Drug Development and Industrial Pharmacy, 34:215–220, 2008

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040701542440

informa
healthcare

Heparin Decreases Permeability of Pig Urinary Bladder Wall Preliminarily Enhanced by Chitosan

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Chitosan significantly increases the permeability of the isolated pig urinary bladder wall by causing urothelial desquamation, the extent of which depends also on the concentration of the polymer. By desquamation permeability barriers of the urothelium are removed. To gain additional insight into the mechanism by which chitosan acts an absorption enhancer into urinary bladder mucosa, we evaluated the influence of a polysaccharide heparin on the permeability of isolated pig urinary bladder wall preliminarily treated with chitosan. Moreover, we aimed to establish whether the effect of heparin depends on its concentration and on the degree of urothelial desquamation caused by chitosan. In the permeability studies performed by the use of diffusion cells, transport of a model drug, pipemidic acid, into the isolated pig urinary bladder wall was determined. Heparin did not have a significant effect on the permeability of the intact urothelium. When applied to the urinary bladder wall, whose permeability was preliminarily enhanced by 0.005% or 0.001% w/v chitosan, heparin decreased the permeation of pipemidic acid into the bladder wall to a level not significantly different from the intact tissue. However, the effect of heparin was not significant at the highest concentration of chitosan tested, where the damage to the urothelium was much more intense compared with that found at lower concentrations of the polymer. The formation of complexes between pipemidic acid and heparin cannot be excluded completely, but it seems that they are not the main reason for the decreased permeation of pipemidic acid in the presence of heparin. By application on the urothelium, damaged by chitosan, heparin is supposed to form a layer on the surface of the urothelium that prevents the transport of the model drug into the bladder wall. In this way heparin probably restores the impermeability properties of the urinary bladder wall to a degree dependent on the urothelial damage.

Keywords chitosan; heparin; urinary bladder; permeability

INTRODUCTION

Urinary bladder epithelium, also called a urothelium, is composed of a layer of superficial cells, several layers of intermediate cells, and a layer of basal cells. The differentiation of the cells increases from the basal to the superficial cells. To prevent the diffusion of substances from urine into the bladder wall, the urothelium provides three types of permeability barriers. Beside tight junctions between superficial cells, the thin layer of glycosaminoglycans on the surface of the urothelium offers an important diffusion barrier (Hurst & Zebrowski, 1994; Lewis, 2000; Lilly & Parsons, 1990; Parsons, Boychuk, Jones, Hurst, & Callahan, 1990). The third permeability barrier are membrane plaques, which cover 70% to 90% of the apical membrane surface of superficial cells and are much thicker than normal membrane regions (Apodaca, 2004; Lewis, 2000).

Chitosan, a polysaccharide composed of glucosamine and N-acetyl glucosamine, is a bioadhesive polymer also known as an absorption enhancer. For enhanced epithelial permeability the positive charge of chitosan is very important as it enables the polymer to interact with the negatively charged epithelial surface via electrostatic interactions. The results of our previous studies (Kerec, Bogataj, Veranič, & Mrhar, 2005; Kerec Kos, Bogataj, Veranič, & Mrhar, 2006) on isolated pig urinary bladders revealed that chitosan increases the permeation of the model drug into the bladder wall. The effect of chitosan on bladder wall permeability depends on a concentration of chitosan as well as on how long the tissue is exposed to the polymer. Within 90 minutes the effect of chitosan on the amounts of the model drug that permeated into the tissue gradually increases and approaches its plateau. In concentrations that significantly enhance the permeability of the bladder wall, chitosan triggers desquamation of the urothelium, however at low concentrations it occurs to a smaller extent. The mechanism by which chitosan influences the permeability of the urinary bladder wall is different from the one proposed for Caco-2 cells, where chitosan was suggested to affect the proteins of tight junctions (ZO-1, occludin) as well as cytoskeletal F-actin (Dodane, Khan, & Merwin, 1999; Schipper et al., 1997; Smith, Wood, & Dornish, 2004).

Heparin is a sulphated glycosaminoglycan. By binding to the urothelium, injured by an acid, a detergent, or protamine sulphate, heparin was reported to restore impermeability as well as antiadherence properties of the urothelium (Gill, Jones, & Ruggiero, 1982; Hanno, Fritz, Wein, & Mulholland, 1978;

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Lilly & Parsons, 1990; Nickel, Downey, Morales, Emerson, & Clark, 1998; Smith, 2004). It can be used in the treatment of interstitial cystitis as a mucosal surface protection (Chancellor & Yoshimura, 2004; Parsons, 1997).

The broader aim of our research is to develop bioadhesive microspheres with chitosan for intravesical instillation into the urinary bladder that would be used for the local treatment of severe bladder inflammations or superficial bladder cancer. Beside mucoadhesive properties, chitosan would increase the permeation of drugs, which are released from microspheres, into the tissue. In the scope of the present work, we aimed to obtain an additional insight into the mechanism by which chitosan increases the permeability of the bladder wall. As it was expected from the literature that heparin would adhere to the urothelial surface (Gill et al., 1982; Nickel et al., 1998), we evaluated its influence on the permeability of the isolated pig urinary bladder wall, preliminarily increased by chitosan. We aimed to establish whether the effect of heparin depends on its concentration and on the degree of the tissue damage caused by application of different concentrations of chitosan. The potential effect of heparin to restore the impermeability of the urothelium could also be applied in the practice to protect the damaged urothelial surface.

MATERIALS AND METHODS

Materials

Chitosan hydrochloride (in further text indicated as chitosan) (Protasan Cl 213, degree of deacetylation 86%, apparent viscosity of 1% w/v aqueous dispersion 95 mPas) was purchased from Pronova Biopolymer, Oslo, Norway. Heparin sodium (in further text indicated as heparin) was obtained from Sigma Aldrich Chemie, Steinheim, Germany. A model drug pipemidic acid was kindly provided by Lek, Ljubljana, Slovenia. For chromatographic determination of pipemidic acid in the tissue samples, methanol and acetonitrile for preparative liquid chromatography (PChromasolv, Sigma-Aldrich Laborchemikalien) were used as well as analytical grade trichloroacetic acid (Merck, Darmstadt, Germany). In the permeability experiments, phosphate buffer saline (PBS) and phosphate buffer (PB) were used. PBS (Ph. Eur. IV) consisted of 0.944 g Na_2HPO_4, 0.19 g KH_2PO_4, and 8 g NaCl in 1 L of deionised water (pH 7.4), while PB consisted of 0.472 g Na_2HPO_4, 0.095 g KH_2PO_4, and 1.6 g NaCl in 1 L of deionised water (pH 4.5).

Permeability Experiments

Until used in the experiments, the pig urinary bladders, obtained from a local slaughterhouse, were kept in PBS saturated with carbogen (95% O_2 and 5% CO_2) and cooled to 5°C. After the bladder corpus was cut into pieces (each approximately 25×25 mm), the tissue pieces were mounted into diffusion cells, developed at the Faculty of Pharmacy, Ljubljana, Slovenia (Kerec et al., 2005). In the diffusion cell the luminal side of the urinary bladder wall was exposed to the tested solution or dispersion. The donor chamber of the diffusion cell had a volume of 10 ml and the tissue exposure area was 4.5 cm^2.

Four series of the permeability experiments were done and they differed mainly in the concentration of chitosan to which the tissue was preliminarily exposed. The solutions that were used in the experiments as well as the time of the tissue exposure to the particular solution are shown in Table 1. In all the experiments, the tissue, mounted into the diffusion cell, was exposed to 8 ml of a tested solution. In most series of the experiments the tissue was first incubated in one solution. Afterwards the tissue was rinsed three times with PB and exposed to the second solution. All tested solutions were prepared in PB and their pH was adjusted to 4.5. The pH of urine can vary from 4.5 to 8, depending on the kind of diet (Burtis & Ashwood, 1986; Lothar, 1998) and the selected pH is within this range. Moreover, it is proven that this pH by itself does not cause any morphological changes of the urothelium (Kerec et al., 2005). Concentration of pipemidic acid was 140 mg/L in all the experiments. The experiments were performed at room temperature.

At the end of the permeability experiments the tissue was first rinsed three times with PB and then was placed between two parallel stainless steel plates whose distance was regulated regarding the tissue thickness. Rapid freezing with liquid nitrogen was performed. Tissue was then sectioned by cryostat (Leica CM 1850, Nussloch, Germany) in sections of 20 μm thickness parallel to luminal surface up to 1.2 mm of the tissue depth. Three consecutive sections were pooled and 250 μL of mobile phase (0.2% TCA/MeOH/ACN, volume ratio 76/4/20) was added to each sample. To ensure complete extraction, the samples were first vortexed until all the tissue sections were sunken into the mobile phase and then shaken (2 hours at 225 cpm, room temperature). After centrifugation of the samples (10 minutes at 45.000 g, room temperature) concentration of pipemidic acid in the supernatant was determined by high performance liquid chromatography. A PRP-1 column (150×4.1 mm, 5 μm particles; Hamilton, Reno, Nevada) and a precolumn of the same type were used. The flow of the mobile phase was 1 ml/minute. Diode array detection at 275 nm was applied. The cumulative amounts of pipemidic acid that permeated into the tissue were calculated.

Evaluation of Pipemidic Acid–Heparin Binding

PB was placed on one side of dialysis cells (Bel-Art-Products, Pequannock, New Jersey), while on the other side of semipermeable dialysis membrane (Spectra/Por, mean molecular weight 3500, Thomas Scientific, United States) a solution of pipemidic acid, a 1% w/v solution of heparin, or a solution of pipemidic acid with 0.5% or 1% w/v heparin was placed. The concentration of pipemidic acid and the pH of all the solutions were the same as in the permeability experiments. In contrast to the diffusion cells used in the permeability experiments, in

TABLE 1
Solutions and Dispersions Used in the Permeability Experiments

Number of the Series	Number of the Diffusion Cell	Tested Solution	
1. series; $n = 6$	1	PPA (45)	
	2	PPA + 0.5 % HEP (45)	
	3	PPA + 1.0 % HEP (45)	
2. series; $n = 6$	1	PB (45)	PPA (45)
	2	0.5% CH (45)	PPA + 0.5% HEP (45)
	3	0.5% CH (45)	PPA + 1.0% HEP (45)
	4	0.5% CH (45)	PPA (45)
3. series; $n = 6$	1	PB (45)	PPA (45)
	2	0.005% CH (45)	PPA + 0.5% HEP (45)
	3	0.005% CH (45)	PPA + 1.0% HEP (45)
	4	0.005% CH (45)	PPA (45)
4. series; $n = 6$	1	PB (45)	PPA (45)
	2	0.001% CH (45)	PPA (45)
	3	0.001% CH (45)	PPA + 0.5% HEP (45)
	4	0.0005% CH (45)	PPA (45)
	5	0.0005% CH (45)	PPA + 0.5% HEP (45)

PB is a phosphate buffer, PPA a solution of pipemidic acid, PPA+HEP a solution of pipemidic acid with 0.5% or 1% w/v heparin, and CH a dispersion of chitosan. 0.5%, 0.005%, 0.001%, or 0.0005% w/v concentration of chitosan was used in the experiments. The labeled concentrations of chitosan or heparin indicate concentrations of the polymers in the solutions that were applied to the luminal surface of the urinary bladder wall, mounted into the diffusion cells. In parentheses the minutes of the tissue exposure to the particular solution are given; *n* indicates the number of the urinary bladders used in the particular series of the experiments; with the exception of the first series of the experiments the tissue was first exposed to one solution, rinsed, and then the second solution was applied.

dialysis cells the semipermeable membrane was placed vertically. After 45, 60, or 120 minutes the concentration of pipemidic acid that diffused into PB was determined spectroscopically (Agilent 8453, UV-Visible Spectrophotometer, Agilent Technologies, United States) at 274 nm. The dialysis was performed at room temperature.

Statistic Evaluation

The data obtained within a particular series of the permeability experiments were assessed for statistically significant differences by ANOVA for repeated measures with the Bonferroni post hoc test ($\alpha = 0.05$). For statistic evaluation of the data obtained in different series of the permeability experiments and to test the data of dialysis, a two-tailed Student's unpaired t-test ($\alpha = 0.05$) was applied.

RESULTS AND DISCUSSION

When applied to the urothelium, injured by an acid, a detergent, or protamine sulphate, heparin was reported to reduce the crystal adhesion, bacteria adsorption, and permeability of the urothelium (Gill et al., 1982; Hanno et al., 1978; Lilly & Parsons, 1990; Nickel et al., 1998; Ruggieri, Hanno, & Levin, 1984; Smith, 2004). The goal of our work was to establish if heparin can also influence the permeability properties of the pig urinary bladder wall preliminarily exposed to chitosan, which increases the permeability of the bladder wall by causing desquamation of the urothelium (Kerec et al., 2005; Kerec Kos et al., 2006).

First we aimed to establish the effect of heparin on the permeability of the untreated urothelium. Therefore, in the first series of experiments we exposed the tissue to the solution of a model drug pipemidic acid or to the solution of pipemidic acid with 0.5% or 1% w/v heparin. As shown in Figure 1, both concentrations of heparin lowered the amounts of pipemidic acid that permeated the urinary bladder wall. However, the decrease was not significant. Intact urothelium by itself prevents the permeation of small molecules into the bladder wall and the effect of heparin was obviously not strong enough to significantly contribute to an additional decrease in the permeation of pipemidic acid into the tissue. This is in accordance with Gill and colleagues (1982), who reported that heparin does not bind to the intact urothelium, but only to the urothelium damaged by an acid or a detergent. Beside its insignificant influence on the

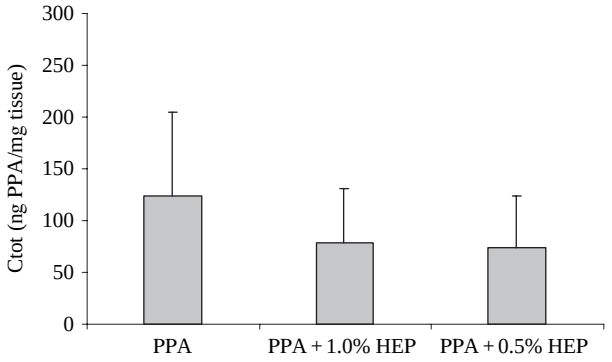

FIGURE 1. The cumulative amounts of pipemidic acid that permeated into the pig urinary bladder wall in the first series of experiments, where the tissue was exposed for 45 minutes to the solution of pipemidic acid (PPA) or the solution of pipemidic acid with heparin (PPA + HEP) ($M \pm SD$, $n = 6$). Heparin was used either in 0.5% or 1 % w/v concentration and both concentrations influenced the permeation of pipemidic acid into the tissue insignificantly ($P > 0.05$).

permeability of untreated urinary bladder wall, revealed also in our study, heparin treatment of intact bladders was reported to slightly, but not significantly, decrease the adherence of four different species of urinary tract pathogens (Ruggieri et al, 1984).

In further permeability experiments the urinary bladders were first exposed to PB or dispersions of different chitosan concentrations and then to the solution of pipemidic acid with or without heparin. The results are shown in Figure 2. In all the experiments where the bladder wall was preliminarily exposed to PB and then to pipemidic acid without heparin (a control), approximately the same amounts of pipemidic acid permeated the tissue. When the tissue was first incubated in the dispersion of chitosan and then in the solution of pipemidic acid only, the permeation of pipemidic acid into the pig urinary bladder wall significantly increased at all concentrations of the polymer

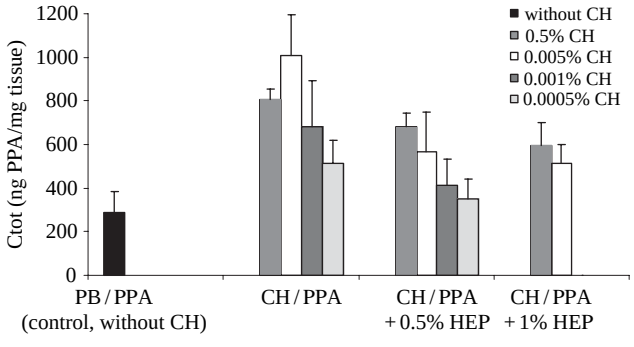

FIGURE 2. The cumulative amounts of pipemidic acid that permeated into the pig urinary bladder wall in the second, third, and fourth series of experiments. The tissue was first exposed for 45 minutes to the phosphate buffer (PB) or the dispersion of chitosan (CH) and then for additional 45 minutes to the solution of pipemidic acid (PPA) or the solution of pipemidic acid with heparin (PPA + HEP) ($M \pm SD$, $n = 6$). Different concentrations of chitosan were tested and heparin was used either in 0.5% or 1% w/v concentration. As a control the average of all three series of the experiments is shown.

used. With decreasing concentrations of chitosan used in the experiments, the effectiveness of chitosan as an absorption enhancer partly diminished, with the exception of 0.5% w/v chitosan, which increased the permeability of the bladder wall less than 0.005% w/v chitosan. In our previous study the influence of chitosan's concentrations on its absorption enhancement effect was studied in detail (Kerec Kos et al., 2006) and there were no significant differences in the cumulative amounts of a model drug, which permeated the tissue that was exposed to 0.005% or 0.5% w/v chitosan. In those experiments different concentrations of chitosan were tested on the urinary bladders of the same animals, while in the present work each concentration of chitosan was tested on the tissue of different animals. Due to the differences in the experiment design mentioned above, it could be concluded that the lower effect of 0.5% w/v chitosan, determined in this work, is a consequence of the variability of the urinary bladders used in the experiments.

After a 1-hour incubation of the urinary bladder wall in 0.5% w/v dispersion of chitosan, a massive desquamation of the urothelium occurs (Kerec et al., 2005; Kerec Kos et al., 2006). By desquamation all three types of urothelial permeability barriers are removed—the layer of glycosaminoglycans on the surface of the urothelium as well as tight junctions and membrane plaques of superficial cells. Consecutively the permeation of pipemidic acid into the bladder wall is enhanced. When heparin was applied to the bladder wall together with pipemidic acid, the permeability of the tissue, preliminarily increased by 0.5% w/v chitosan, was partly reduced (Figure 2). However, the decline was not significant at tested concentrations of heparin. Moreover, the permeability of the bladder wall was still significantly increased in comparison with the control tissue. When applied to the luminal side of the preliminarily damaged urinary bladder wall, due to the incubation in the dispersion of chitosan, heparin probably forms a layer on the urothelium that mimics the naturally occurring highly negatively charged glycosaminoglycans present on the apical surface of superficial cells. At 0.5% w/v concentration of chitosan the damage of the urothelium is obviously so extensive that this layer of heparin cannot restore the impermeability of the bladder wall to a significant extent.

When the permeability of the bladder wall was preliminarily increased by 0.005% w/v chitosan, the addition of heparin to the solution of pipemidic acid significantly reduced the permeation of the model drug into the tissue (Figure 2). Moreover, in the presence of heparin the tissue amounts of pipemidic acid were not significantly different from the control tissue. There were no significant differences in the effect on the tissue between both concentrations of heparin used in the experiments.

0.5% and 1% w/v heparin significantly reduced also the diffusion of pipemidic acid through semipermeable membrane (Figure 3). The reason for lower diffusion of pipemidic acid in the presence of heparin could be the formation of complexes between pipemidic acid and heparin that cannot pass the membrane. At 120 minutes the diffusion was decreased to a higher

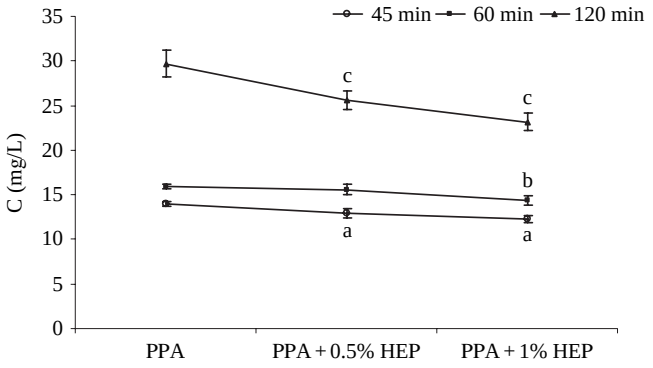

FIGURE 3. The concentration of pipemidic acid ($M \pm SD$, $n = 4$) determined in phosphate buffer (PB) after the dialysis through semipermeable membrane from the solution of pipemidic acid (PPA) with or without heparin (HEP). Tested concentrations of heparin are labeled. The time of dialysis was 45, 60, or 120 minutes; a, b and c indicate a significant effect of heparin on the concentration of pipemidic acid that diffused in PB at a particular time of dialysis. Concentrations obtained in the presence of heparin were compared with concentrations obtained in the experiments with pipemidic acid only.

extent than it was at 45 and at 60 minutes. In the permeability experiments pipemidic acid and heparin were present on the surface of the urinary bladder wall for 45 minutes only. Therefore, the results obtained after 45 and 60 minutes of dialysis are more relevant. Due to the differences in the design of the dialysis cells and the diffusion cells used in the permeability experiments, the results of dialysis can not be applied directly. However, it is evident that in the presence of heparin the relative decrease of pipemidic acid transport is in the case of dialysis smaller than the decline determined in the permeability experiments. Therefore, heparin lowered the permeability of urinary bladder wall, preliminarily enhanced by chitosan, to a higher extent as would be expected from the results of dialysis. Moreover, heparin had a smaller effect on the diffusion of pipemidic acid through semipermeable membrane than on the permeation of pipemidic acid into intact urinary bladder wall, although in both cases the effect of heparin was insignificant.

It was shown in our previous work (Kerec Kos et al., 2006) that 0.005% w/v chitosan triggers desquamation of the urothelium to a lower extent compared with 0.5% w/v concentration of the polymer, although the tissue permeability was not significantly different when the urinary bladder wall was exposed to 0.5% or 0.005% w/v chitosan. Due to the minor damage of the urothelium, the layer of heparin on the surface of the desquamated urothelium is already able to prevent the permeation of the model drug into the bladder wall in significant quantities. The fact that heparin can reduce the permeability of the pig urinary bladder wall, preliminarily enhanced by chitosan, is in accordance with the literature data. The solution of heparin instilled into the urinary bladders of volunteers, whose surface layer of glycosaminoglycans was preliminarily impaired by quaternary amine protamine sulphate, decreased the permeation of urea from the bladder lumen (Lilly & Parsons, 1990). Moreover, in the study performed by Nickel and colleagues (1998) it was demonstrated that instillation of heparin into the rabbit urinary bladders pretreated with protamine sulphate significantly reduced the uptake of ^{14}C-urea in the bladder wall as well as in the blood when compared with the control.

In experiments so far, there were no significant differences between the effects that both concentrations of heparin had on tissue permeability. We assumed that 0.5% w/v heparin forms a layer on the surface of the urothelium that is thick enough to reduce the permeability of the bladder wall in a significant manner. Therefore, only 0.5% w/v heparin was used in further experiments. The tissue was preliminarily exposed to 0.001% or 0.0005% w/v dispersion of chitosan. Both concentrations of the polymer significantly enhanced the bladder wall permeability (Figures 2 and 4). Permeability was increased by 0.0005% w/v chitosan to a lower degree in comparison with the higher concentrations of the polymer tested. However, an increase was still significant regarding the control tissue. In our previous study scanning electron micrographs showed that after a 1-hour incubation of the urinary bladder wall in 0.001% or 0.0005% w/v dispersion of chitosan, large areas of the urothelium became desquamated. However, at these concentrations desquamation occurred to lower degree than at 0.5% w/v concentration of chitosan (Kerec Kos et al., 2006). The permeability of the tissue preliminarily enhanced by 0.001% or 0.0005% w/v chitosan (Figure 4) was reduced by 0.5% w/v heparin. In the case of 0.001% w/v chitosan, the decrease was significant. Moreover, after exposure of the bladder wall to heparin, the permeability of the tissue did not significantly differ from the control tissue. The layer of heparin on the surface of the damaged urothelium obviously compensates for the urothelial permeability barriers removed after the incubation of the tissue in 0.001% w/v dispersion of chitosan. When the tissue was first exposed to 0.0005% w/v chitosan, the effect of heparin was not significant. The permeation of pipemidic acid into the bladder wall was enhanced to a lower extent by 0.0005% w/v chitosan

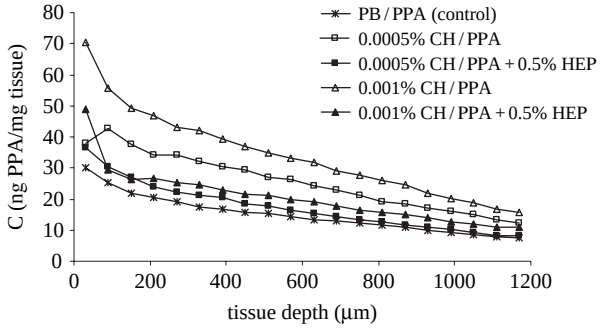

FIGURE 4. The amounts of pipemidic acid that permeated into the pig urinary bladder wall in the fourth series of experiments as a function of the tissue depth (M, $n = 6$). The tissue was first exposed for 45 minutes to the phosphate buffer (PB) or the dispersion of chitosan (CH) and then for additional 45 minutes to the solution of pipemidic acid (PPA) with or without heparin (HEP). Tested concentrations of chitosan and heparin are labeled.

compared with 0.001% w/v chitosan, and the influence of heparin on the permeability of the damaged tissue was consecutively harder to prove statistically.

CONCLUSIONS

In concentrations that significantly enhance the permeation of pipemidic acid into the pig urinary bladder wall, chitosan triggers desquamation of the urothelium. By desquamation the urothelial permeability barriers are removed. In the case of the intact urothelium, which was in advance not exposed to chitosan, the influence of heparin on tissue permeability was not significant. When applied to the urothelium, preliminarily treated with chitosan, heparin significantly decreased the permeability of the bladder wall. The permeation of the model drug into the bladder was in the presence of heparin not significantly different from the intact tissue. However, at the highest concentration of chitosan tested, where the damage of the urothelium was much more intense compared with the lower concentrations of the polymer, the effect of heparin was observable, but not statistically proven. The formation of complexes between pipemidic acid and heparin cannot be excluded completely, but it seems that they are not the main reason for the decreased permeability of the urinary bladder wall in the presence of heparin. Heparin is supposed to form a layer on the surface of the damaged urothelium that compensates for the urothelial permeability barriers, which normally prevent the transport of substances from urine into the bladder wall. The impermeability of the urinary bladder wall is restored, but to a degree dependent on the urothelial damage.

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