samples were then analyzed by HPLC using a C-8 column and 20 mL samples, which were eluted with acetonitrile as the mobile phase.

**Liposome Preparation**

Liposomes were generated using phosphatidylcholine 75 % (Nanosolv/Lipoid GMBH/part 776095-1/Gerbras) as the phospholipid (PL). PL and capsaicin solution (4.6 mg.mL⁻¹) were homogenized in a 1:10 molar ratio in ethanol. The final concentration of capsaicin was 3 mM. The ethanol was removed from the mixture by rotary evaporation to form thin lipid films on the flask wall. The dried lipid film was hydrated in 50 mL phosphate buffer (Avelino and Cruz 2000, Frézard and Schettini 2005). The final dispersion (CAPLIP) was filtered through a nylon membrane with 0.22-µm pores (Millipore®). The same procedure was performed without capsaicin to prepare the control formulation (LIP).

**Liposome Characterization**

The zeta potentials (Zetasizer Nanoseries, Malvern Instruments, UK) of LIP and CAPLIP were determined by measuring the electrophoretic mobility of the charged particles at 25 °C. The samples were diluted in distilled water at a ratio of
1:10, and the procedure was performed 24 h, 7 and 14 days after liposome preparation.

The size and polydispersity index of LIP and CAPLIP were assessed by dynamic light scattering (DLS) using a computerized system (Malvern Zetasizer Nanoseries (Nano-ZS - Malvern, UK). The samples were diluted at a ratio of 1:10 with distilled water and analyzed at 633 nm at room temperature (25 °C).

The morphological characterization of LIP and CAPLIP was performed by transmission electron microscopy. Liposome suspensions were imaged, using a transmission electron microscope (TEM) JEOL (JEM-2010) microscope at 200 kV. A few droplets of an ultrasonically dispersed suspension of liposomes in water were deposited on a copper grid with lacey ultrathin carbon film (400 mesh) and then dried at ambient conditions for TEM characterization.

An in vitro drug delivery study was performed at 37 °C using dynamic dialysis (Zhu et al. 2014). Briefly, the CAPLIP dispersion (2 mL) was placed in a dialysis tubing cellulose membrane (33 mm; Sigma-Aldrich) immersed in 40 mL of pH 7.2 buffer solution. At a regular time intervals, 3.5 mL of receiver solution was withdrawn. The capsaicin released from the liposome was measured at each sampling time by UV spectrophotometry, using calibration curve of standard capsaicin (λ= 270 nm; linearity 20 to 100 µg/mL; y=0.0082x-0.0148; r=0.991).

The in vitro release study was carried out at 37 °C using dynamic dialysis technique ref. Briefly, the CAPLIP dispersion (2 mL) was kept in a dialysis membrane (MD34, 8000–14,000, Sigma-Aldrich) and this system was immersed in 40 mL of pH 7.2 buffer solution. At a regular time intervals, 3.5 mL of receiver solution was withdrawn. In order to calculate of capsaicin released from liposome, was measured at sampling time by spectrophotometry UV, using the same calibration curve.

**Biological Assay**

This study was approved by our Animal Care and Use Committee (register # 060208) the National Research Council prior to beginning the experiments. In accordance with the institution’s guidelines outlined in «Guide for the Care and Use of Laboratory Animals», all animals received humane care throughout the study.

The experiment utilized 24 female rats (*Rattus norvegicus albinus*, Wistar strain, weighing 190 ± 30 g, approximately 8 weeks of age) that were housed in clear plastic cages with loose hardwood chip bedding in a temperature and humidity-controlled environment and supplied with food and water *ad libitum*. The animals were divided into four groups (n=6): CTR (treated with saline solution), LIP (treated with liposomes only), CAP (treated with capsaicin only) and CAPLIP (treated with capsaicin-loaded liposomes).

The animals were subcutaneously anesthetized with ketamine (70 mg/kg) and midazolam (4 mg/kg). Intramuscular administration of ceftriaxone (57 mg/kg) was performed to prevent further cystitis due to manipulation of the urinary tract. After 30 min, the urethral meatus was disinfected with 5% PVPI solution, and vesical catheterization was carried out using an intravenous catheter (22, Jelco), which was fully inserted in order to reach the bladder. Next, the formulations were instilled into the bladder according to experimental group: saline solution (CTR), 3 mM capsaicin solution (CAP), 30 mM liposomes only (LIP) and 30 mM capsaicin-loaded liposome solution (CAPLIP).

The animals were euthanized in a CO₂ chamber 24 h later, and the bladder of each animal was removed and fixed in formalin for 24 h. The specimens were cleared with xylene, embedded in paraffin and cut in a longitudinal plane. Then, six semi-serial histological sections (3-µm thick) were prepared and stained in hematoxylin/eosin (HE) and Sirius Red (SR).
HE-stained histological sections were scored for inflammatory response as follows: 0 (absence of inflammatory response), 1 (inflammatory cells representing less than 10 % of the cell population observed within the wound area), 2 (inflammatory cells representing between 10 % and 50 % of the cell population observed within the wound area), and 3 (inflammatory cells representing more than 50 % of the cell population observed within the wound area). We also classified the inflammatory profile (IP) as acute (predominance of polymorphonuclear cells) or chronic (predominance of mononuclear cells).

SR-stained histological sections were viewed under polarized light to assess collagen fibers in the bladder wall connective tissue. Collagen fibers were evaluated according to their morphological appearance (wavy or stretched, thin or thick, short or long), spatial arrangement (reticular, parallel or interlaced) and birefringence pattern (type III, green/yellow-green fibers; or type I, orange/orange-red fibers).

Statistics

Statistical analysis was performed using one-way analysis of variance (ANOVA) and post-

Results and Discussion

The capsaicinoids, such as capsaicin and dihydrocapsaicin, possess analgesic, anti-inflammatory, anti-tumor and antioxidant potential. Structural characteristics of capsaicinoids, termed also as vanilloids, are responsible for biological activity. The main active group is the vanillyl (4-hydroxy-3-methoxybenzyl) (Kuzma et al. 2015). Capsaicin and dihydrocapsaicin, like other vanilloids, has a benzene ring and long hydrophobic carbon tail with a polar amide group.

These compounds have comparable pharmacodynamic and pharmacokinetic properties. Intravesicular application of capsaicin and analogs, like dihydrocapsaicin, increases bladder capacity and reduces urge incontinence in humans with detrusor hyper-reactivity (Cruz 2004).

Liposome efficiency depends on physicochemical parameters, such as composition, size, polydispersity index and zeta potential (Zhu et al. 2014, Zhao et al. 2011). Figure 1 shows the zeta potentials of LIP and CAPLIP. The LIP zeta potential was decreased after 7 days (-40.9 mV), which is suggestive of system stabilization. After 14 days, the zeta potential showed a slight increase (-36 mV). CAPLIP zeta potential values were constant (around -32 mV) throughout the 14-day experiment, which indicates s colloidal dispersion. According to Pham et al. (2012), negative zeta potential values are usually recognized to indicate good suspension stability.

Vesicle size is another important parameter for colloidal system stability. Except for the LIP formulation, which showed a slight increase at 7 days, vesicle size was unchanged after 14 days. The CAPLIP formulation contained smaller vesicles (68-78 nm) than LIP (78-106 nm), but the average size for both was less than 100 nm (Figure 2), which...
The polydispersity index (PdI) determines the homogeneity profile of vesicles with regard to size. Values lower than 0.2 indicate homogeneously sized vesicles, and those higher than 0.3 suggest size heterogeneity (Zhu et al. 2014). The PdI values of LIP and CAPLIP remained constant (minimal variation from 0.257 to 0.279), indicating stable homogeneity of vesicle size over 14 days.

The TEM images of LIP and CAPLIP showed the formation of nanometer-sized, oligo and multi-lamellar vesicles of spherical shape (Figure 3).

In our experiments, we used a high concentration of capsaicin (10^{-3} M), considering that the drug delivery would be retarded. We can observe in in drug delivery study that this slow release occurred, delivering 26.6 % of capsaicin after 24 hours and 33.8 % after one week. The slow delivery guaranteed the effective doses to biological action, spreading the drug among the tissue, avoiding the adverse effect for drug accumulation in specific local.

In regard to the biological assay, we did not observe any changes in the histological architecture or cytological features of the transitional epithelium and connective tissue in CTR- or LIP-treated bladders, except for very mild lymphocyte infiltration observed in one specimen, which was likely due to mechanical irritation caused by catheter insertion. These findings suggest that the phospholipids do not trigger inflammatory or degenerative processes in bladder tissues. Conversely, we found intense infiltrate composed of polymorphonuclear neutrophils and lymphocytes in the CAP group, especially near the epithelial lining, as well as intense exudative changes, hyperemia and interstitial edema. Extensive areas of ulceration of the transitional epithelium were also noted (Figure 4). The intensity of the inflammatory response was significantly increased in CAP compared to the other groups (p<0.05). Intense bladder tissue aggravation following intravesical capsaicin administration was previously reported by Hsu et al. (2013), suggesting that capsaicin has remarkable irritative properties. However, only minor vascular changes

Figure 2 - Vesicles size of liposomes LIP (a) and liposomes containing capsaicin CAPLIP (b).
Figure 3 - Transmission electron microscopy image of LIP (a) and LIPCAP (b).

Figure 4 - Histological sections of the bladders 24 h after the instillation of the different formulations. CTR and LIP show no change in the usual features of the transitional epithelium (TE) and connective tissue. Intense hyperemia (arrows) is showed in CAPLIP, but the normal architecture of the tissue is sustained. In CAP, replacement of the transitional epithelium by extensive ulceration (U), recovered by polymorphonuclear neutrophils, as well as marked exsudative changes (EC) of the connective tissue are observed (HE, 200x).
were observed in tissues treated with liposome-loaded capsaicin (CALIP). Likewise, reduced toxic effects of capsaicin released by liposomal systems within bladder tissue were described by Tyagi et al. (2004), and such protective properties were related to the slow incorporation of liposomes into the cell phospholipid bilayer, resulting in gradual release of the active chemical compound. Moreover, liposomes seem to protect the vesical epithelium; they enhance the phospholipid urothelial barrier, improving the resistance to aggravating substances (Chuang et al. 2009).

Collagen molecules play a fundamental role in maintaining the architecture, resistance, and cell-cell/cell-extracellular matrix interactions of different organs and tissues (Yuan et al. 2014). Suitable post-injury collagenization is considered one of the most relevant biological factors for successful tissue recovery (Franz et al. 2011). As seen in Figure 5, CTR, LIP and CAPLIP induced similar patterns of collagenization, comprised mostly of type-I collagen fibers, which were shortened and interlaced in sub-epithelial areas but long and outstretched in the bladder periphery. CAP, on the other hand, induced mostly short and irregularly arranged type-III collagen fibers that were associated with larger interfibrillar spaces throughout the specimens.

Wound healing studies carried out in experimental rodent models have demonstrated that type-III collagen fibers are initially deposited and are supposed to orientate proliferating and migrating endothelial cells to form granulation tissue as the first step of connective tissue repair (Albuquerque-Júnior et al. 2009, Ribeiro et al. 2009). These fibers are gradually replaced by type-I

Figure 5 - Histological sections of the bladders 24 h after the instillation of the different formulations. CTR, LIP and CAPLIP show predominance of type I collagen fibers, whereas CAP presents predominance of type III collagen (SR, 200x).
collagen, which gives the newly repaired tissue mechanical resistance and tensile strength (Nunes et al. 2011, Dantas et al. 2011). Therefore, the high level of sparsely arranged type-III collagen fibers observed in CAP, as opposed to the bimodal pattern of dense type-I collagen deposition observed in CTR, suggests that free capsaicin disrupted normal collagen disposition and that granulation tissue formation had yet to occur at the time the bladders were harvested. Nevertheless, the histological similarity observed in the collagen fiber disposition seen in CTR and CAP supports the hypothesis that the irritative properties of capsaicin were attenuated by loading the substance into liposomes.

In conclusion, we demonstrated that the liposomal formulation remained stable for 14 days after sample preparation and contained vesicles smaller than 100 nm. We also provided histological evidence that encapsulating capsaicin into liposome vesicles prevents bladder irritation 24 h after catheter administration.

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