

Activity of different phospholipids in attenuating hyperactivity in bladder irritation

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OBJECTIVE

To evaluate the effect of liposomes prepared from various natural and synthetic lipids in a rat bladder injury model in the absence or the presence of cholesterol and to elucidate the key structural elements necessary for the efficacy of liposomes required for alleviating bladder hyperactivity.

MATERIALS AND METHODS

The intravesical pressure was recorded using a transurethral catheter in adult female Sprague-Dawley rats anaesthetized with urethane (1.0 g/kg subcutaneously).

Continuous cystometrograms (CMGs) were obtained by slowly filling the bladder with solutions of varying compositions after obtaining a baseline CMG with saline. Rat urothelium was injured with protamine sulphate (PS) and irritated by subsequent infusion of KCl (500 mM) for 1 h. Thereafter, liposomes prepared in KCl using several natural and synthetic phospholipids were infused for 2 h. The percentage reduction in bladder contraction frequency (BCF) was used as a comparative variable for judging the activity of different phospholipids.

RESULTS

Exposure of rat bladder to sequential infusion of PS and KCl increased its BCF and empty liposomes of uncharged zwitterionic phospholipids markedly attenuated the

PS-induced irritation and decreased the raised BCF. But empty liposomes prepared with either cationic or anionic charged lipids were not able to achieve the same effect. Addition of cholesterol did not significantly increase their efficacy. Optimal efficacy of liposomes was achieved with phosphatidylcholines with longer acyl chains and saturation in only one of the two acyl chains.

CONCLUSIONS

These *in vivo* studies show that phospholipids attenuate the bladder irritation from KCl after PS-induced bladder injury.

KEYWORDS

liposomes, phospholipids, bladder irritation, protamine sulphate

INTRODUCTION

Numerous studies on interstitial cystitis (IC) agree that disruption in the permeability barrier of the urinary bladder might induce the pathogenesis of IC [1]. A compromised permeability barrier has been shown in the feline model of IC as well as in most patients with IC [2–4]. A dysfunctional permeability barrier can allow the migration of solutes, such as potassium, which can depolarize subepithelial afferent nerves and provoke inflammation [1]. The resulting tissue irritation causes increased afferent excitability, which ultimately leads to increased urinary frequency and urgency in an effort to reduce the effect of urine on the bladder wall [5]. The permeability barrier of the human bladder is erected through the unique anatomy of umbrella cells in the urothelium [6]. Umbrella cells are characterized by the exceptionally low permeability of their apical membrane, which has also been shown to possess a

smaller surface area than its basolateral membrane [7].

Studies on membrane biology frequently use liposomes as a membrane model, which are vesicles, composed of concentric lipid bilayers separated by aqueous compartments. Investigations on the apical membrane of canine kidney cells imply asymmetrical lipid composition for the leaflets of lipid bilayer in the apical membrane of umbrella cells. The outer exofacial leaflet is enriched with choline-containing lipids such as phosphatidylcholine (PC), and cholesterol whereas the inner cytoplasmic leaflet contains mostly amino-containing phospholipids such as phosphatidylserine and phosphatidylethanolamine [5,7]. Interestingly, removal of choline containing lipids from liposomes formulated with the lipid composition obtained from the exofacial leaflet in the apical membrane of dog kidney cell showed an increased permeability towards water, protons, and nonelectrolytes [7].

In a previous study on rats reported from our laboratory [8], intravesical infusion of liposomes formulated with egg PC and cholesterol was effective in reducing bladder hyperactivity caused by bladder injury induced by protamine sulphate (PS). Bladder treated with PS showed necrosis and sloughing of umbrella cells on electron microscopic examination and compromised bladder permeability [9]. The previous report published from our laboratory, showed that liposomes produce an immediate decrease in bladder contraction frequency (BCF) even in the presence of provoking agent, KCl [8]. The present study was designed to elucidate the charge and necessary elements in the chemical structure of lipids required for alleviating bladder hyperactivity. We evaluated the effect of liposomes prepared from various pure natural and synthetic lipids in a rat bladder injury model in the absence or the presence of cholesterol. In addition, synthetic lipids having acyl chain lengths similar to natural lipids but varying in degree

of saturation and charge on the headgroup were also tested for efficacy in the bladder injury model (Fig. 1).

MATERIALS AND METHODS

Pure (>99%) lipids, L- α -phosphatidylcholine (egg PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (OSPC), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Liposomes were prepared using the thin film hydration method as described previously [10,11]. Briefly, lipids in chloroform were spread in a round bottom flask under a stream of nitrogen and then dried under vacuum for 4 h. Solid lipid films were hydrated with saline containing 500 mM KCl by sonication for >5 min at a temperature exceeding the transition temperature of the respective lipids. Liposomes were prepared with a final lipid concentration of 2 mg/mL using 500 mM KCl as a vehicle.

The bladder reflex activity of female Sprague-Dawley rats (200–250 g) was studied by cystometry performed under anaesthesia with urethane (1.0 g/kg s.c.). Their body temperature was maintained in the physiological range using a heating lamp. A transurethral polyethylene bladder catheter (PE-50) connected by a three-way stopcock to a pressure transducer and to a syringe pump was used to record intravesical pressure and to infuse solutions into the bladder. A control cystometrogram (CMG) was obtained by slowly filling the bladder with saline (0.04 mL/min) to elicit repetitive voiding. The BCF of the reflex bladder contractions was recorded. The animal studies were done under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

After obtaining control CMGs with saline infusion for 3 h, PS (Sigma Chemical; 10 mg/mL) was infused at 0.04 mL/min for 1 h to increase epithelial permeability followed by infusion of KCl (500 mM) for 1 h. Subsequently liposomes prepared in KCl were

infused for an additional 2 h. The percentage reduction in bladder hyperactivity after liposome infusion was calculated using the following formula:

$$(1 - [\text{BCF during liposomes} + \text{KCl/BCF during KCl}]) \times 100$$

Quantitative data are expressed as the mean (SEM). Multiple comparisons among the different groups were analysed by a single-factor ANOVA, followed by *post hoc* comparisons with Newman-Keuls test. Differences among groups were considered statistically significant at $P < 0.05$.

RESULTS

As shown in Fig. 2, the normal rat bladder contracts ≈ 3 –4 times/h (top tracing of Fig. 2) in the absence of any irritation and accordingly its BCF was very low. We allowed the baseline CMG to run for 2–3 h for bladder contractions to stabilize and achieve regular intervals between two micturition peaks. Infusion of PS (10 mg/mL) into the rat bladder for 1 h induced injury and there was a slight change in the CMG but no significant difference from saline infusion. The bladder injury caused by PS infusion at the concentration used in these experiments has been well documented [9,12]. However, infusion of KCl after PS infusion produced an irritative effect on the bladder and the BCF was markedly increased as shown in the bottom tracing of Fig. 2. The bladder injury induced by PS and irritation from KCl causes the changes in bladder contractions; the peak pressure is raised and the duration of bladder contraction is slightly longer. The changes in BCF after KCl infusion vary from rat to rat and for this reason, the reduction in BCF after liposome infusion is expressed as a percentage of BCF before liposome infusion. Liposomes prepared using different phospholipids were infused in the presence of KCl and the percentage reduction in BCF was calculated using the formula above. The infusion of liposomes produces more pronounced changes in urinary frequency or BCF and other cystometric variables were not altered by liposomes, i.e. peak pressure and contraction duration. This effect of liposomes on cystometric variables suggest that liposomes block urinary irritants from acting on the afferent branch of the micturition reflex and do not alter the efferent branch.

Each phospholipid is named by the increasing carbon chain length and degree of

FIG. 1. Pure natural lipid egg PC and its synthetic analogues varying in acyl chain length and saturation.

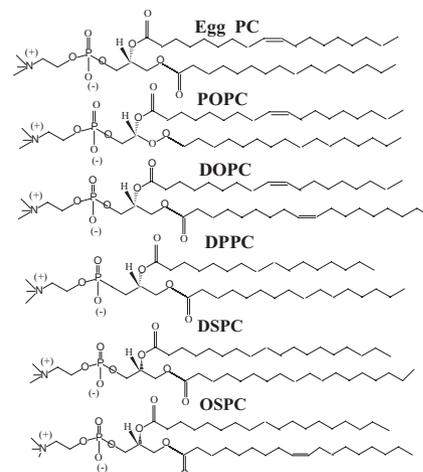
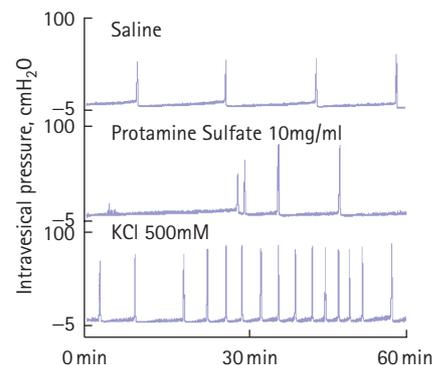


FIG. 2. Representative CMG of normal rat infused sequentially at 0.04 mL/min with saline, PS (10 mg/mL) and KCl 500 mM for 1 h. BCF during saline infusion was very low and it is markedly increased during KCl infusion after PS injury. The higher BCF in third panel shows the bladder hyperactivity after bladder irritation.



unsaturation. They contain two fatty acids in the *sn*-1 and *sn*-2 positions that establish the complete identity of each phospholipid (Fig. 1). Synthetic PCs with increasing length or saturation of the acyl chains and charged lipids with and without cholesterol were tested for their activity to reduce BCF in this rat model.

We first determined the effect of the charge on the lipid headgroup in reducing bladder activity. Liposomes prepared with zwitterionic lipid egg PC were compared with lipids having positive (DOTAP) or negative (POPS) polar headgroups. Liposomes made from egg PC were able to suppress the bladder

FIG. 3. Effect of the charge carried on the lipid headgroup in reducing bladder hyperactivity. The black arrow marks the start of infusion of liposomes in presence of the irritant 500 mM KCl (panel A). KCl alone was infused in the period before the black arrow. Zwitterionic lipid egg PC was significantly better than lipids having a negative (POPS) or a positive (DOTAP) charge in their headgroup in reducing bladder hyperactivity (panel B). $P < 0.01$ (five rats in each group).

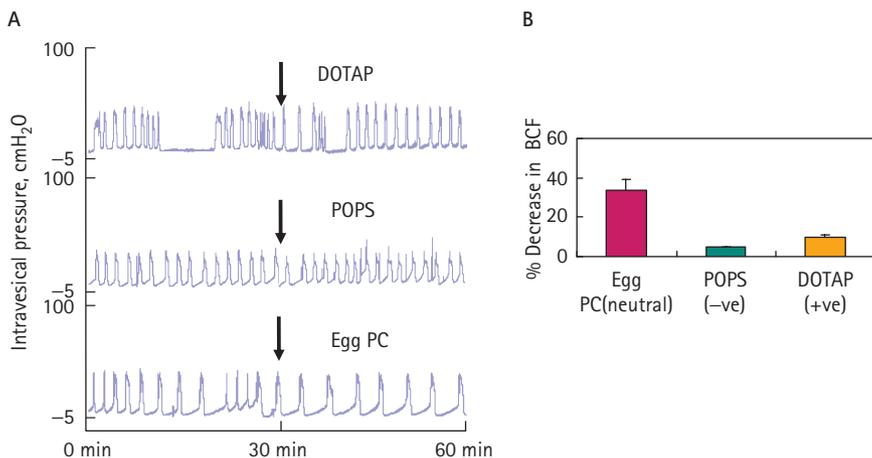
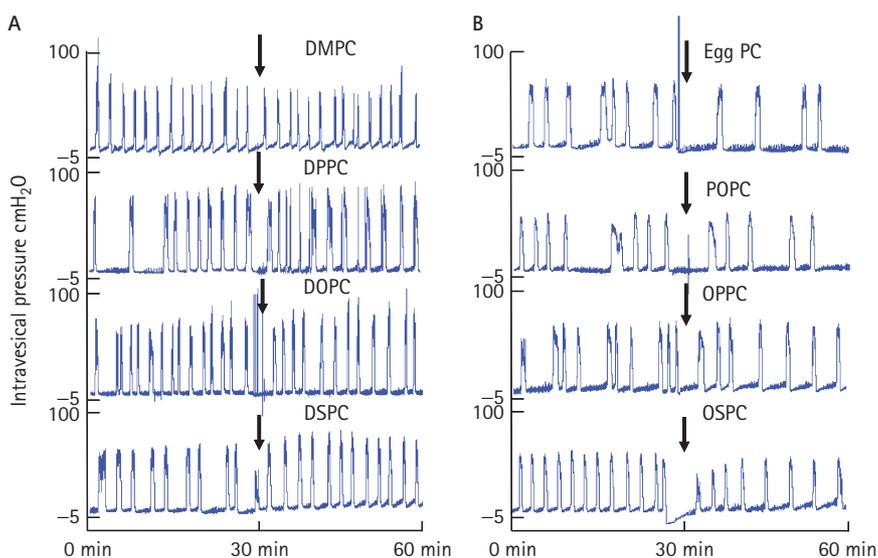


FIG. 4. The effect of the acyl chain length and saturation in reducing bladder hyperactivity. Rats infused with PCs of increasing length DMPC, DPPC, DOPC and DSPC failed to show any improvement in bladder hyperactivity after the start of liposome infusion (black arrow) compared with the BCF before infusion (panel A). Lipids of increasing length with saturation patterns similar to egg PC such as POPC, OPPC and OSPC were effective in reducing bladder hyperactivity as evident from the decreased BCF after the start of liposome infusion (black arrow) compared with the BCF before infusion (panel B).



hyperactivity significantly more than either DOTAP or POPS (Fig. 3A,B). These results indicate that lipids with a neutral charge in the headgroup are more effective than those with a charged headgroup.

When liposomes were prepared with natural lipid found in cell membranes such as pure

egg PC or its synthetic analogue POPC; they were able to significantly reduce bladder hyperactivity (Fig. 4; top two tracings in Panel B). Liposomes made from POPC and egg PC produced mean reductions of $\approx 35\%$ in BCF compared with the BCF before infusion of liposomes. POPC (C16:0, C18:1) is the major chemical species present in pure egg PC.

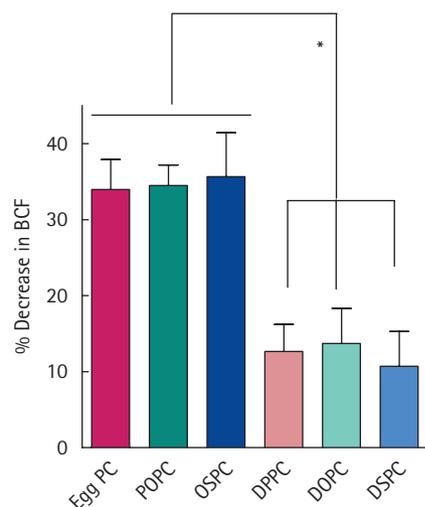
PCs with increasing lengths of acyl chains were tested for their activity in attenuating bladder hyperactivity caused by KCl. DMPC (di C14:0) has saturated acyl chain lengths shorter than those found in natural lipids of mammalian cell membranes (Fig. 4; top tracing of Panel A). DMPC (di C14:0) was unable to reduce the bladder hyperactivity and same was true for liposomes prepared with higher homologues DPPC (di C16:0) and DSPC (di C18:0) indicating that chain length is not important (Panel A; Fig. 4). The presence of saturation (DPPC and DSPC) in both the acyl chain linked to the *sn*-1 and *sn*-2 position was detrimental to the efficacy of liposomes (Fig. 4; Panel A). The same was true for the presence of unsaturation in both chains, as DOPC liposomes were significantly worse at reducing bladder hyperactivity (Fig. 4; third tracing in Panel A). Switching the position of unsaturation from one chain to the other did not drastically reduce the efficacy of POPC, as was evident from the activity of OPPC (Fig. 4; third tracing in Panel B). Similarly, the efficacy of OSPC was comparable with POPC, as saturation was absent in one of the chains (Fig. 4; bottom tracing in Panel B). These results indicate that pure synthetic lipids possessing acyl chain lengths and saturation similar to that of natural egg PC also have a similar activity to that of natural egg PC (Fig. 5). There was optimal activity when only one of the linked acyl chains was unsaturated. The amount of *cis* double bonds in the acyl chains of phospholipids is known to have dramatic effects on the order and dynamics of the membrane.

Liposomes prepared from a pure natural lipid (egg PC) and inactive synthetic lipid DOPC were tested for the effect of cholesterol in reducing BCF to the same extent as in presence of cholesterol (Fig. 6; Panel A). Inclusion of cholesterol in the liposomal formulation at 2:1 molar ratio did not significantly change the activity in both lipids (Fig. 6; Panel B). This indicates the efficacy of liposomes in hyperactive bladder was primarily due to the effect of PC.

DISCUSSION

Our laboratory previously reported the development of an effective rat model of bladder injury, which can simulate the condition of the breached bladder permeability barrier in patients with IC [13]. PS is a cationic peptide found in the sperm of

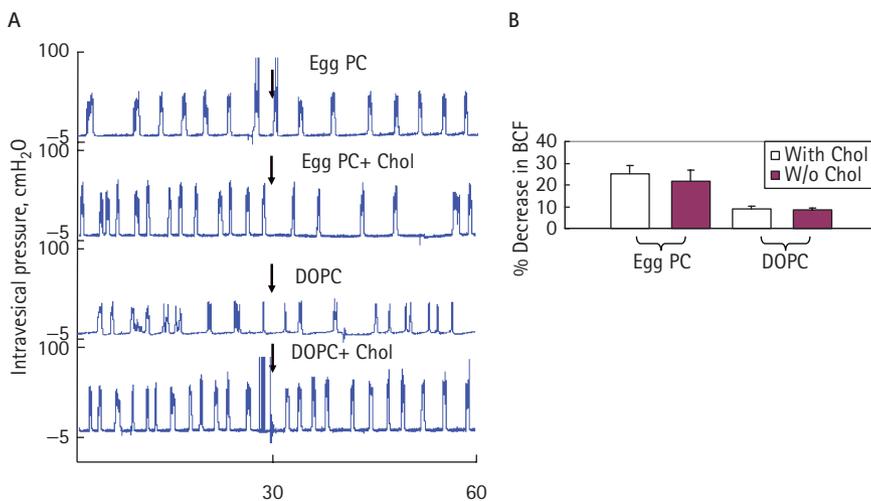
FIG. 5. The mean percentage decrease in BCF of the rat groups (seven rats in each group) infused with the different lipids. POPC and OSPC behaved similarly to egg PC with no significant difference between them but these three were significantly different from DPPC, DOPC and DSPC ($P < 0.05$). The values are expressed as the mean (SEM). Multiple comparisons among the different groups were analysed by a single-factor ANOVA, followed by post hoc comparisons using the Newman-Keuls test.



animals, which can interact with anionic layer of surface mucopolysaccharide glycosaminoglycans (GAG) found on urothelial cells or with specific lipids present in the apical, but not in the basolateral, membrane [14,15]. It has been shown that PS can induce nonselective pores in the apical membrane, leading to irreparable cell damage in the rabbit bladder [16].

Studies claim that the GAG layer on the surface of transitional cells improves the bladder impermeability barrier function [15]. The goal of restoring the defective GAG layer found in some patients prompted intravesical administration of heparin, hyaluronic acid, or pentosan polysulphate [17]. Although effective in a third of patients with IC, oral administration of heparinoid-based therapy (pentosan polysulphate sodium) might require several months or more to provide any relief of pain and urgency. Intravesical administration of GAG analogues such as pentosan polysulphate sodium also fails to produce immediate and sustained relief of IC symptoms [18]. Lilly and Parsons [15] reported the immediate reversal of PS-induced bladder permeability damage by the GAG analogue

FIG. 6. The effect of cholesterol inclusion in the liposomes prepared from active or inactive lipid. The black arrow marks the start of infusion of liposomes in presence of the irritant 500 mM KCl (Panel A). The liposomes in the absence of cholesterol were able to reduce bladder hyperactivity as evident from a decreased BCF after the start of liposome infusion (black arrow) compared with the BCF before infusion. There was no significant difference after the addition of cholesterol (Chol) to the phospholipids (Panel B), egg PC and DOPC (three rats in each group).



heparin. By contrast, our studies evaluated the effect of liposomes on the secondary outcome of increased urinary frequency after bladder damage and we observed that infusion of liposomes produced immediate decrease in the BCF of the injured bladder irritated with KCl [8]. The study design followed in our study used sequential administration of PS followed by KCl and then KCl + liposomes. We choose this method as it allows unrestricted injurious effect of PS on the bladder surface to occur before evoking frank irritation with KCl. The beneficial effect of liposomes is then evaluated by relative decrease in BCF compared with infusion of KCl alone.

The lipid used in our previous study [8] was impure ($\approx 60\%$ purity) egg PC. In the present study, infusion of liposomes prepared with only pure natural lipid egg PC was also able to reduce BCF comparable to liposomes composed of crude impure egg PC [8]. The present study was undertaken to elucidate the key structural elements necessary for the efficacy of liposomes required for alleviating the hyperactive bladder. The bladder injury model used in our earlier study was also used in the current study to evaluate the liposomes prepared from lipids with variation in the charge of the polar headgroup and variation in hydrocarbon domain of the lipid. As reported earlier, we found that neutral

zwitterionic lipids are significantly effective in reducing bladder activity compared with anionic and cationic liposomes [8]. Charged molecules are known to aggravate the bladder irritation [19]. Previous studies also used liposomes containing cholesterol [8]. The results were similar with infusion of liposomes from pure synthetic lipids in the absence or presence of cholesterol (Fig. 6). This led us to infer that cholesterol is unnecessary for the effect of the liposomes prepared with choline headgroup phospholipids in hyperactive bladder. The lack of difference in the activity of egg PC and DOPC following inclusion of cholesterol indicates that the fluidity of the bilayer might not be an important factor in the effects of liposomes in bladder.

It appears that liposomes prepared from lipids having acyl chain lengths shorter than those found in natural lipids found in cell membranes do not have efficacy in hyperactive bladder. This suggestion was supported by the inactivity of liposomes prepared from DMPC. Synthetic lipids possessing average saturation state in acyl chains similar to natural lipids were preferred for reducing bladder hyperactivity. Absence or presence of saturation in the acyl chain linked at *sn*-2 position of POPC led to parallel changes in the activity of liposomes. Absence or presence of saturation in the acyl chain

linked at *sn*-2 position of POPC led to parallel changes in the activity of liposomes. Phospholipids having fatty acids shorter than 16 carbons are usually absent in egg PC, but it does contain minor amounts of lipids having one of the acyl chains derived from stearic acid. There was a similar pattern of difference in biological activity with respect to the degree of saturation in the chains when comparing OSPC and DSPC (Fig. 5). It was determined that acyl chain length is not important for the efficacy of liposomes in hyperactive bladders. Symmetric unsaturation does not favour efficacy and optimal activity is obtained with asymmetrical unsaturation in the acyl chains. The possibility of a specific lipid-protein interaction seems unlikely from the similar activity of OPFC and POPC involving a change in positioning of the double bond.

The nature of acyl chain content can dramatically alter the biophysical properties of liposomes and biomembranes. Phospholipids with saturated fatty acid chains are known to form more rigid and stable membranes than natural phospholipids. The presence of double bonds imposes different packing constraints, creates disorder in hydrophobic region of the lipid bilayer and leaves more free volume space for water and other solutes to permeate through. Unsaturation in the acyl chains of phospholipids increases membrane fluidity by having higher degrees of freedom and they are less rigid from their lower hydrogen-hydrogen interactions. In a previous study, liposomes prepared from phospholipids of natural origin (egg PC) could inhibit generation of superoxide anion O²⁻ in neutrophils more than liposomes prepared with saturated lipids such as DPPC [20].

The fatty acid distribution of egg PC is heterogeneous with a degree of unsaturation varying up to 0.35 *ci*-double bonds per molecule. A ratio of 0.82 exists between saturated and unsaturated fatty acids with 34% of palmitic, 32% of oleic acid and 11% of stearic acid and 16% of linoleic acid. A recent report suggested that urinary glycoprotein Tamm-Horsfall has cytoprotective properties that can prevent bladder irritation from small molecular weight cations such as K⁺ by chemically binding with them [21]. Probably, the liposomes used in the present study blunted the KCl-induced irritation by preventing their access to the bladder surface.

Studies on other inflammatory disorders such as pancreatitis, acute lung injury have also reported the protective role of PC and oxidized phospholipids [22,23]. Free phospholipids generated from cell membrane at the site of injury can attenuate inflammation and augment membrane barrier function [22,23]. Based on those reports, we speculate that bladder injuries stimulate urothelial lipid synthesis to augment barrier function and intravesical exposure of natural phospholipids probably augments that process. The present results imply that liposomes probably improve the barrier function of the injured bladder and the specific changes in cystometric variables caused by liposomes suggest that liposomes block urinary irritants from acting on the afferent branch of the micturition reflex and not acting on the efferent branch [24]. The peak pressure of the bladder contraction peaks in CMG and the duration of bladder contraction is determined by the efferent activity to the detrusor muscle and as there was no change in those variables, we think that liposomes do not change the efferent activity. The time interval between bladder contractions is mainly determined by afferent activity originating in the bladder and these results suggest that liposomes act as a barrier in the afferent activity induced by bladder irritation, as they remain unchanged. Phospholipids with a choline headgroup and asymmetrical saturation in acyl chains seem to readily interact in the biochemical milieu of bladder injury [25,26].

In conclusion, the nature of the acyl chain content and type of headgroup can dramatically alter the biophysical properties of liposomes and their therapeutic effect on injured rat bladder. Synthetic lipids possessing an average saturation state in the acyl chains similar to that of natural lipids were better for reducing bladder hyperactivity. The results of the present study can be utilized for selecting lipids for formulating drugs for intravesical treatment of IC.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: IC, interstitial cystitis; CMG, cystometrogram; PS, protamine sulphate; BCF, bladder contraction frequency; PC, phosphatidylcholine; egg PC, L- α -phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; OSPC, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]; GAG, glycosaminoglycan.