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Effects of formulation variables on nasal epithelial cell integrity: Biochemical evaluations

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Abstract

The effects of pH, osmolarity, type and concentration of buffers on the nasal mucosal epithelium have been investigated in rats using an in situ nasal perfusion technique. Traditionally, histological approaches which are qualitative and not predicitive of nasal mucosal sensitivity, have been used to assess the damage to the nasal mucosa. A biochemical approach has been used in this report to assess irritation to the nasal mucosa which may provide a priori indication of nasal sensitivity to chronic use of nasal formulations. The nasal mucosal irritation may be predicted by determining the amount of total protein and two enzymes, lactate dehydrogenase (LDH, EC 1.1.1.27), a cytosolic enzyme and 5′-nucleotidase (5′-ND, EC 3.1.3.5), a membrane-bound enzyme released during perfusion. To determine the effect of pH on the nasal mucosa, phosphate buffers ranging in pH from 2 to 12 were utilized. Solutions within a pH range of 3–10 caused minimal release of the biochemical markers whereas solutions of pH above 10 caused significant membrane and intracellular enzyme release. Acetate buffers (pH 4.75) at three different concentrations, 0.07, 0.14 and 0.21 M, were used to study the effect of buffer concentration on the nasal mucosal integrity. The results indicate that the alteration to the nasal mucosal cells by buffers is concentration dependent. To study the effect of buffer type, four different buffers, i.e., acetate, adipate, citrate, and phosphate (0.07 M, pH 4.75) were studied. The acetate buffer was found to have the most irritation potential when compared to adipate, citrate, and phosphate buffers. To determine the effects of unionized and ionized species of a buffer, 0.025 M benzoate buffers at pH 3.2 and 5.2 were studied. The results indicate that the unionized species of benzoic acid causes more cellular perturbation than the ionized species. Hypertonic and isotonic sodium chloride solutions caused minimal mucosal cell aberrations while hypotonic solutions caused extensive leakage of LDH. These results along with other results from our laboratory may help in designing well tolerated nasal formulations for chronic use.

Keywords: Nasal perfusion; pH; Tonicity; Buffer composition; Protein release; Lactate dehydrogenase; 5′-Nucleotidase; Nasal irritation

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1. Introduction

In recent years, we have witnessed a rapid advancement in the understanding and development of nasal drug delivery systems. The compounds that have been studied for nasal delivery range from highly lipophilic compounds such as steroids (Hussain et al., 1981) to extremely hydrophilic drugs such as clofilium (Su et al., 1984), sodium cromoglycate (Fisher et al., 1985) as well as peptides and proteins. The rate and extent of nasal drug absorption may depend on a number of physicochemical factors, i.e., the aqueous to lipid partition coefficient of the drug, pK_a, molecular weight, instilled solution pH, initial drug concentration, and the overall design of the dosage form. Although a considerable amount of formulation research has been conducted in this area, some basic factors concerning effects of formulation variables on nasal drug absorption, irritation potential and sensitivity are still poorly understood.

Various methods have been used to test the effects of drugs and additives on the nasal mucosa and the mucociliary system. Traditionally, histopathological examination of the pre-fixed membrane specimen is regarded as the indicator of cytotoxicity. This method suffers from some disadvantages like inspector subjectivity and length of time and cost. This technique does not highlight the subtle changes that occur in the nasal mucosa and does not provide the nasal sensitivity tolerance measurement in response to a particular formulation. Therefore, to determine minute changes occurring in the nasal mucosal tissue due to exposure to formulations, a more sensitive technique needs to be utilized. A biochemical approach has been developed by Shao and Mitra (1992) and Shao et al. (1992) to assess toxicity to the nasal membrane by various groups of absorption enhancers. Total protein, phosphorus, lipid phosphorus, nasal membrane-bound and intracellular enzyme releases have been examined to provide a wide range of information concerning the extent of nasal irritation or epithelial cell damage. The extent of release of total protein and the enzymes, lactate dehydrogenase (LDH) and 5′-nucleotidase (5′-ND), indicate directly the extent of damage sustained by the nasal mucosa. Membrane-bound 5′-ND release in the nasal perfusate gives an indication of the level of membrane perturbation while LDH, being a cytosolic enzyme, indicates the amount of cell leaching and/or lysis thereby providing additional information about the intracellular damage sustained by the nasal mucosa. The total protein release data although not very specific in the type of damage, provide a general indication about the extent of irritation.

It would only be fair to note that these assays will not pick up more subtle, deleterious changes such as transmembrane and transcellular ion conductances. Some cell functions like permeability, mucus and fluid secretion, etc., may be even more sensitive to the formulation variables tested. Nevertheless, these biochemical methods provide an alternative way to quantitatively determine the subtle changes occurring in the nasal mucosa as opposed to the gross changes needed to assess damage using the histopathological method. Such methods correlate quite well with the histopathological studies when the changes to the nasal mucosa by a particular formulation are relatively severe. The biochemical evaluations definitely offer an additional mechanism to assess mucosal toxicity and may complement the standard histopathologic studies. Also, long-term acceptance of a formulation may be judged by this method due to the nature of these evaluations.

Usually during the development process, the completed formulation is evaluated for a toxicological response following chronic dosing, but not for less damaging sensitivity reactions. However, using the biochemical method described in this article, certain basic parameters of a nasal liquid formulation like pH, ionic strength, osmolarity, charge and concentration of buffer species can be evaluated with respect to nasal epithelial response and integrity. The findings of this study will aid the pharmaceutical scientist in formulating a nasal delivery vehicle which does not irritate the nasal mucosa and which in turn may provide more clinically acceptable nasal formulations to patients for chronic use.

2. Materials and methods

2.1. Chemicals

Lactate dehydrogenase (LDH, EC 1.1.1.27) and 5′-nucleotidase (5′-ND, EC 3.1.3.5) kits were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and used as received.

2.2. In situ nasal perfusion method

The perfusion medium was prepared by dissolving the required reagents in deionized double-distilled water. All solutions were made isotonic (300 mOsm/kg H_2O)by the addition of sodium chloride except when determining osmolarity effects.

The rat in situ perfusion technique developed by Hirai et al. (1981) and Huang et al. (1985) was used in this investigation due to the relatively simple nature of the experimental setup and good reproducibility of the method. It allows for multiple sampling of the nasal perfusate, enabling the estimation of time dependent membrane protein and enzyme marker release. Male Sprague-Dawley rats weighing 250–350 g were used in these experiments. The rats were anesthetized by an intraperitoneal injection of 0.1 ml/100 g body wt of a ketamine (90 mg/ml) and xylazine (10 mg/ml) mixture, followed by an additional 0.1 ml/rat administered every 30–45 min to maintain the anesthetic state. After an incision was made in the neck, the trachea was cannulated with a polyethylene tube (PE-200, Intramedic, Clay Adams, NY) to maintain respiration. Another PE-200 tube was inserted through the esophagus toward the posterior part of the nasal cavity and ligated. The passage of the nasopalatine tract was sealed with an adhesive agent (Instant Jet, Cal Goldberg Models Inc., Chicago, IL) to prevent drainage of the solution from the nasal cavity to the mouth. The cannula served to deliver the solution to the nasal cavity.

5 ml of the perfusion medium was circulated at a rate of 2 ml/min by means of a peristaltic pump and collected into a reservoir. An aliquot (200 μl) was sampled every 15 min for a maximum of 2 h and a correction factor was used to account for the volume change that occurred due to sampling. The temperature of the reservoir was maintained at 37 ± 0.5°C during the course of an experiment.

2.3. Assay procedures

2.3.1. Protein content

The protein contents in the perfusate at various sampling points were determined by the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A standard curve was constructed in conjunction with the samples. Absorbance at 750 nm was measured with a Beckman DU-7 UV/Vis spectrophotometer (Irvine, CA).

2.3.2. Enzyme activity assays

The activity of a nasal epithelial intracellular enzyme, lactate dehydrogenase, was determined according to the method of Cabaud and Wroblewski (1958). The activity of a nasal membrane-bound marker enzyme, 5′-nucleotidase, in the perfusate was analyzed according to a kinetic method reported by Arkesteijn (1976).

3. Results and discussion

3.1. Effect of solution pH on the rat nasal epithelium

To determine the effect of solution pH on the nasal mucosal integrity, 0.07 M phosphate buffers ranging in pH from 2 to 12 were perfused for 120 min and total protein released in the perfusate was then measured. The data plotted in Fig. 1 clearly demonstrate that the pH 12 buffer caused the maximum protein release followed by the pH 2 buffer. Buffers within pH values of 3 and 10 caused very low and essentially similar amounts of total protein release. The pH of the rat nasal mucosa is reported to be 7.39 (Hirai et al., 1981) and the pH of the human nasal mucosa is in the range of 5.5–6.5 (Chien et al., 1989). Ohwaki et al. (1985) have studied the effects of pH (pH 2–8) on the nasal absorption of secretin in rats. They

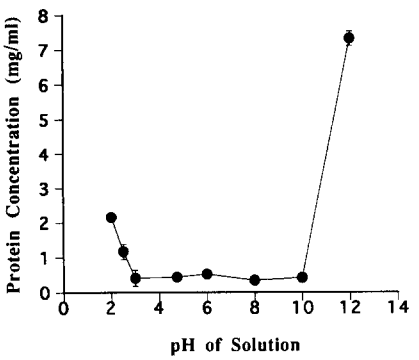

Fig. 1. Release profiles of protein from the rat nasal cavity as a function of pH at the end of 120 min perfusion. Phosphate buffers (0.07 M) within pH ranges of 2–12 were used. Values represent means ± SD (n = 3–4).

reported that increased absorption of the peptide takes place at low pH especially at pH 2.1 and 2.94, leading them to postulate that the increased absorption could be due to mucosal damage caused by the pH of the solution. Subsequent histological studies also revealed structural changes in the nasal epithelial cells by the pH 2.94 solution (Ohwaki et al., 1987).

Phosphate buffers at three pH values (pH 3, 8, 11) were subsequently chosen to examine the time-dependent release of lactate dehydrogenase (LDH), an intracellular enzyme and 5′-nucleotidase (5′-ND), a membrane-bound enzyme during an in situ perfusion experiment. Fig. 2 depicts the amount of 5′-ND released with time when solutions of pH 3, 8, and 11 were perfused. The results indicate that solutions of pH 11 caused the most damage as compared to pH 3 and 8 buffer solutions. No statistical difference between pH 3 and 8 was observed for the release of 5′-ND by the rat nasal mucosa during perfusion. The LDH enzyme loses its activity below pH 4, therefore, this particular enzyme cannot be accurately monitored for solutions below pH 4. Solutions of pH 11 caused large amounts of LDH release (1500 ± 60 U/l at 90 min) as compared to pH 8 (130 ± 30 U/l at 90 min).

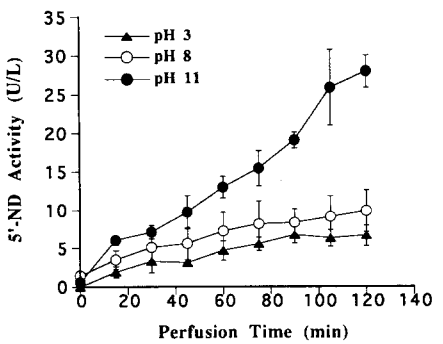

Fig. 2. Release profiles of 5′-ND from the rat nasal cavity as a function of perfusion time. Phosphate buffers (0.07 M) of pH 3, 8, and 11 were perfused. Values represent means ± SD (n = 3–4).

The release of 5′-ND and LDH from the nasal mucosa exposed to pH 11 indicates that this pH caused both membrane and intracellular damage. Van de Donk et al. (1980) have studied the effect of pH on the nasal ciliary beat frequency, another indicator of mucotoxicity. Their results appear to indicate that a solution of pH 11 causes 80–90% decrease in the beat frequency. Also, solutions of pH 7 and 10 caused very little decrease in beat frequency. Their results are very consistent with the results obtained by the biochemical methods described here.

In summary, the results indicate that phosphate buffers with pH values between 3 and 10 cause minimal protein and enzyme release, while buffers with pH values above 10 and below 3 seem to produce both membrane and intracellular damage.

3.2. Effect of buffer species on the rat nasal epithelium

3.2.1. Buffer type

To determine the effect of buffer species on the rat nasal mucosa, four different buffers, acetate, adipate, citrate, and phosphate at pH 4.75 and a concentration of 0.07 M were perfused. Fig. 3 depicts the extent of protein release as a function of perfusion time. The results show that acetate buffer causes the largest degree of protein release, which could probably be due to the strong lipid partitioning behavior of unionized acetic acid at pH 4.75. The unionized acid partitions into the mucosal cell due to its lipophilicity and may cause increased protein release by decreasing the intracellular pH. Hirai et al. (1981)

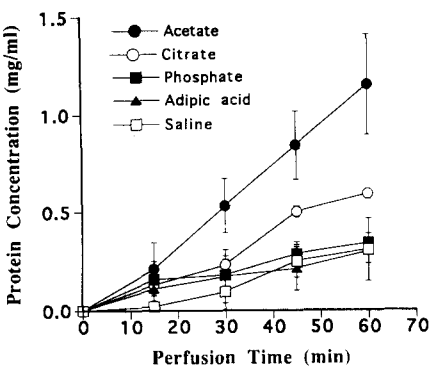

Fig. 3. Release profiles of protein from the rat nasal cavity as a function of perfusion time with different buffers at 0.07 M and pH 4.75. Values represent means ± SD ($n = 3$–4). Saline was the control solution.

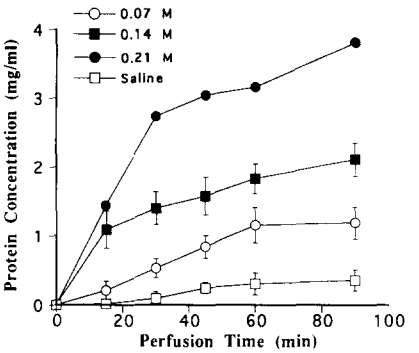

Fig. 4. Release profiles of protein from the rat nasal cavity as a function of perfusion time with acetate buffers (pH 4.75) at 0.07 M, 0.14 M and 0.21 M. Values represent means ± SD ($n = 3$–4).

have determined the absorption rate constants of salicylic acid and aminopyrine at different pH values and found that the absorption rate constants increased with an increase in the amount of unionized species of both compounds. A linear relationship between the rate constant for the nasal absorption of hydralazine in rats and the fraction of its undissociated species at various pH values has also been reported (Chien, 1992). Nevertheless, if partitioning were the only factor, adipic acid at pH 4.75 ($pK_1 = 4.41$, $pK_2 = 5.27$) would also have been quite damaging to the nasal mucosa. Therefore, there appears to be an intrinsic factor unique to the buffer species alone which is responsible for damage to the nasal mucosa in addition to the unionized and ionized forms of the buffer species (Table 1). It may be related to the ability of anionic buffer species to form tight ion pairs with appropriate cations and then undergo membrane transport. Citrate buffer caused intermediate protein release while phosphate and adipate buffers caused very little damage to the nasal mucosa.

Table 1
Percent of ionized and unionized species of buffers at pH 4.75

Buffer type	% A	% HA	% H_2A	% H_3A
Acetate	49.60	50.40	NA	NA
Adipate	17.30	57.20	25.50	NA
Citrate	1.06	47.15	50.51	1.28
Phosphate	negligible	0.34	99.42	0.23

3.2.2. *Buffer concentration*

Acetate buffers (pH 4.75) at three different concentrations (0.07, 0.14 and 0.21 M) were used to study the effect of buffer concentration on the rat nasal mucosa. The damage assessment was carried out by measuring the amount of total protein in the perfusate. Fig. 4 depicts the amount of protein released with the three different concentrations of acetate buffers. The results clearly indicate that the damage to the nasal mucosa by acetate buffers is concentration dependent.

3.2.3. *Ionization of buffer species*

The effect of ionized vs unionized buffer species on the nasal mucosa is currently under investigation in our laboratory. The first candidate for this study is benzoate buffer at pH 3.2 and 5.2, i.e., one unit below and one unit above the pK_a of benzoic acid which is 4.2. Due to the poor solubility of benzoic acid at pH below 4, the concentration of the buffer was kept at 25 mM. The results indicate that the unionized acid species is able to extract significantly more mucosal protein than the ionized species. Fig. 5 shows the amount of protein released with time when these two buffers were perfused through the rat nasal cavity. As depicted in Fig. 5, benzoate buffer at pH 3.2 (% unionized: 90.92) causes

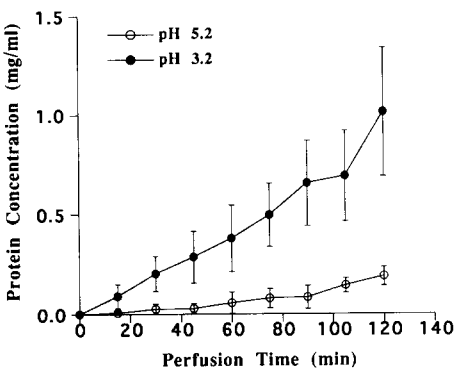

Fig. 5. Release profiles of protein from the rat nasal cavity as a function of perfusion time with benzoic acid buffers (0.025 M) at pH 3.2 (unionized) and 5.2 (ionized). Values represent means ± SD (n = 3–4). Saline was the control solution.

more cellular aberrations than the buffer at pH 5.2 (% unionized: 9.1). The results clearly indicate that the presence of unionized species in the buffer causes greater nasal irritation and damage than the ionized species.

The amount of 5′-ND released with time for both the solutions was quite similar (results not shown). While the unionized species of benzoate may partition into the cell cytoplasm and cause release of 5′-ND, the ionized benzoic acid molecules probably cause cell membrane disruption resulting in the release of 5′-ND. Since the unionized species partition into the cell cytoplasm, they lead to greater release of protein (Fig. 5) than the ionized species of benzoic acid. Results with tartarate ($pKa = 3.6$) buffers at pH 2.6 and 4.6 are consistent with these observations. Huang et al. (1985) determined the extent of nasal absorption of benzoic acid as a function of pH of the perfusate. The results indicate that the extent of absorption is pH dependent. The absorption of benzoic acid is higher at a pH lower than its pK_a and absorption decreases with an increase in pH of the perfusion medium. Two events could be occurring here; first, due to the ionization of the molecule, absorption could be decreasing with increasing pH and second, it is possible that the increased damage to the nasal epithelium at a pH below its pK_a leads to increased absorption of benzoic acid. A combination of these two factors may be responsible for the increased absorption of benzoic acid reported by Huang et al. (1985).

In summary, the buffer type, species, and concentration are important parameters not only from the point of view of drug stability and absorption but also from the standpoint of mucosal damage and long-term irritation potential.

3.3. Effect of osmolarity on the rat nasal epithelium

Sodium chloride solutions with osmolarity ranging from 0 to 600 mOsm/kg H_2O were perfused through the rat nasal passage to study the effect of osmolarity on the nasal epithelium. Due to the varying osmolarities of the solutions, the perfusion volume changed during perfusion because of water uptake by (or release from) the rat nasal cavity depending on the osmolarity of the solution. Since it is rather difficult to determine the volume of water absorbed at a given point during the perfusion experiment, the volume change correction is not possible at each time point. Therefore, only data from the end points were taken into account to determine the effect of osmolarity on the nasal mucosa.

Fig. 6 exhibits the amount of LDH release at the end of 105 min perfusion as a function of the osmolarity of the solution. The results show that pure water of zero osmolarity causes the most mucosal marker leakage of all the solutions. Isotonic and hypertonic solutions demonstrate very

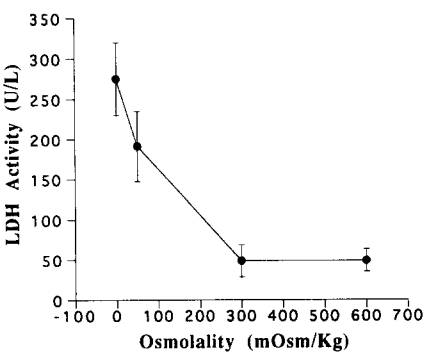

Fig. 6. Release profiles of lactate dehydrogenase from the rat nasal cavity (at the end of 105 min perfusion) as a function of formulation osmolarity. Values represent means ± SD (n = 3–4).

little effect on the LDH release from the nasal epithelium. The reason for the high amount of LDH release with solutions of low osmolarity could be due to the fact that these solutions cause leaching of LDH from the cells due to swelling of the epithelial cells as a result of increased water uptake. On the other hand, hypertonic solutions cause shrinkage of the cells (Ohwaki et al., 1987), therefore the chances of protein or enzyme marker release are quite small leading to the conclusion that isotonic and hypertonic solutions are less damaging than hypotonic solutions.

The amount of protein (mean: 0.585 mg/ml) and 5'-ND (mean: 5.82 U/l) released with solutions of varying osmolarities was similar. Van de Donk et al. (1980) have studied the effect of osmolarity on the nasal ciliary beat frequency thus, nasal mucociliary clearance. Their results indicate that the ciliary beat is best preserved in an isotonic NaCl solution.

In summary, hypertonic and isotonic solutions seem to produce minimal mucosal cell aberrations while hypotonic solutions cause extensive release of LDH from the nasal mucosa.

4. Conclusion

The results from these studies suggest that buffers within a pH range of 3–10 are best suited for nasal formulations. The concentration and type of buffer are very important parameters including the buffer capacity of the buffer. Choosing the lowest acceptable concentration of the buffer will cause the least intolerance. The state of ionization of a buffer species seems to be related with the damage, therefore, acidic buffers that are ionized are recommended. The optimal osmolarity of the nasal formulation appears to be > 300 mOsm/kg while buffers with an osmolarity > 50 mOsm/kg may be acceptable.

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