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Original Research Article

Investigation of betahistine dihydrochloride biocompatibility and nasal permeability *in vitro*

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ABSTRACT

Betahistine dihydrochloride, which is widely prescribed for the treatment of symptoms associated with Meniere's syndrome, is generally administered orally in solid or liquid formulations. There is a strong need of profound investigation of alternative routes of administration of betahistine to overcome difficulties related to oral administration. The aim of this study was to evaluate betahistine cytotoxicity and permeability in vitro and to assess the drug's relevance for incorporation in drug delivery systems for nasal administration. RPMI epithelial model was used to evaluate drug permeability in vitro. The cytotoxicity of betahistine was assessed by MTT test. Chitosan microspheres were used as a betahistine delivery system. RPMI 2650 formed a thick, impermeable cell layer on the apical side of the filter inserts and developed enough TEER values to confirm confluence. According to the obtained results, BET showed high permeability coefficients (Papp values in the range 2.3×10^{-5} to 19×10^{-5}) and could, therefore, be successfully used in nasal drug delivery formulations. Also, BET exhibited a good safety profile regarding nasal epithelium toxicity. A dose-dependent reduction in cell viability was observed. The microspheres as drug delivery systems affected BET permeation profiles due to the presence of chitosan as an absorption enhancer.

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Introduction

Betahistine dihydrochloride (BET) is a histamine-like agent used for the symptomatic treatment of vestibular disorders associated with Meniere's syndrome. Generally, the drug is prescribed in the form of tablets (8, 16 and 24 mg) or a solution (8 mg/dose) for oral administration three to four doses daily. BET is known for its short plasma half-life (3–4 h) which necessitates frequent administration and may lead to noncompliance, especially in elderly patients (Lacour et al., 2007). Moreover, BET acts like histamine agonist on H_2 receptors localized on secretory cells of the gastric mucosa (Della Pepa et al., 2006). Therefore, oral administration by patients suffering gastritis, ulcer, etc. should be avoided. On the other hand, typical of Meniere's syndrome symptoms such as persistent nausea and vomiting, or neurovegetative disorders such as swallowing difficulty and also patient incapability to move are other limitations for the oral administration of BET. These facts could serve as a strong argument for studying alternative routes for BET application in the treatment of Meniere's syndrome.

Nasal delivery with its undeniable advantages and various areas of application is a reasonable alternative as it provides fast systemic drug absorption and rapid onset (Bitter et al., 2011; Jadhav et al., 2007). To reduce the duration and frequency of attacks related to the disease and to achieve favorable prognosis regarding hearing, the rapid onset is of considerable importance. Not exclude the possibility of absorption of BET in the cerebrospinal fluid and achieving rapid onset through the mechanism of direct "nose-to-brain" delivery via the olfactory neurons (Bommer, 2007).

An adhesive system for transdermal administration of BET was developed (Heda et al., 2010) as an approach to overcome the problems related to oral administration and the potential for detailed studies on its transdermal absorption was uncovered. Transdermal delivery of BET encapsulated in microemulsion formulations was studied as a promising and reliable alternative

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(Hathout and Nasr, 2013). Literature does not reveal any data about investigations on other alternative routes of administration of BET, including intranasal. It was thus advantageous to evaluate biocompatibility and nasal permeability of BET.

In vitro cell culture models have gained significant importance in biocompatibility and drug transport studies since they allow a rapid analysis of a large number of substances at a reasonable price and also suggest a possibility for the simultaneous study of different variables avoiding the controversial usage of animal models (Dimova et al., 2005; Lin et al., 2007, 2005). RPMI 2650 cell line is the only commercially available nasal human cell line. It was derived from pleural effusion of 52-year old male with nasal septum carcinoma (Moorhead, 1965). RPMI 2650 epithelial model has exhibited features such as high stability throughout culturing, production of mucoid material on the cell surface and ability to form a tight barrier layer with a transepithelial electric resistance (TEER) close to physiological conditions (Kreft et al., 2015). RPMI 2650 has been widely explored for its suitability as a permeation study model (Harikarnpakdee et al., 2006; Kreft et al., 2015; Kurti et al., 2013; Reichl and Becker, 2012; Wengst and Reichl, 2010).

The ability of the living cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) to formazan product after treatment with xenobiotics is a routine technique applied for the assessment of substances' toxicity (Mosmann, 1983). This test is widely used in many studies to demonstrate the toxic effect of drugs on different cell lines. No data was found in the scientific literature on studies of BET epithelial toxicity using *in vitro* nasal models.

The aim of our research was to study *in vitro* biocompatibility and permeability of BET incorporated in bioadhesive chitosan microspheres as a potential drug delivery system for nasal administration.

Materials and methods

Betahistine dihydrochloride, chitosan (from shrimp shells, low viscosity, degree of deacetylation >75%), sorbitan monooleate 80 (Span 80), petroleum ether, fetal bovine serum (FBS), trypsin, EDTA, dimethyl sulfoxide (DMSO) and penicillin were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). RPMI 2650 cell line and Eagle's Minimum Essential Medium (EMEM) were purchased from ATCC (USA). MTT and streptomycin were obtained from AppliChem (Germany). All other reagents and solvents were provided by Alfa Aesar (Germany).

Preparation of BET loaded microspheres

Microsphere formulations were prepared by single emulsion/solvent evaporation method. Four models labeled M1–M4 were developed by varying drug/polymer concentration and ratio. The formulated microspheres were analyzed for size, shape, drug loading, entrapment efficiency, drug/polymer compatibility and mucoadhesion. The obtained results have been extensively described in our previous research paper (data not present). The evaluated formulations M1–M4 varied in size, drug loading and entrapment efficiency as given in Table 1.

Permeation study

Cell cultivation

In vitro cell culture model was applied using RPMI 2650 cell line derived from human nasal septum carcinoma. Cells were seeded onto matrices at density 2×10^6 cells/mL and cultivated in Eagle's minimal essential medium enriched with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C, 5% CO_2 and high humid conditions. At 80–90% confluence the cells were trypsinized using trypsin – EDTA solution, suspended in EMEM to obtain density 2×10^6 cells/mL and used for the prospective studies.

RPMI 2650 cell culture model

100 μL of the initial RPMI 2650 cell suspension at a density of 2×10^6 cells/mL were cultivated onto Thincert® filter inserts (polyethylene terephthalate membrane, 0.4 μm pore size, 10.34 mm diameter, 33.6 mm^2 surface area, Greiner bio-one, Germany) for 9 days to attain confluence. Cultivation medium was replaced in apical and basolateral chambers of the inserts every two days.

TEER measurement

Measurement of transepithelial electric resistance (TEER) is a routine technique in various *in vitro* and *in vivo* studies. As an indicator for tight junctions' permeability for sodium ions in culture conditions, it is widely applied for rapid and reliable assessment of monolayer integrity (Benson et al., 2013; Lin et al., 2007).

The barrier role of cell monolayer was established by daily measuring of TEER with specially designed equipment. The appliance was completed with a couple of thin (0.8 mm) Ag/AgCl electrodes. It used alternating current in order to overcome the adverse polarizing effects of the constant electric field. In sterile conditions the inserts were allowed to reach room temperature, TEER values were measured and after filtering and analog-to-digital conversion TEER changes were recorded in a two-dimensional array: resistance (Ω) – time (s). The obtained values were expressed relative to the surface area of cell monolayers (Ω cm^2). Drug transport was evaluated after stable, unchanging TEER values were obtained.

Drug transport study

The test was performed after formation of a confluent monolayer confirmed by TEER measurement. Cell cultures were washed with tempered medium prior to each experiment. All transport studies were carried out in HBSS (Hank's balanced saline solution). A certain amount of microspheres, corresponding to 750 μg BET, was added to the apical chamber of the inserts containing 350 μL HBSS. At predetermined time intervals basolateral compartment content (1200 μL) was withdrawn and was replaced with fresh transport medium. BET concentration was analyzed spectrophotometrically using an Ultrospec 3300 pro UV/Visible Spectrophotometer (Biochrom Ltd., Cambridge, UK) at a wavelength of 261 nm with calibration correlation coefficient 0.9999. Also, permeability study was performed for BET not incorporated in polymer particulate delivery system as a standard. For the purpose 350 μL BET solution

Table 1
Composition and physicochemical properties of BET loaded microspheres (*$n = 6$).

Model	Drug concentration (%)	Polymer concentration (%)	Drug/polymer ratio	Drug loading (% ± SD)*	Entrapment efficiency (% ± SD)*	Mean particle size (μm ± SD)*
M1	1	1	1:1	44.78 ± 0.78	69.37 ± 0.91	3.82 ± 0.14
M2	1	1.5	1:1.5	41.55 ± 1.34	93.02 ± 0.98	4.49 ± 0.24
M3	1	2	1:2	33.97 ± 0.89	93.85 ± 2.51	4.52 ± 0.33
M4	2	2	1:1	58.78 ± 1.76	98.27 ± 0.73	7.69 ± 0.33

at concentration 850 μg/mL was added to the apical compartment of the inserts. Similarly, drug transport was studied across cell-free filter inserts.

Tight junction integrity study

To evaluate polymer influence on the tight junction integrity 0, 15, 30, 60, 120, 180 and 240 min after treatment in the permeability test changes in TEER were monitored. As a negative control, untreated filter insert with confluent monolayer was used. The study was performed in HBSS at room temperature.

Permeation coefficient calculation

The apparent permeation coefficients of BET through the cell monolayer (P_{app}) were calculated according to the equation:

$$Papp = \frac{dQ}{dt} \frac{1}{CoA}$$

where *dQ/dt* is the cumulative drug content in the basolateral compartment after a certain time interval (μg/s), A is the surface area (cm^2), C_0 is the initial donor concentration of the substance (μg/mL).

Cell viability test

The ability of cells to reduce the yellow dye MTT to purple formazan product after treatment with a particular substance is a measure of intactness and functionality of mitochondria and thus of substance toxicity (Mosmann, 1983).

RPMI 2650 were seeded in flat-bottomed 96-well plates (Cellstar®, Greiner bio-one, Germany) at a density of 4×10^6 cells/mL and were cultivated in EMEM for 24 h. Then, the cells were treated with BET (0.5, 5 and 50 μg/mL) and incubated for another 24 h. To each well 100 μL of dye solution were added to attain a final concentration of 500 μg/mL MTT and the cells were incubated for 2 h. The formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm using ELISA Sunrise microplate reader (Tecan, Switzerland). The reference wavelength 690 nm was used to correct nonspecific background values. Cell viability was calculated as a percentage of viable cells and averaged from minimum seven determinations, following the equation:

$$Cell\ viability\% = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells(Control)} * 100$$

Statistical analysis

All presented results are expressed as means ± standard deviation (SD). Where appropriate, ANOVA and Student's *t*-test were applied using the SPSS Statistics 11.5 to determine the statistical significance at the significance level alpha = 0.05.

Results

BET loaded microspheres in a previous study Four samples of drug-loaded microspheres (Table 1) were investigated in our previous experiments and certain impact of chitosan concentration on particle size, drug loading, and entrapment efficiency was revealed. The increase of chitosan concentration in the sample caused the formation of microspheres of a larger size probably due to an enhanced frequency of collisions, which resulted in a fusion of semi-formed particles. Moreover, higher polymer concentration in the emulsion droplets led to an improved drug entrapment efficiency. Probably, the higher viscosity of chitosan solution tended to restrict diffusion of the drug in the surroundings and enhanced the drug entrapment efficiency.

BET permeation study

RPMI 2650 cell culture model

RPMI 2650 cells were cultivated onto inserts for 7–8 days until a confluent monolayer was formed. The presence of mononuclear epithelial cells was established and confirmed by optical microscopy (Fig. 1). It was visible that RPMI 2650 cells developed a thick monolayer but development of a multilayer was also possible under continuous cultivation.

TEER measurement

Bioelectric parameters were measured throughout cell cultivation onto Thincert® plates as an indicator for tight junction integrity and thus for the barrier role of the cultivated monolayer. Maximum TEER values ($197 \pm 9\ \Omega\ cm^2$) were measured after 7 day's cultivation (Fig. 2). Thus, drug transport study was carried out 9 days after seeding the cells onto the inserts.

In vitro *permeability through RPMI 2650 cell monolayer*

The results of drug transport study are presented as permeability profiles of BET solution (850 μg/mL), M1, M2, M3 and M4

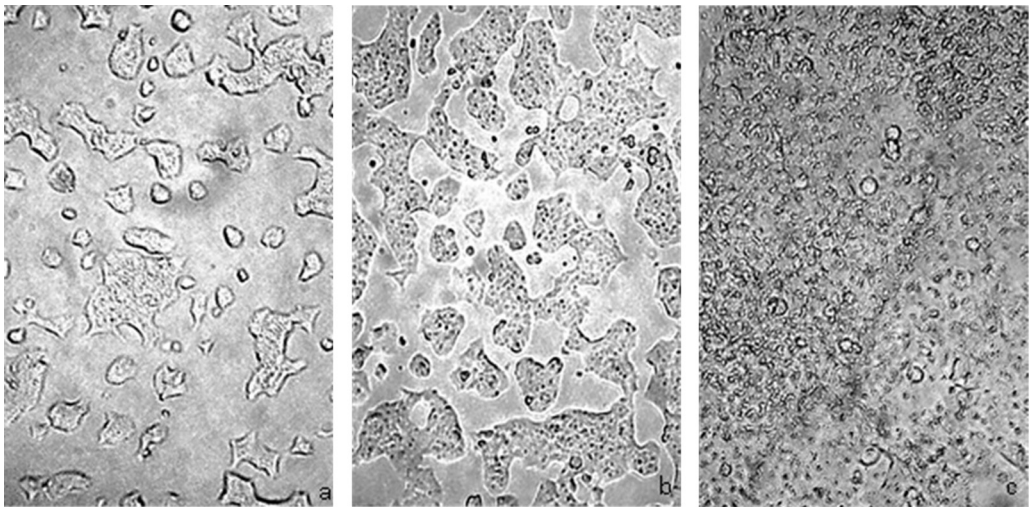

Fig. 1. Photomicrographs of RPMI 2650 cells after one (a), three (b) and nine (c) day's cultivation onto Thincert® filter inserts (×40).

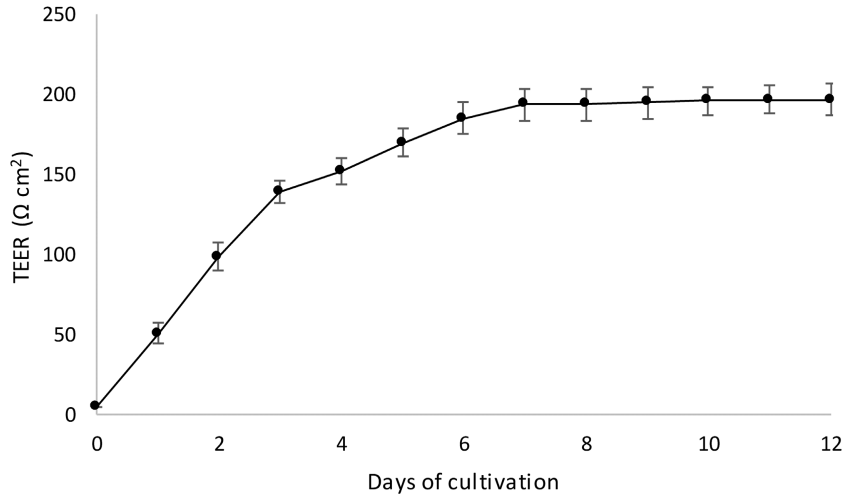

Fig. 2. TEER values measured throughout RPMI 2650 cell cultivation onto Thincert® filter inserts (n = 7, mean ± SD).

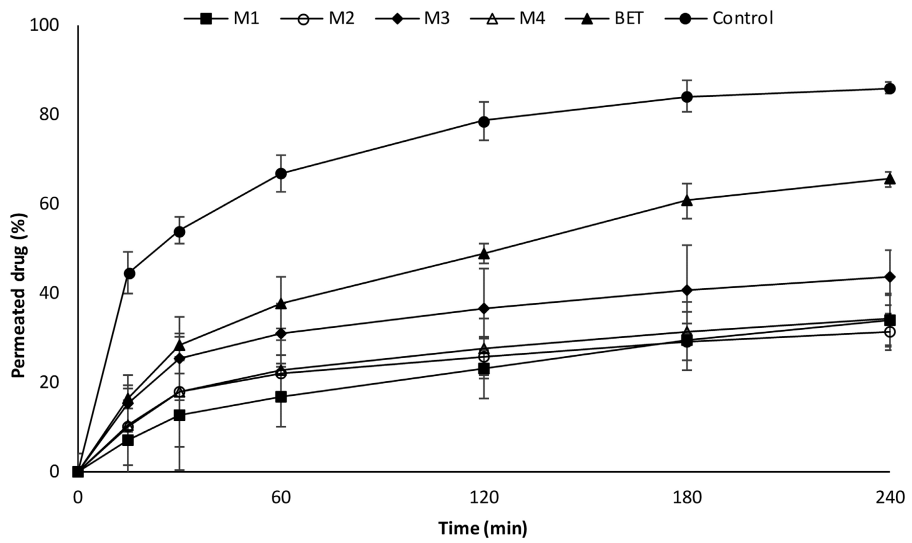

Fig. 3. Permeability profiles of BET delivered by microsphere formulations M1–M4 and BET solution (850 μg/mL) through RPMI 2650 cultivated monolayer onto Thincert® filter inserts. Each data point represents mean value ± SD; n = 7 experiments. A filter insert without cells was used as a control.

microsphere formulations (Fig. 3). According to data, BET permeability was three times lower than the control (cell-free filter inserts) which confirmed the barrier role of the cultivated layer. The average permeated BET was 20% in the first 30 min and slowly increased till the end of the experiment. A considerable similarity between the permeability profiles and *in vitro* dissolution profiles (Fig. 4) of BET obtained in a previous study was found.

Permeability coefficients

Permeability coefficients of BET (P_{app}) were calculated and the results are presented in Fig. 5. P_{app} were of high values in the first 15–30 min ranging from 7.4×10^{-5} to 17.5×10^{-5} and gradually decreased with time. By the end of the four hours' experiment P_{app} lowered to 2.3×10^{-5}.

Impact of microspheres on tight junction integrity

To investigate the influence of the polymer microspheres on the cell monolayer permeability changes in the TEER values throughout drug transport study were monitored. Statistically significant differences in TEER values were found in RPMI 2650 cell model incubated with mucoadhesive chitosan microspheres (64.1–72.3% from the initial TEER) compare to the untreated controls (97.5% from the initial TEER) (Fig. 6). A rapid decrease in TEER (18–32%) in the presence of microspheres was detected which was a signal of their impact on the integrity of tight junctions between cells. Four hours' additional incubation resulted in a further gradual decrease in TEER.

RPMI 2650 cell viability study

After exposure to growing concentrations of BET (0.5, 5 and 50 μg/mL) the percentage of surviving cells decreased. The results from MTT assay are presented in Fig. 7. The observed effect was concentration-dependent. Cell survival ranged from 105 to 80%. The lowest concentration tested – 0.5 μg/mL did not show any cytotoxicity but the percentage of dead cells increased when higher concentrations were applied. The highest concentration used – 50 μg/mL decreased the percentage of cell survival to 80%.

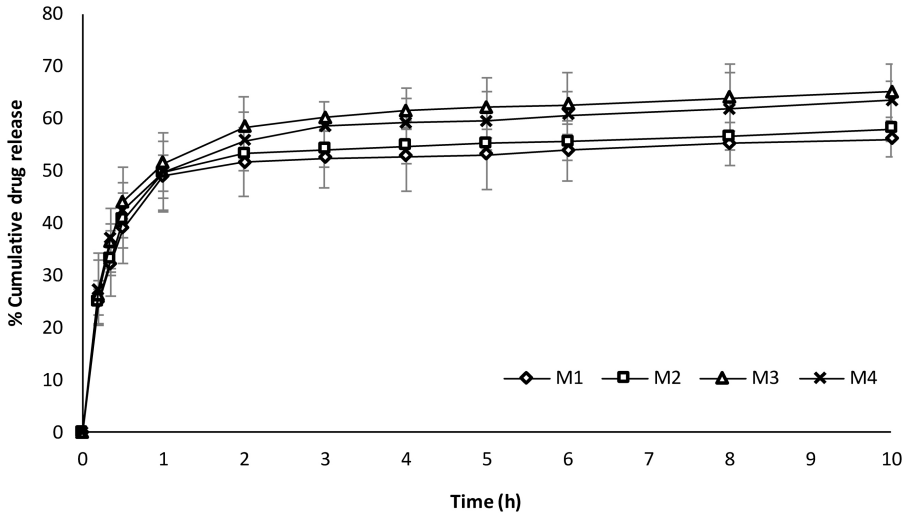

Fig. 4. Dissolution profiles of BET delivered by microsphere formulations M1–M4. Each data point represents the mean value ± SD; n = 7 experiments.

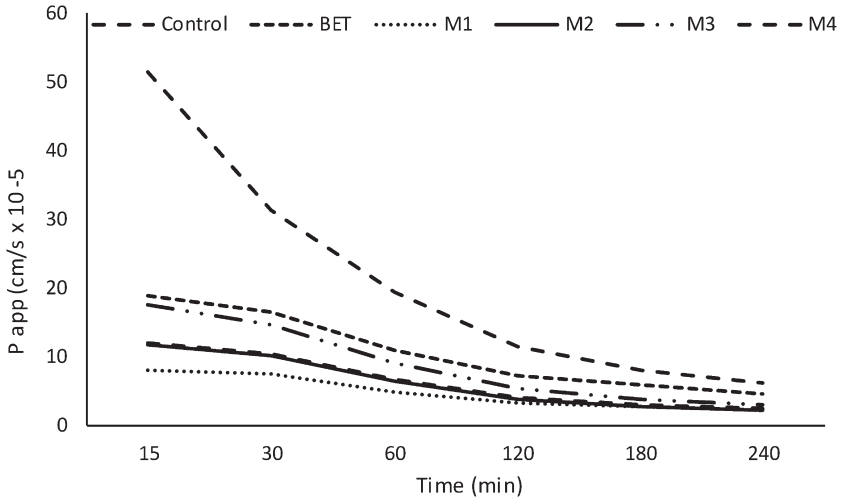

Fig. 5. Changes in permeability coefficients of BET with time during drug transport study through RPMI 2650 monolayer cultivated onto Thincert® filter inserts (n = 7). A filter insert without cells was used as a control.

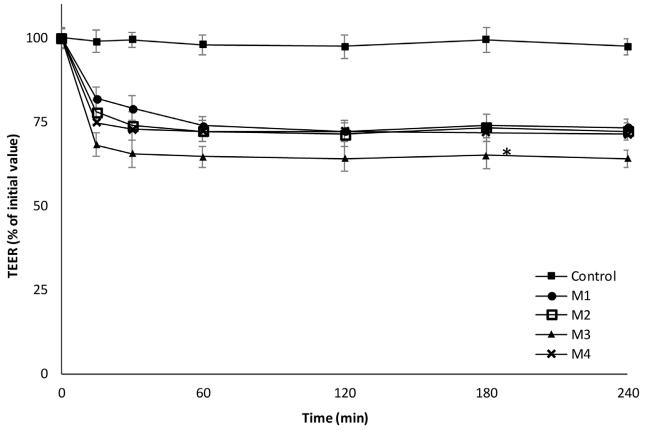

Fig. 6. The impact of chitosan microsphere formulations M1-M4 on TEER values after treatment of RPMI 2650 cultivated cell monolayer (n = 7), *- a statistically significant difference ($p < 0.05$) compare to the other formulation models.

According to the results of MTT test the cells retained their viability after treatment with BET in low and medium concentrations. Appreciable cytotoxicity was found at concentrations higher than 50 μg/mL which is rather unfeasible to be achieved under *in vivo* conditions.

Discussion

In vitro cell culture models have attracted significant attention in biocompatibility and drug transport studies since they have important advantages such as opportunity for rapid screening of a large number of substances, possibility for the simultaneous study of different variables and also avoiding the controversial use of animal models (Cho et al., 2010). RPMI 2650 cell line used in this study is the only commercially available human nasal cell line. Despite its neoplastic nature, it has similar characteristics to those of normal epithelial cells. Although RPMI 2650 cell line is claimed to be unsuitable for transport studies (Dimova et al., 2005), its application for assessment of drug permeability has been reported (Harikarnpakdee et al., 2006; Kurti et al., 2013; Reichl and Becker,

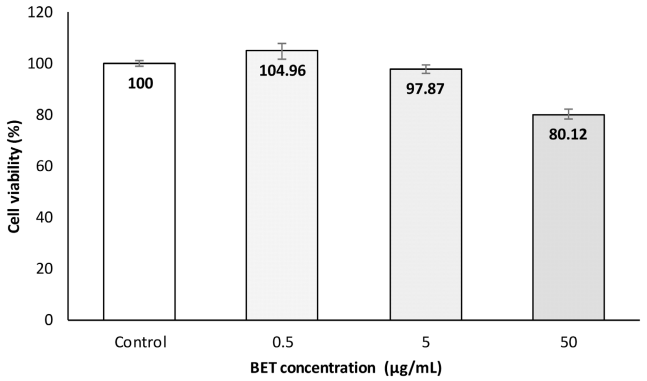

Fig. 7. The viability of RPMI 2650 cells after treatment with BET at concentrations 0.5, 5 and 50 μg/mL analyzed by MTT test (n = 7, mean ± SD).

2012; Wengst and Reichl, 2010). RPMI 2650 formed a thick, impermeable cell layer on the apical side of the filter inserts and developed enough TEER values to confirm confluence. The highest TEER values ($197 \pm 9\,\Omega\,cm^2$) were measured after cultivation for 7 days. Throughout drug transport study BET was detected in the basolateral compartment of the filter inserts which confirmed its ability to permeate the RPMI 2650 barrier layer. This is a prerequisite for the reliable delivery of BET via the nasal route. The permeation profiles revealed much higher influx of BET delivered by M3 microspheres compared to the other models. Also, M3 permeation profile was quite similar to that of BET solution, especially in the first 30 min. This phenomenon could be explained by the influence of the polymer chitosan on the integrity of the tight junctions between cells. M3 was reported to have the lowest drug loading among the models examined (Table 1) which determined the use of a larger mass of microspheres from this model. Probably, the greater amount of chitosan that was applied on RPMI 2650 cell layer after treatment with M3 was the reason for the significant impact on the cell layer integrity and thus on BET permeability. When the microspheres come into contact with the cell monolayer, they absorb water and swell, which results in dehydration of the epithelial cells and causes a rapid separation of the intercellular tight junctions. Furthermore, the microspheres prepared with the natural polymer chitosan, have a high capacity for binding of Ca^{2+} and have the ability to induce an increase in the permeability of tight junctions by translocation of intracellular proteins from the cell membrane to the cytoskeleton (Davis and Illum, 2003; Karasulu et al., 2008; Smith et al., 2004). Our suggestions were confirmed by the measurement of TEER after exposure to the microspheres. The results from this study consolidate the role of chitosan as an absorption enhancer and a reliable carrier for the formulation of nasal drug delivery systems.

The permeation profiles of BET through the cultivated cell layer resembled the *in vitro* dissolution profiles; permeation coefficients of BET had high initial values (probably due to the extensive "burst effect" that was observed in our previous experiments), gradually decreasing with time, which indicated that the release of BET from the polymer matrix is rate-limiting step in the nasal absorption.

A statistically significant difference in TEER values measured after cell exposure to microspheres from M3 and the other models was observed. It comes to show that drug/polymer ratio in the microspheres and drug entrapment efficiency strongly influence drug permeability through the cultivated barrier layer.

The cytotoxicity of BET was investigated by MTT test. A dose-dependent reduction in cell viability was observed. According to Marín et al. (2015) the mean BET plasma concentration in healthy volunteers detected after oral administration of 24 mg BET solid dosage form was in the range 1742.5–1772.8 ng/mL which is far below the maximum concentration (50 μg/mL) evaluated in our cytotoxicity assay. Practically, any concentration below 50 μg/mL might be considered harmless for the nasal epithelium. This is essential for the safe use of BET formulations intended for nasal administration and could serve as a prerequisite for BET compatibility with epithelial cells after nasal drug delivery.

Conclusion

In vitro permeability of betahistine dihydrochloride was accomplished through cultivated RPMI 2650 nasal cell monolayer. The role of chitosan as an absorption enhancer in microparticulate drug delivery was demonstrated. Also, a good safety profile of betahistine, regarding nasal epithelium toxicity, was estimated.

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