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# In vivo and in vitro characterization of novel microparticulates based on hyaluronan and chitosan hydroglutamate

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**ABSTRACT** This study examined the application of previously characterized microparticles composed of hyaluronan (HA) and chitosan hydroglutamate (CH) as well as novel microparticles consisting of both polymers (HA/CH) to improve the nasal delivery of a model drug. The rabbit bioavailabilities of gentamicin incorporated in HA, CH, and HA/CH microparticles were increased 23-, 31-, and 42-fold, respectively, compared with the control intranasal solution of gentamicin, indicating that all test microparticles were retained for longer periods on the nasal mucosa of the rabbits as supported by previous in vitro dissolution as well as frog palate mucoadhesion studies, thereby improving drug absorption. The higher bioavailabilities of CH-based formulations (CH and HA/CH) suggest the penetration-enhancing effects of CH may also be partially responsible for the improvement. A model was developed, based on a glass impinger device, to deliver dry powder formulations reproducibly onto the surface of cultured cell monolayers. In vitro permeability and fluorescence microscopy studies on the tight junctions of the 16HBE14o- cell lines further confirmed the ability of CH-based formulations to enhance penetration. Furthermore, the in vitro absorption profile from cell culture studies was consistent with those determined from in vivo studies. The complementary effect from the mucoadhesive nature of HA coupled with the penetration-enhancing effects of CH makes the novel HA/CH formulation a promising nasal delivery system.

**KEYWORDS:** Nasal delivery, microparticles, hyaluronan, chitosan

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## INTRODUCTION

The bioadhesive properties of a wide range of materials have been evaluated over the last decade, and synthetic polymers such as carbopol and polycarbophil display excellent adhesion when tested in vitro [1,2]. However, in vivo, such performance cannot be replicated [3], which explains why relatively few bioadhesive delivery systems have become commercially available [4,5]. Furthermore, as with any formulation excipient, bioadhesives have the potential of inducing biological toxicity [6].

The mucoadhesive properties of naturally occurring polymers such as hyaluronan (HA) have previously been investigated by Pritchard et al [7] for various grades of esterified and nonesterified HA using in vitro weight detachment studies and frog palate studies. The authors concluded that nonesterified HA had superior mucoadhesive properties compared with its esterified counterparts. Another naturally occurring polymer that has been of much interest over the last decade is chitosan, owing to its good biocompatibility, nontoxicity, and biodegradability. In addition to its mucoadhesive properties, chitosan has been shown to enhance drug absorption through tight junctions via the paracellular route [8].

Hence, the aim of this study was to examine the in vivo and in vitro properties of novel microparticles composed of unesterified HA and a combination of HA and CH (chitosan hydroglutamate). CH microparticles alone were also included in the study. This study also aims to develop and characterize a simple but reproducible system to aerosolize dry powdered microparticulate formulations onto the surface of Transwell inserts to examine their toxicity and effects on epithelial permeability and tight junctions, to determine the transport of entrapped drug, and to compare the results with those found in vivo.

## MATERIALS AND METHODS

### Materials

Sodium hyaluronate (KZ 60254, molecular weight 8.5 × $10^5$ Da), was supplied by Kiwahako (Osaka, Japan). CH (Protasan G210, molecular weight 1.8–2.3 × $10^5$ Da) was supplied by Pronova (Oslo, Norway). Minutesimal essential medium, fetal bovine serum (FBS), trypsin (0.25% wt/vol), EDTA (0.22% wt/vol) solution, MTT [(3-4,5-dimethylthiazol-2-yl)]-2,5-diphenyl tetrazolium bromide (Thiazoyl blue), Hank's balanced salt solution (HBSS), rabbit anti-rat IgG labeled with TRITC (Tetramethylrhodamine-5(-6-)isothiocyanate, Triton X-100, rabbit serum, ammonium acetate, and paraformaldehyde were obtained from Sigma-Aldrich Co Ltd (Dorset, UK). Rat anti–Zonula occludens-1 (ZO-1) antibody (Mab1520) was obtained from Chemicon International Ltd (Hertfordshire, UK). Vitrogen was obtained from Celtrix (Palo Alto, CA). Gentamicin sulfate was obtained from Fluka (Dorset, UK). 16HBE14o- cells were kindly donated by Deiter Gruenert at the Cardiovascular Research Institute (University of California, San Francisco).

### Preparation of microparticles

Microparticles were prepared by a water/oil emulsification solvent evaporation technique, as described previously [9]. Novel microparticles of HA (positive control) and HA/CH were prepared along with CH.

### In vivo studies

Animal studies were carried out in accordance and approved by the Animals (Scientific Procedures) Act of 1986. Thirty-two New Zealand white rabbits with a mean weight of 2.7 ± 0.17 kg were fasted overnight and weighed before administration of the formulation. A catheter was inserted into the left marginal ear vein for blood sampling, and microparticle formulations, equivalent to approximately 6 mg of gentamicin, were administered to the rabbits with one half of the dose (approximately 3 mg) being introduced to each nostril, the exact amount being adjusted according to the weight of the animals. The total weight of microparticle formulation administered as powder per nostril ranged from 5 mg to 15 mg. Placebo animals underwent the same procedures but were not given any gentamicin.

Blood samples (0.5 mL) were collected, at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, and 360 minutes after drug administration. The total blood volume withdrawn during the study was 6 mL for each rabbit, and serum was separated by centrifugation at 13 000 rpm for 5 minutes, then collected and stored at -20°C until assayed by using a fluorescence polarization immunoassay as described previously [9].

Calculations of the area under the curve to time t ($AUC_t$) and to infinity ($AUC_8$) were calculated using the linear trapezoidal rule. The intranasal bioavailabilities (*F*) were calculated relative to the intravenous (IV) administration where IV bioavailability was considered 100%, as shown in equation 1.

$$\%F = \frac{[AUC_{IN}]\,[Dose\ (mg)]_{IV}}{[AUC_{IV}]\,[Dose\ (mg)]_{IN}} \times 100 \quad (1)$$

IN represents intranasal administration. The maximum plasma concentration ($C_{max}$) and the time to achieve maximum concentration ($t_{max}$) were also obtained from each serum concentration curve and statistical analysis on the differences among $AUC_t$, $AUC_8$, $C_{max}$, and $T_{max}$ were performed using analysis of variance.

### In vitro deposition apparatus

A glass adapted twin impinger (ATI) device was developed for this study based on apparatus A of the British Pharmacopoeia (BP) [10] (Figure 1). It was employed with a view to deliver dry powder formulations to the epithelial cell-bearing surface of a 1 $cm^2$ Transwell diffusion chamber. The dimensions of the neck (C) and upper impingement chamber (D) of the twin impinger apparatus were modified to accommodate the attachment of the Transwell diffusion chamber (I), as shown in Figure 1.

### Culture of cells

16HBE14o- cells (passage 60–89) were grown as a monolayer in MEM (minimum essential medium) supplemented with 10% vol/vol FBS. The cells were subcultured once weekly by gentle trypsinization with 0.22% EDTA/0.25% trypsin and maintained at 37°C in a humidified atmosphere of 95% air:5% $CO_2$. For transport experiments, the cells were seeded at a density of 4.5 ´ $10^5$ cells/$cm^2$ onto Vitrogen-precoated polycarbonate filters (0.4 $\mu$m pore size, 1 $cm^2$ growth area) inside Transwell cell culture chambers, grown at an air:liquid interface after 48 hours and used experimentally between 6 and 8 days.

a) b)

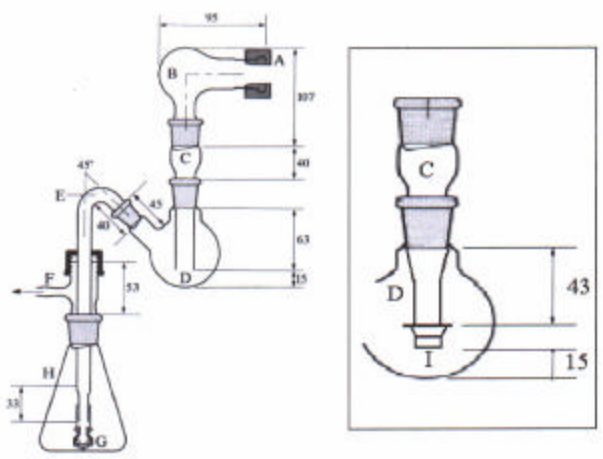


**Figure 1. Schematic of the apparatus showing the a) standard glass adapted twin impinger (ATI) and b) a closeup of the ATI showing the modified dimensions (mm) and position of the Transwell insert. A. Mouthpiece adapter. B. Throat (round-bottomed flask). C. Neck (modified glass adapter). D. Upper impingement chamber (modified round-bottomed flask). E. Coupling tube. F. Screwthread side arm adapter. G. Lower jet assembly. H. Lower impingement chamber. I. Transwell insert. Modified from British Pharmacopoeia Commission. Apparatus A (glass impinger). Appendix XIIF. London: British Pharmacopoeia, The Stationary Office London. 1999;A209–A210.**

## *Deposition experiments*

Approximately 6.5 ± 0.5 mg of gentamicin-loaded microparticles of HA, HA/CH, CH, or physical mixtures (1:1) of gentamicin/lactose powder were loaded into size 3 hard gelatin capsules and aerosolized by initially drawing air through the apparatus (vacuum pump) for 2 seconds, followed by rapid breakage of the capsules in a Rotahaler at a flow rate of 60 ± 5 L/min for a further 5 seconds. The percentage deposition delivered was determined by weighing the Transwell inserts as shown in equation 2; the distribution of the particles over the surface was assessed by optical microscopy at magnification ×10.

$$\% \text{ Deposition} = ((I–W)/W) \times 100 \quad (2)$$

I is the weight of the Transwell insert after deposition, and W is the initial weight of the Transwell insert.

The ability of the cells to withstand the forces (from vacuum and impact of microparticulates) during and after powder deposition was assessed by monitoring cellular metabolism and monolayer permeability as indicated by the transepithelial electrical resistance (TEER).

## *Transepithelial electrical resistance study*

The pre-experimental TEER was measured at intervals of 10, 20, 45, 60, 90, 120, 180, and 240 minutes. At 120 minutes, the apical and basolateral chambers were washed 3 times with HBSS to remove the formulation from the cell surface. TEER was measured after applying 1% water/vol polymer solutions of HA, HA/CH, CH, gentamicin solution, and aerosolization of gentamicin-loaded and empty microparticles (approximately 2 mg to 2.7 mg) of HA, CH, and HA/CH onto the cell monolayers with the inclusion of a simulated deposition (with empty capsule) and a control.

## *Effects of HA and CH on tight junctions (fluorescence microscopy)*

To observe the effects of the polymers on the tight junctions of the cell monolayer, 16HBE14o- cells were seeded ($4.5 \times 10^5$ cells/cm$^2$) onto sterile 6-mm glass cover slips placed in cluster wells and were allowed to grow to confluence. The cells were treated with solutions of HA (1% water/vol) and CH (2% water/vol) for either 30 or 60 minutes and washed with phosphate buffered saline (PBS). The cells were then fixed with 1 mL of 4% paraformaldehyde for 10 minutes and permeabilized by incubating them with 1 mL of 0.2% Triton X-100 in PBS at 37°C for 3 minutes; the aldehyde group autofluorescence was quenched by the addition of 0.5 mL 50 mM ammonium acetate. The cells were then washed 3 times with PBS, incubated for 1 hour with 1 mL of 10% rabbit serum to block nonspecific binding, washed again with PBS, and incubated for an additional hour with a 1:50 dilution of a 1 mL rat anti–ZO-1 antibody (Mab1520). After rinsing with PBS, the cells were incubated with a 1:100 dilution in PBS of 50 $\mu$L rabbit anti-rat IgG labeled with TRITC. The cover slips were removed from the cluster wells and mounted on a microscope slide and observed under a fluorescence microscope at a wavelength of 555 nm.

## *Toxicity testing (MTT assay)*

Microparticle formulations were deposited onto the apical cell surface using the ATI and transferred to a cluster well and incubated at an air-liquid interface. After 30 minutes, MTT (tetrazolium salt) (2 mg/mL)

in HBSS was added and incubated at 37°C for 1 hour. The cells were washed twice with HBSS, and acidified isopropyl alcohol (1% vol/vol concentrated sulfuric acid in isopropyl alcohol) was added. The filters of the Transwell inserts were pierced to mix the solution of the apical and basolateral chamber, and the inserts were incubated in a shaking incubator for 30 minutes at 37°C. Absorbance was measured at 570 nm and corrected for background absorbance at 630 nm. The MTT was performed on cells exposed to a simulated deposition procedure (using an empty capsule) and on cells that were washed and incubated with a transport buffer (HBSS) but not exposed to the deposition process or formulations (control). To test the effect of the formulations, the MTT assay was performed 1) after incubation with CH (1% wt/vol) and HA (1% wt/vol) solutions in the apical chamber and 2) after deposition of HA, HA/CH, and CH microparticles. A solution of 0.6 mM sodium dodecyl sulfate (1 mL) was also added as a control. The MTT assay was subsequently performed on completion of all absorption studies.

### Gentamicin transport

The permeability characteristics of gentamicin across the cell monolayer were determined by measuring both apical to basolateral and basolateral to apical transport. The appearance of gentamicin in the receptor (apical or basolateral) chamber was measured over 180 minutes. The apparent permeability ($P_{app}$) of gentamicin applied as a solution was calculated and compared in the apical to basolateral and basolateral to apical directions (equation 3).

$$P_{app} = (dQ/dt)/(C_o \times A) \quad (3)$$

In this equation, $dQ/dt$ is the transport rate of gentamicin, Co is the concentration of gentamicin in the donor chamber, and $A$ is the area available for transport.

### Absorption of gentamicin from microparticle formulations

The absorption profiles of the gentamicin following the deposition of the gentamicin-encapsulated microparticles of HA, HA/CH, and CH were studied after delivery of the microparticles to the epithelial surface of the cells using the ATI. After particle deposition had occurred, the Transwell insert was removed immediately and replaced into its respective cluster well. Samples (200 $\mu$L) were removed from the chamber at 15, 30, 45, 60, 90, 120, 180, 240, 360, 420, and 500 minutes and replaced with the same volume of warmed (37°C) transport buffer.

## RESULTS

### In vivo studies

The mean serum concentration curve of gentamicin administered IV, intramuscularly, and IN is shown in Figure 2. The dry powder formulation of gentamicin-lactose mixture produced only low levels of serum gentamicin (0.35 ± 0.1 $\mu$g/mL) (Figure 2). Figure 3 shows that the HA formulation gave rise to a significantly higher ($p < .05$) mean peak serum gentamicin level (0.61 ± 0.12 $\mu$g/mL) at 60 minutes, which decreased below the limit of detection after 6 hours. The CH-based formulations, both CH and HA/CH microparticles, gave much higher maximum mean serum gentamicin concentration at 30 minutes (1.53 ± 0.35 $\mu$g/mL) and 60 minutes (1.29 ± 0.34 $\mu$g/mL) respectively (Figure 3), compared with the gentamicin/lactose mixture.

The $AUC_8$, $AUC_t$, F, $C_{max}$, and $T_{max}$ are shown in Table 1. The IN administration of gentamicin as a gentamicin-lactose dry powder formulation exhibited a 2-fold increase in bioavailability compared with a simple solution of gentamicin. Gentamicin formulated in HA microparticles further increased the bioavailability by approximately 11-fold in comparison to the gentamicin/lactose solution. The bioavailability of gentamicin was approximately 31 and 42 times greater for CH and HA/CH microparticles, respectively, in comparison to that obtained after IN administration of gentamicin as a solution.

### In vitro studies

Observation of the deposition of formulations using the ATI device on the Transwell inserts under optical microscopy at magnification ´10 for all microparticle formulations showed a distribution across the cells that was considered broadly uniform (Figures 4A-4C, Table 2). Between 31% and 40% of the initial capsule content of powder was reproducibly deposited in the Transwell insert (coefficient of variation of less than 3.1%) for each individual formulation. No significant difference ($p > .05$) was observed in percentage deposition (by weight) for repeated depositions of the same formulation (n = 6) for all the formulations tested.

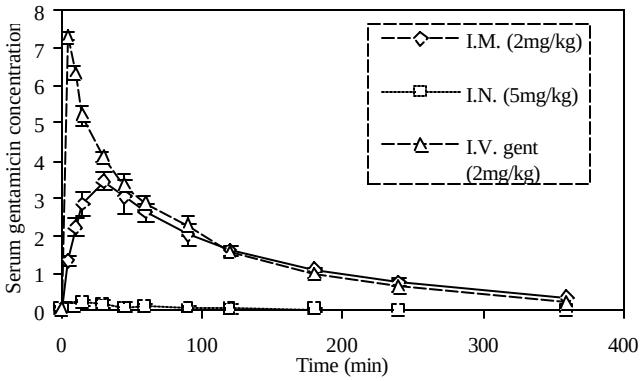


**Figure 2. Mean serum concentrations of gentamicin (µg/mL) in rabbits after intravenous (IV), intramuscular (IM), and intranasal (IN) administration of solutions, mean ± SD (n = 4).**

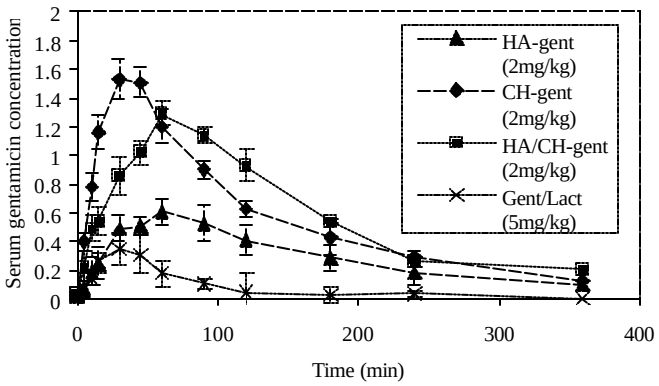


**Figure 3. Mean serum concentrations of gentamicin (µg/mL) from microparticle formulations administered intranasally, mean ± SD (n = 4).**

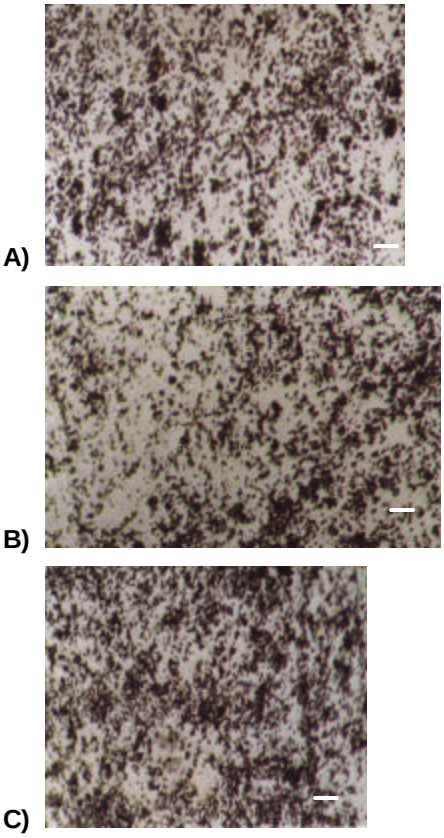


**Figure 4. Optical micrograph showing the distribution of microparticles. A. Hyaluronan (HA) microparticles. B. Chitosan hydroglutamate (CH) microparticles. C. HA/CH microparticles on a Transwell insert after aerosolization from the adapted twin impinger (ATI) (magnification ×10). Micrometer bar represents 100 µm.**

**Table 1. Area under the curve from time 0 to 360 minutes and to infinity ($AUC_8$)**

| Formulation | $C_{max}$ (µg/mL) | $T_{max}$ (min) | $AUC_{t=360}$ (µg/min/mL) | $AUC_{\infty}$ (µg/min/mL) | F (%) |
|---|---|---|---|---|---|
| IV | 7.4 ± 0.7 | 5 | 560.4 ± 20.7 | 617.1 ± 21.5 | 100 ± 4.1 |
| IM | 3.45 ± 0.4 | 30 | 484.4 ± 26.9 | 614.7 ± 24.3 | 99.6 ± 4.0 |
| IN gent/soln | 0.21 ± 0.1 | 15 | 11.1 ± 6.4 | 17.31 ± 5.9 | 1.1 ± 0.6 |
| HA | 0.61 ± 0.1 | 60 | 102.6 ± 2.6 | 143.56 ± 2.1 | 23.3 ± 1.3 |
| CH | 1.53 ± 0.3 | 30 | 193.6 ± 10.1 | 205.7 ± 12.7 | 31.4 ± 2.7 |
| HA/CH | 1.29 ± 0.3 | 60 | 203.9 ± 10.3 | 264.58 ± 9.9 | 42.9 ± 3.5 |
| Gent/lact | 0.35 ± 0.1 | 30 | 22.0 ± 5.2 | 31.98 ± 5.8 | 2.1 ± 0.6 |
| Placebo | - | - | - | - | - |

**$AUC_t$ indicates area under the curve from time 0 to 360 minutes ($AUC_t$); $AUC_{\infty}$, area under the curve to infinity; F, bioavailability; $C_{max}$, maximum serum concentration; $T_{max}$, time at which maximum serum concentration is achieved following administration of gentamicin microparticulate formulations in rabbits, mean ± SD (n = 4); Gent/lact, gentamicin/lactose; IN gent/soln, intranasal administration of gentamicin solution; HA, hyaluronan; CH, chitosan hydroglutamate.**

**Table 2. The effect of initial capsule content (mg) on the percentage deposition from the glass adapted twin impinger (ATI) device**

| Formulation | Amount Deposited on Filter (mg) | Capsule Content (mg) | Percentage Deposition |
|---|---|---|---|
| **HA** | **2.93** | **9.51** | **30.81** |
| **HA** | **3.04** | **9.59** | **31.71** |
| **HA** | **3.11** | **9.62** | **32.36** |
| | | | |
| **HA** | **1.06** | **3.47** | **30.76** |
| **HA** | **1.02** | **3.46** | **29.56** |
| **HA** | **1.13** | **3.59** | **31.41** |
| | | | |
| **CH** | **3.19** | **9.01** | **35.44** |
| **CH** | **3.17** | **9.03** | **35.16** |
| **CH** | **3.06** | **9.18** | **33.33** |
| | | | |
| **CH** | **1.32** | **3.99** | **33.22** |
| **CH** | **1.02** | **3.07** | **33.11** |
| **CH** | **1.36** | **3.98** | **34.11** |
| | | | |
| **HA/CH** | **3.16** | **9.05** | **34.87** |
| **HA/CH** | **3.29** | **9.07** | **36.24** |
| **HA/CH** | **3.08** | **9.00** | **34.26** |
| | | | |
| **HA/CH** | **1.04** | **3.00** | **34.67** |
| **HA/CH** | **1.07** | **3.11** | **34.53** |
| **HA/CH** | **1.43** | **3.98** | **35.95** |

**HA indicates hyaluronan; CH, chitosan hydroglutamate.**

The cell layers used in these studies had a TEER of 300 ± 6.7 ohm.cm$^2$cm$^2$ (n = 6). Figure 5A shows the effect of the various formulations on the TEER of 16HBE14o- cells. No significant difference ($p > .05$) in TEER was observed for the controls, cell layers subjected to a simulated deposition, or cell layers to which gentamicin solution had been applied over a 240-minute incubation. In general, the TEERs of the deposition's cell layers of all microparticle formulations and polymer solutions were observed to fall after 45 minutes. Of the 3 microparticle formulations, gentamicin-loaded and empty HA/CH and CH were seen to significantly reduce the TEER of the cell monolayer to almost 50% of the initial TEER after 45 minutes, after which the TEERs started to recover. The TER of the cell layers was restored after the cells were washed with HBSS after 120 minutes (Figures 5B and 5C). Similar results were observed when polymer solutions were added to the apical surface of the cells (Figure 5D).

Immunofluorescent staining of control epithelial layers and layers that were incubated with HA or CH solutions are shown in Figures 6A-6H. Immunofluorescent staining showed clear bands of ZO-1 outlining the perimeter of 16HBE14o- cells in control layers at 30 and 60 minutes (Figures 6A and 6B). Immunofluorescent staining of cell layers 30 minutes after application of a 1% wt/vol HA solution did not show any marked alterations in the ZO-1 staining, although a slight dilation of the junctions between the neighboring cells was observed after 60 minutes (Figures 6C and 6D). However, a 2% wt/vol solution of CH after 30 and 60 minutes showed a clear dilation of the junctions between neighboring epithelial cells and a redistribution of ZO-1 across the cell layer (Figures 6E and 6F). Upon removing both polymers (CH and HA) after washing with HBSS, the original distinct bands of ZO-1 staining around the perimeter of the cells was once again apparent (Figures 6G and 6H, respectively).

Results of the MTT test (Table 3) showed that there was no significant difference ($p > .05$) in absorbance readings between the metabolism profile of cell layers subjected to a simulated deposition of microparticles and control. Furthermore, no significant difference ($p > .05$) in absorbance was found between the control layers and after simulated deposition in comparison to the layers onto which the 3 microparticle test formulations had been deposited. Furthermore, upon completion of absorption studies (500 minutes), no signs of reduction in metabolism were observed and TER studies showed no significant difference ($p > .05$) to controls (data not shown). The application of SDS (sodium dodecyl sulphate) (0.6 mM) to the cell layers demonstrated a significant difference ($p < .05$) in absorbance after application of MTT when compared to the control cell layers and after application of the other formulations (Table 3).

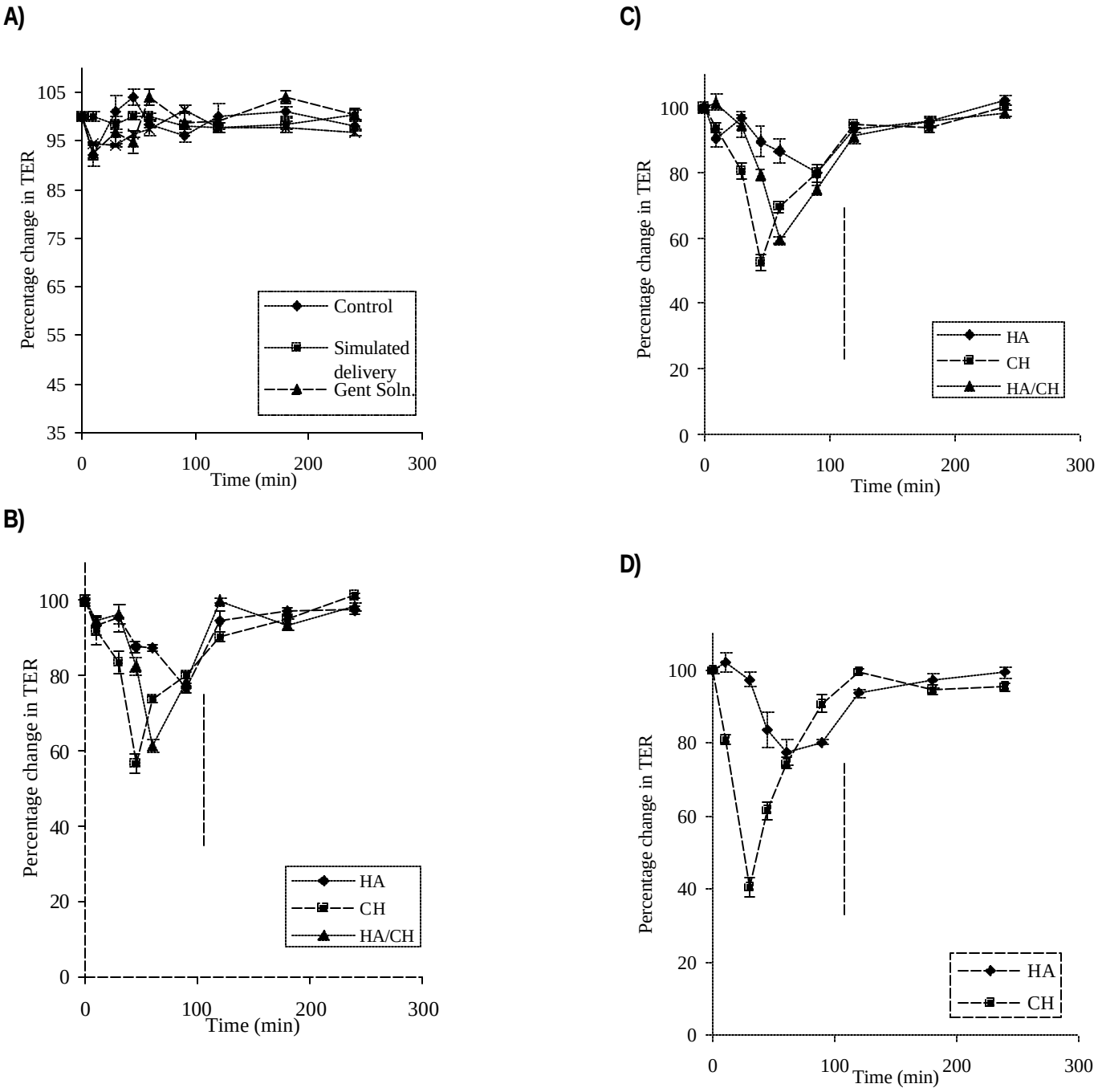


**Figure 5. Effect of formulations on the percentage change in TER over time, mean ± SD (n = 4). A. Cells treated with control, simulated delivery, and gentamicin solution. B. Cells treated with empty microparticles. C. Cells treated with gentamicin-loaded microparticles. D. Cells treated with polymer solutions. Arrows show time at which cells were washed with phosphate buffered saline.**

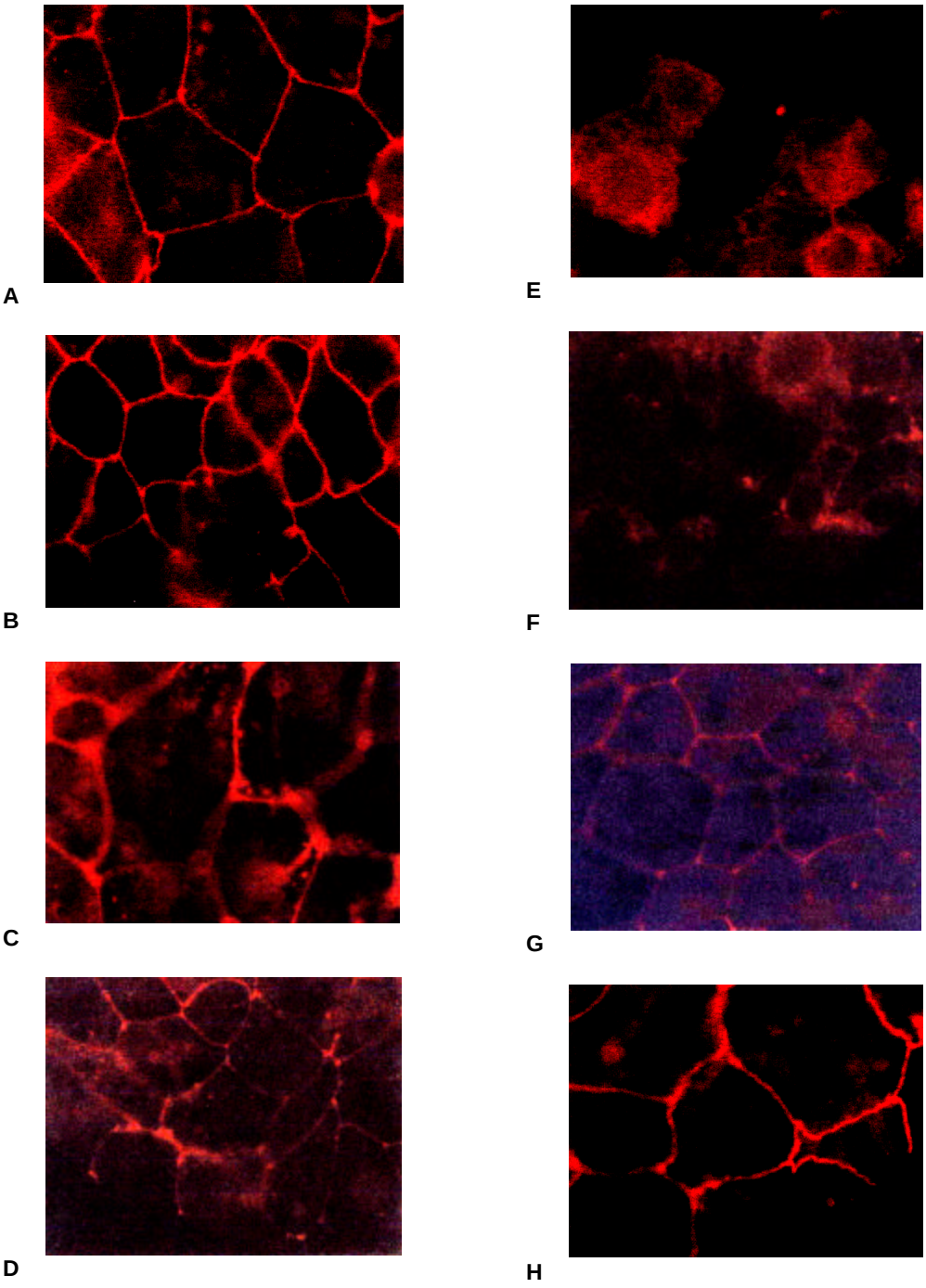


**Figure 6. Typical immunofluorescent images of the apical side of 16HBE14o- monolayers. A, B. Control cells after 30 and 60 minutes, respectively. C, D. Cells treated with HA 1.0% wt/vol after 30 and 60 minutes, respectively. E, F. Cells treated with CH 2.0% wt/vol after 30 and 60 minutes, respectively. G and H. Cells 30 minutes (after washing) after treatment with (hyaluronan) HA 1% wt/vol and chitosan hydroglutamate (CH) 2% wt/vol, respectively.**

**Table 3. MTT test data showing the absorbance at 570 nm for empty (E), gentamicin-loaded (G) microparticle formulations after 30 minutes and 30 minutes after completion of TER studies (270 minutes) and controls**

| Formulation | Average Absorbance at 570 nm Corrected for Background at 630 nm; Mean ± SD (n = 4); Exposure t = 30 Minutes | Average Absorbance at 570 nm Corrected for Background at 630 nm; Mean ± SD (n = 4); Exposure t = 270 Minutes |
|---|---|---|
| Control | 0.409 ± 0.017 | 0.412 ± 0.021 |
| Simulated Deposition | 0.411 ± 0.011 | 0.402 ± 0.031 |
| Lactose/ Gentamicin | 0.389 ± 0.041 | 0.401 ± 0.013 |
| Gentamicin Solution | 0.396 ± 0.024 | 0.417 ± 0.011 |
| HA (E)<br>HA (G) | 0.398 ± 0.053<br>0.377 ± 0.032 | 0.369 ± 0.43<br>0.398 ± 0.12 |
| CH (E)<br>CH (G) | 0.400 ± 0.030<br>0.409 ± 0.007 | 0.385 ± 0.33<br>0.413 ± 0.09 |
| HA/CH (E)<br>HA/CH (G) | 0.378 ± 0.027<br>0.371 ± 0.032 | 0.403 ± 0.29<br>0.412 ± 0.34 |
| Sodium Dodecyl Sulfate (0.6 mM) | 0.156 ± 0.098 | 0.149 ± 0.12 |

**HA indicates hyaluronan; CH, chitosan hydroglutamate.**

The apparent permeability of gentamicin across the cell layers was similar in the apical to basolateral and basolateral to apical direction at $6.7 \times 10^{-7}$ cm $s^{-1}$ (n = 8) (Figure 7). The influence of 3 types of microparticle formulations on gentamicin permeability across the cell monolayer is shown in Figure 8. The time taken for 50% of the applied gentamicin to diffuse across the cell layer ($t_{50}$) was found to be fastest (50 minutes) after application of the CH microparticles, while the $t_{50}$ values for HA and HA/CH were markedly prolonged at 120 and 170 minutes, respectively.

## DISCUSSION

Intranasal administration of gentamicin in solution resulted in very low absorption (bioavailability of approximately 1% compared with the IV dose). Such findings were in agreement with those of another study involving the nasal administration of gentamicin in sheep where less than 1% bioavailability was obtained when gentamicin was administered IN as a solution [11].

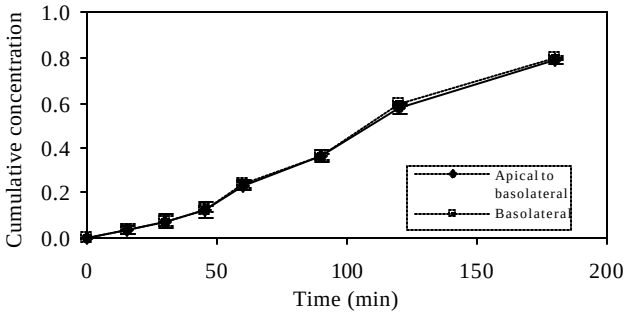


**Figure 7. Cumulative concentration (µg/mL) of gentamicin transport across the apical to basolateral and basolateral to apical chamber, mean ± SD (n = 4).**

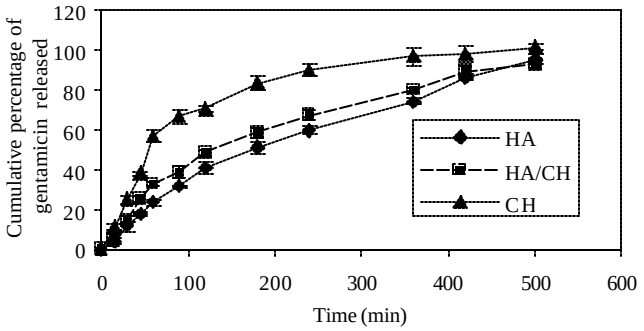


**Figure 8. Comparison between cumulative percentages of gentamicin encapsulated formulations permeated across epithelial layer, showing t50 values mean ± SD (n = 4).**

Morimoto et al [12] demonstrated that the use of HA solution as a vehicle for IN administration increased absorption of vasopressin and a vasopressin analogue. In the present study, HA microparticles as a nasal delivery system for gentamicin resulted in a 2-fold increase in $C_{max}$ of gentamicin and 11-fold increase in bioavailability when compared with an IN gentamicin/lactose mixture. The peak plasma time from such microparticles was delayed until 60 minutes, reflecting the prolonged release of gentamicin, further supported by the $t_{1/2}$ being extended from 3.12 hours to 4.27 hours. In a similar study, HA ester microspheres were investigated for the nasal delivery of insulin in sheep by Illum et al [13]. The mean bioavailability of insulin of HA ester microspheres was found to be 10%, almost half of that achieved by the HA microparticles employed in the current study (23.3%). Such a difference is most likely to be accounted for by the larger molecular weight of insulin compared with that of gentamicin and the greater bioadhesive properties of the nonesterified HA employed in this study [14]. However, direct

comparisons are difficult to make because of the differences in animal and drug models used. The CH containing microparticles used in the current study provided a significant increase ($p > .05$) in bioavailability of IN-administered gentamicin compared with IN-administered gentamicin solution. This finding can be supported by an earlier study in which the nasal application of insulin with chitosan led to a significant reduction in blood glucose levels in sheep and rats [13]. More recently, it was found that chitosan formulations significantly enhanced the bioavailability of goserelin (11.6%, 25.6%, and 36.6% for the chitosan solution, powder, and microsphere formulation, respectively) when compared with that of a simple nasal solution [8].

Such findings may explain the increased bioavailability of gentamicin from a combination of HA and CH formulated as a microparticulate system in which the bioavailability of gentamicin from the HA/CH formulation was found to be significantly greater ($p < .05$) than from either HA or CH microparticulate formulations. These results suggest a synergistic effect between HA and CH in the improvement of bioavailability when gentamicin was formulated in HA/CH microparticles.

Delivery of dry powder onto cell monolayers in most cell culture studies has not been reported in the literature. To reproducibly deposit dry powder formulation onto cell monolayers, an impinger device, originally designed to estimate the respirable fraction of aerosolized drug in the lungs [10], was modified. It was demonstrated that by using such a procedure, it was possible to uniformly and reproducibly deposit dry powder formulations; therefore, this method was subsequently employed to deliver all dry powdered formulations onto cell layers grown on Transwell inserts.

TER in this study was not compromised by the delivery of the gentamicin/lactose physical mixture, suggesting that the ATI apparatus had no effect on the cell integrity. The results of this study also show that both the CH and HA/CH formulations were potent absorption enhancers. The ability of CH to act as a penetration enhancer has been well documented [15–17]. Such properties are believed to be due to an interaction of the positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes, which induces a redistribution in cytoskeletal F-actin, thereby resulting in a structural reorganization of tight junction associated proteins such as ZO-1 [15,16]. The ability of HA/CH formulations to reduce TEER significantly may be due to the presence of CH because HA formulations alone had only a minor effect on the cell integrity. However, the addition of HA to CH in HA/CH microparticulates has demonstrated a prolongation of the dilation of tight junction (Figure 5B). HA might reduce TEER by causing cell shrinkage by hydration, which could withdraw water from the neighboring cells. Such a phenomenon has previously been shown by Edman et al [17], who used starch microspheres.

The TEER results for CH and HA were supported by the results obtained from immunofluorescent staining studies. Again, in agreement with the initial TEER studies, Figures 6A and 6B showed that the protein ZO-1 was present exclusively at the perimeter of the cell membrane. However, upon the introduction of CH after 30 minutes, dilated tight junctions were visible, suggesting a reorganization in ZO-1. A 1.0% wt/wt HA solution did not show any marked difference in the distribution of ZO-1 in comparison to the controls 30 minutes after immunofluorescent staining. However, after 60 minutes, slight dilation of the tight junctions was observed, suggesting a redistribution of ZO-1, possibly resulting from the hydration of HA as discussed earlier. Interestingly, as with TEER upon washing, the integrity of the cell layer returned to normality (Figures 6G and 6H), suggesting the effects are reversible.

This study also employed a biochemical toxicity test, using tetrazolium salt 3-(4,5 dimethyl) thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assays that assess the cells' metabolic competence. The dissolved MTT is converted into an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes [18]. Toxicity measurements on 16HBE14o- cells have been described previously [19]. In the current study there appears to be no cellular toxic effects to the cell layers as assessed by carrying out the deposition procedure using the ATI device in the absence of microparticulates or indeed directly after application of any of the formulations to the cell layers as assessed by MTT reduction. In addition, toxicity testing (MTT) after completion of all the TER and absorption experiments did not result in a significant difference ($p > .05$) between the rate of MTT reduction before initial toxicity testing of the

formulations and controls and after completion of the experiments. Such results indicate that the cells were still viable after the incubation with these polymers and that all the polymers and microparticulates are broadly nontoxic using the above procedure.

The cumulative absorption profiles of gentamicin from the various formulations show that the HA microparticle formulation produced the most prolonged release of gentamicin. The absorption profile from CH microparticles showed that permeability was increased, probably as a consequence of the epithelial penetration-enhancing properties of CH. These absorption profiles relate closely to those obtained in the in vivo studies. Similarly, both the in vivo and in vitro studies show the HA/CH formulation to have a complementary effect as a result of the mucoadhesive and epithelial penetration-enhancing nature of HA and CH, respectively.

## CONCLUSIONS

A previous in vitro study [9] on gentamicin release and mucoadhesion indicated the potential of these microparticulate formulations. The in vivo studies demonstrated that synergistic effects in combining HA and CH to form HA/CH microparticles can be employed intranasally to obtain a high bioavailability and prolonged release of a drug. The study also demonstrated that the aerosolization procedure employing the ATI did not affect the integrity of the cell layer and proved to be acutely nontoxic as well as reproducible. Such results suggests that this model might be appropriate for studying nasal absorption in vitro after presentation of the drug as a powder. The influence of chitosan as an epithelial permeation enhancer was demonstrated with formulations comprising HA/CH and CH gentamicin-loaded microparticles. The current in vitro results compare favorably with the in vivo results and provide an additional insight into the mechanisms of action of the formulation.

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