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Normal flora: living vehicles for non-invasive protein drug delivery

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Abstract

Feasibility to use probiotic bacteria as a living protein delivery system through oral route was assessed in vitro. *Lactococcus lactis* transformed with a plasmid to express and secret β-lactamase was used to deliver β-lactamase through Caco-2 monolayer, an intestine epithelium. Transport of β-lactamase through Caco-2 monolayer was carried out in the transwells. The viability and integrity of the cell monolayers co-cultured with *L. lactis* was examined by trypan blue exclusion method and by measuring the transport of mannitol and propranolol as well as the transepithelial electrical resistance (TEER). Results show that it is feasible to use cell culture technique to evaluate the drug delivery by normal flora. The transport rate of β-lactamase when delivered by *L. lactis* was $2.0 \pm 0.1 \times 10^{-2}\,h^{-1}$ ($n = 9$) and through free solution form was $1.0 \pm 0.1 \times 10^{-2}\,h^{-1}$. When co-cultured with *L. lactis*, Caco-2 cell viability decreased to 98, 96, and 94% at 6, 8, and 10 h, respectively. Transport of mannitol through Caco-2 cell monolayer was significantly increased and the transport of propranolol through Caco-2 cell monolayer was significantly decreased in the presence of *L. lactis*. Increase in the amount of protein delivered is probably due to the concentrate of the protein by *L. lactis* on the monolayer (absorption surface) and the opening of the tight junction of Caco-2 monolayer by *L. lactis*.

Keywords: Caco-2; *Lactococcus lactis*; TEM β-lactamase; Protein delivery; Probiotic bacteria; Cell integrity

1. Introduction

Oral delivery of protein drugs is always a problem as: (1) proteins are extensively degraded in the digestive tract; (2) proteins are poorly absorbed due to their large sizes and polar nature. These two problems are also encountered in most of the other non-invasive routes. So far non-invasive delivery system for proteins that is highly efficient, easy to administer and patient friendly is still not available. Studies have shown that adequate absorption could be achieved if a large amount of protein is present right at the site of absorption with or without the help of absorption enhancers. For example, when insulin was given orally together with enzyme

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inhibitors and absorption enhancers, its bioavailability was significantly increased (Piard, 1998). However, idea of using enzyme inhibitors does not appear practical due to their adverse effects. For the same reason, the amount of absorption enhancers that can be used is also a concern.

We have thought that the normal flora existing in the open alimentary tracts of the human body may be utilized for protein delivery to overcome the problems associated with the non-invasive administration of the protein drugs. The normal flora can be genetically modified to synthesize and secrete the protein drugs. Their natural tendency to adhere tightly to the epithelial cell surface of the channels where they normally reside will result in delivering large amount of protein at the site of absorption and will also minimize the enzymatic and bacterial degradation of the protein drugs (Ouwehand et al., 1999). In addition, due to their interaction with the epithelial cells, the normal flora may open the tight junctions of the epithelial cells to a certain degree, which will facilitate the protein drug absorption. Therefore, we have hypothesized that the utilization of normal flora as a novel delivery system will overcome the two major problems associated with the oral protein delivery, namely, extensive degradation and poor absorption.

In the present study, *Lactococcus lactis* (*L. lactis*) is used as a delivery system for β-lactamase, a model protein. *L. lactis* is a probiotic and considered as the safest strain in the genera of lactic acid bacteria (LAB), which are usually regarded as generally recognised-as-safe (GRAS) (Salminen et al., 1998). Use of LAB dates back to the time immemorial, this group comprises of a remarkably diverse and heterologus group of gram-positive bacteria. Lactobacillus strains have been administered to cure diarrhea and other intestinal disorders. The mechanism of action is not very clear. It is probably because they may interfere with the colonisation of *Helicobacter pylori* and of enteropathogenic microorganisms (Sullivan and Nord, 2002). There have been some studies on the probiotic activity of *Lactococcus* strains. In a study, nine strains of the genus *Lactococcus* were examined for their probiotic properties, such as adherence to human enterocyte-like Caco-2 cells and tolerance to acid and bile (Kimoto et al., 1999).

Caco-2, an epithelial cell line isolated from a human colon adenocarcinoma (Fogh et al., 1977) has been used extensively by the pharmaceutical industry for the prediction of in vivo intestinal drug absorption. The use of this cell line is based essentially on the following observations: (i) Caco-2 cells differentiate into enterocytes and form polarized monolayers thus having distinct apical and basolateral sides when cultured on permeable membrane; (ii) the apical side has well-developed microvilli (brush border) with transport systems, ion channels and tight junctions similar to those found in the intestinal epithelial cells (Pinto et al., 1983).

The objectives of the present study are to explore the possibility of using *L. lactis* as the vehicle in the in vitro absorption studies on drug transport through the Caco-2 cell monolayer; to study the transport of β-lactamase through Caco-2 monolayer delivered by *L. lactis*, to study the mechanism for the increased delivery efficiency by *L. lactis*, and to access the safety of *L. lactis* in the in vitro cell culture studies.

2. Material and methods

2.1. Materials

Caco-2 cell line (passage no. 28) was purchased from ATCC (Rockville; MD, USA). *L. lactis* subsp. *lactis*, transformed with plasmid ss80 containing the gene of β-lactamase was generously provided by Dr. Soile Tynkleynen (Valio Ltd., Helsinki, Finland). Dulbecco's Modified Eagle Medium (DMEM), trypsin 0.25% with EDTA, fetal bovine serum (FBS), sodium pyruvate (100 U/ml) and non-essential amino acids were obtained from Hyclone (Logan, UT); Transwell® inserts, tissue culture treated (0.4 μm pore size, 4.7 cm^2 surface area), and culture flasks (growth area 25 and 75 cm^2) were purchased from Costar Corporation (Cambridge, MA); Bacto M17 broth and Bacto agar were purchased from Becton Dickinson (Sparks, MD). D-Mannitol-[1-^{3}H(N)] (20 Ci/mmol) and DL-propranolol-[4-^{3}H] hydrochloride (33 Ci/mmol) were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Assay methods

2.2.1. Bacteria viability assay

Viable count of *L. lactis* in suspension culture was carried out by plating serial dilutions of the bacterial

culture onto the agar plates, and incubated at 30 ± 2 °C for 24 h. The bacteria density in the culture was also assayed by measuring the optical density (OD) at 600 nm wavelength.

2.2.2. β-Lactamase assay

Fluorimetry was used to quantitatively analyze β-lactamase (Baker, 1999). Briefly, 0.2 ml of β-lactamase sample (in culture media) was mixed with 0.8 ml of 6.25 mM ampicillin at 25 °C. The reaction was allowed to last for exact 10 min and then 0.1 ml of 8% trichloro acetic acid at 4 °C was immediately added to cease the reaction. This solution was diluted to 5 ml with 0.5 M acetate buffer at pH 4 and then heated at 100 °C for 30 min. After cooling to room temperature, 0.1 ml of each sample was placed in 96 well plates and the fluorescence was read by a microplate reader at an excitation wavelength of 340 nm and an emission wavelength of 465 nm. The activity of β-lactamase was then calculated according to the standard curves. Pure cell culture media was used as the blank control. The supernatant of the culture of Caco-2 cell line without the addition of the bacteria or β-lactamase solution, were also tested for any β-lactamase like activity in order to determine the potential interference on the assay from the culture supernatant. This assay method was validated in terms of within-day variation, day-to-day variation, precision, and fluorescent stability over the storage at 4 °C in dark.

2.2.3. Tumor cell viability assay

The Caco-2 cell monolayer in the transwell was rinsed twice with phosphate buffer saline (PBS) solution and then treated with 500 μl of trypsin 0.25% with EDTA solution and neutralized after 5–10 min with 500 μl of *s*-DMEM. Fifty microliters of this solution was diluted with equal volume of 0.4% trypan blue dye (Sigma). Then the cell viability was counted in a Neubauer chamber under the microscope.

2.2.4. Assay on radio-labelled mannitol and propranolol

Samples containing the radio-labelled mannitol or propranolol (100 μl) were placed in the scintillation vials and 10 ml of scintillation cocktail (Sigma) was added. These samples were counted for total counts per minute (cpm) on a Packard Tricarb LSC. A serial standard solutions were made. A standard curve of the cpm and concentration was generated from these standard solutions.

2.3. Selection of the media for co-culture of L. lactis and Caco-2 cells

L. lactis was cultured in M17 broth, DMEM supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin-100 μg/ml streptomycin, sodium pyruvate (100 U/ml) and 1% (v/v) non-essential amino acids (*s*-DMEM) or the *s*-DMEM without antibiotics at 37 °C, 5% CO_2, and relative high humidity. At predetermined time points, samples were taken for the measurement of the bacterial growth and the β-lactamase activity in the culture media. Caco-2 cells were grown in M17 broth or *s*-DMEM at 37 °C, 5% CO_2, and relative high humidity. Cell growth was monitored at predetermined times.

2.4. Culture of Caco-2 cells in transwells

Caco-2 cells cultivated in 75 cm^2 flasks in *s*-DMEM were harvested by the treatment of 0.25% trypsin with EDTA and then seeded onto the polycarbonate filters of the transwells at a density of 0.3×10^5 cells/cm^2 and further incubated under normal cell culture conditions. The growth media was replaced every other day and TEER was monitored periodically by using a Millicell® ERS meter (Millipore, Bedford, MA), connected to a pair of chopstick Ag/AgCl electrodes. The monolayer became confluent and ready for transport studies after about 21–24 days when TEER reading reached a plateau.

2.5. Effect of L. lactis on the viability and the integrity of the Caco-2 monolayer

2.5.1. Effect on the viability

After the Caco-2 cells formed confluent monolayer in the transwells, the culture media was removed and the cells were washed twice with PBS. Then 2.6 ml of *s*-DMEM without antibiotics was added to the apical side. An amount of 1.5 ml freshly made *L. lactis* suspension in antibiotic-free *s*-DMEM was added to the apical side ($\sim 4 \times 10^7$ bacterial cells/well). The plate was centrifuged at 45 × *g* for 5 min (Beckman GPR Centrifuge) and was placed inside the incubation chamber. The Caco-2 cell viability was determined right

before the addition of *L. lactis* (0 h) and at 6, 8, and 10 h after the addition. This study was repeated thrice in total nine wells for each time point.

2.5.2. Effect on the integrity

The confluent Caco-2 monolayers on the transwells were washed twice with PBS. To the basolateral side 2.6 ml of antibiotic-free *s*-DMEM was added, and to the apical side, 1.5 ml of the freshly made *L. lactis* suspension ($\sim 4 \times 10^7$ bacterial cells/well) in antibiotic-free *s*-DMEM containing either 35.5 nM D-mannitol-[1-^{3}H(N)] or 20.0 nM DL-propranolol-[4-^{3}H] hydrochloride was added. At each time interval, 100 μl of the media was taken from the basolateral side and replaced with equal amount of fresh media. Samples were withdrawn to 10 h. At the end of this experiment, 100 μl of sample was also taken from the apical side. All the samples were assayed for radioactivity. TEER was also monitored during the study.

2.6. Transport of β-lactamase through the Caco-2 monolayer

After the Caco-2 monolayer became confluent in the transwells, the basal media was replaced by 2.6 ml of fresh *s*-DMEM without antibiotics in all six wells of each plate. The apical media in half of the wells was replaced by 1.5 ml of freshly made *L. lactis* suspension in antibiotic-free *s*-DMEM ($\sim 4 \times 10^7$ bacterial cells/well), while to the other half of the wells, the apical media was replaced by 1.5 ml antibiotic-free *s*-DMEM containing 1000 mU of β-lactamase. The plate was centrifuged at $45 \times g$ for 5 min and cultured at normal cell culture conditions. At the predetermined time periods, 50 and 200 μl samples were taken from the apical and the basal sides for β-lactamase assay and were replaced by equal volume of the fresh *s*-DMEM without antibiotics. The samples were withdrawn up to 10 h. The $AUC_{0–10\,h}$ under the β-lactamase total amount-time curve at the apical side was calculated by the linear trapezoid method to determine the total β-lactamase exposure to the monolayer at the apical side. The transport of β-lactamase was normalized by the AUC because the concentration at the apical side is not the same for all the wells. In order to make a comparison on the transport rate of β-lactamase in different wells, AUC was used as a representative of the total drug exposure to the apical side.

TEER was monitored throughout the absorption studies. At the end of this study Caco-2 cell viability was also analyzed. This transport study was repeated thrice using one plate every time. In each plate, three wells were used as the control and three wells for *L. lactis*.

2.7. Data analysis

Student *t*-test was used to compare the difference between the data of interest. Wherever possible, the data is presented as mean ± standard deviation.

3. Results and discussion

3.1. Selection of the media for co-culture of Caco-2 cells and L. lactis

In order to culture *L. lactis* and Caco-2 cells together, selection of a common media in which both *L. lactis* and Caco-2 cells can grow well is very important. Regular media for Caco-2 cells and *L. lactis* are *s*-DMEM and M17, respectively. The viability of *L. lactis* in *s*-DMEM was significantly reduced because of the presence of the antibiotics. When the antibiotics were deleted from the *s*-DMEM media, the growth of *L. lactis* was very comparable to its growth in M17. The Caco-2 viability reduced to 60% when cultured in M17 for 6 h. Therefore, the antibiotic-free *s*-DMEM was selected for the co-culture of *L. lactis* and Caco-2 cells.

3.2. Characterization of L. lactis growth and β-lactamase secretion

The β-lactamase secretion profile by *L. lactis* was similar in M17 broth and antibiotic-free *s*-DMEM. As shown in Fig. 1, the growth of *L. lactis* and secretion of β-lactamase correlated with each other: the amount of β-lactamase in the culture media increased at the initial log-growth phase, then reached a plateau at the stationary growth phase and then decreased at the decline phase. It has been shown in the previous studies that some proteases are secreted by *L. lactis* that may metabolize β-lactamase resulting in decrease in the amount of protein in the media at the decline phase (Foucaud-Scheunemann and Poquet, 2003).

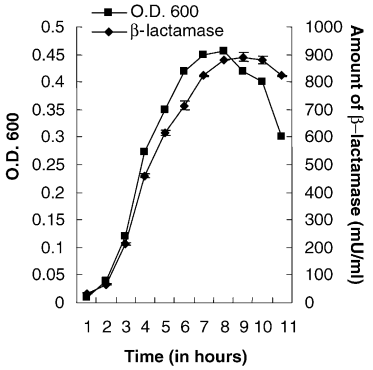

Fig. 1. Growth and β-lactamase secretion by *L. lactis* in *s*-DMEM without antibiotics. *L. lactis* were cultured in *s*-DMEM without antibiotics at 37 ± 2 °C. At the predetermined time points, optical density at wavelength 600 nm was observed, and the β-lactamase in the culture supernatant was measured by fluorometry ($n = 3$).

3.3. Effect of L. lactis on the viability of the Caco-2 monolayers

The cell viability was monitored by trypan blue exclusion method. The results are shown in Fig. 2. The cell viability decreased to 98, 96, and 94% at 6, 8, and 10 h, respectively. This is probably due to the large amount of bacteria competing with the Caco-2 cells for the limited amount of nutrients. There was no statistical significant difference ($P > 0.05$) in the cell viability between the control wells (absence of *L. lactis*) and the wells containing *L. lactis*.

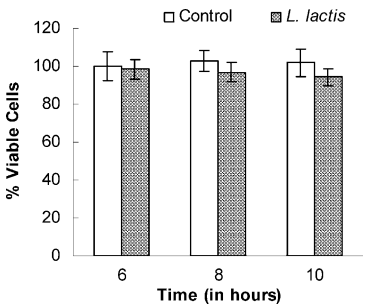

Fig. 2. Caco-2 cell viability when co-cultured with *L. lactis* in *s*-DMEM without antibiotics. *L. lactis* suspension in *s*-DMEM without antibiotics was added to the confluent Caco-2 cell monolayer in each well (4×10^7 bacterial cells/well) at time zero. To the control wells, only the medium was added. The plates were briefly centrifuged and then incubated at normal cell culture conditions. Caco-2 cell viability was examined at the predetermined time points, and standardized against the viability at time zero (mean ± S.D., $n = 9$).

3.4. Effect of L. lactis on the integrity of the Caco-2 monolayer

The effect of *L. lactis* on the Caco-2 monolayer was further investigated by using both paracellular transport marker (D-mannitol-[1-^{3}H(N)]) and transcellular transport marker (DL-propranolol-[4-^{3}H] hydrochloride). In these studies, 1.5 ml of the bacteria suspension added into the apical side containing either 35.5 nM D-mannitol-[1-^{3}H(N)] or 20 nM DL-propranolol-[4-^{3}H] hydrochloride. The results are shown in Figs. 3 and 4. It is very obvious that after 4 h the transport of mannitol from the Caco-2 cell monolayer was significantly increased at the presence of *L. lactis*. On the contrast, the transport of propranolol through Caco-2 cell monolayer was significantly decreased at the presence of *L. lactis*. Mannitol has log *P* (partition coefficient) value and molecular weight as −2.50 and 182.2, respectively. Since mannitol is transported through paracellular route (Cogburn et al., 1991), the increase of its transport indicates the opening of the tight junction of the Caco-2 cells by *L. lactis*. The opening of tight junction is desired for protein drug as well as other hydrophilic drug delivery, because these drugs cannot diffuse through cell membranes and they usually take paracelluar route through the junctions between the cells. Propranolol is a lipophilic compound with a

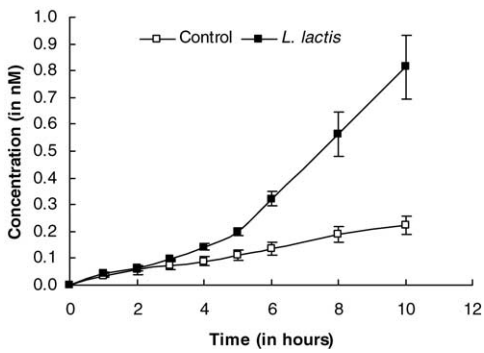

Fig. 3. Accumulated mannitol concentration at the basolateral side of the Caco-2 monolayer. An amount of 1.5 ml of *L. lactis* suspension in the radiolabelled mannitol solution (35.5 nM) was added to the apical side of three wells (4×10^7 bacterial cells/well) at the beginning. To the other three wells (the control), only the radiolabelled mannitol solution (without *L. lactis*) was added. The plates were briefly centrifuged and then incubated at normal cell culture conditions. Samples (100 μl) were withdrawn from the basal side at different time points (mean ± S.D., $n = 9$).

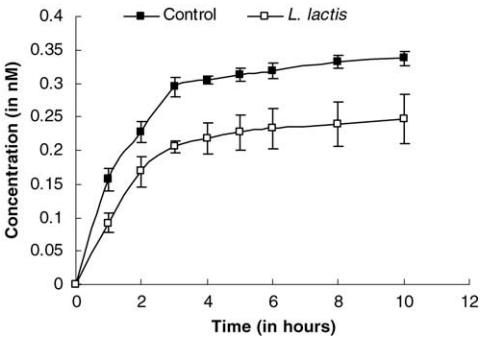

Fig. 4. Accumulated concentration of propranolol at the basolateral side of the Caco-2 monolayer. An amount of 1.5 ml of *L. lactis* suspension in the radiolabelled propranolol solution (20 nM) was added to the apical side of three wells (4×10^7 bacterial cells/well) at the beginning. To the other three wells (the control) only the radiolabelled propranolol solution (without *L. lactis*) was added. The plates were briefly centrifuged and then incubated at normal cell culture conditions. Samples (100 μl) were withdrawn from the basal side at different time points (mean ± S.D., $n = 9$).

log *P* value of 2.53 and molecular weight of 259.3. It undergoes passive transcellular transport (Cogburn et al., 1991). The decreased transport rate of propranolol at the presence of *L. lactis* compared with the control wells (without *L. lactis*) indicates that the Caco-2 cell monolayer integrity was maintained well (if the monolayer had been disrupted, then the transport of propranolol would have been increased). The decreased transport of propranolol is probably because of the adhesion of *L. lactis* on the monolayer hindered the diffusion of propranolol through the cells. Based on the above studies, we can conclude that even though *L. lactis* might slightly decrease the Caco-2 viability, the Caco-2 monolayers were well maintained throughout the experiment period (10 h) and *L. lactis* was able to open the tight junctions between the Caco-2 cells which is desired for protein drug delivery.

Previous studies have shown that *L. lactis* has good adhesion properties to the cell monolayers. Many studies have been carried out to elucidate the interaction between *L. lactis* and the cell layer. It is believed that the structure, chemical composition, and physicochemical properties of the surface of bacteria are important factors. However, the true mechanism for the opening of the tight junction is still unknown.

At the end of the transport study, both apical and basal media were taken and analyzed to calculate the total radioactivity. The results are shown in Table 1 and it is very clear that the addition of *L. lactis* to the monolayer just very slightly reduced the recovery of the radioactivity (only additional 2.5 and 2.0% loss for mannitol and propranolol, respectively). Thus the transport studies of mannitol and propranolol did not understate the amount of the marker going to the basal side due to the loss of radioactivity to the cell monolayer or adsorption to the transwell surface. This study also shows that in transwells alone, without any Caco-2 monolayer, the loss of radioactivity was more for propranolol than for mannitol. This loss may be due to the adsorption of propranolol by the transwells because of the lipophilic nature of propranolol, thus the total radioactivity reduced to 96.8%.

3.5. *Transport of* β-*lactamase through Caco-2 monolayers*

The results are presented in Fig. 5. The area under the β-lactamase total amount-time curve ($AUC_{0–10\,h}$) at the apical side from beginning to 10 h was calculated by linear trapezoid method to determine the total β-lactamase exposure to the monolayer at the apical side. The accumulated amount transported through the Caco-2 monolayer was normalized by this $AUC_{0–10\,h}$. The normalized transport rate was $2.0 \pm 0.1 \times 10^{-2}\,h^{-1}$ ($n = 9$) and $1.0 \pm 0.1 \times 10^{-2}\,h^{-1}$ ($n = 9$) when

Table 1
Recovery of mannitol and propranolol from the transwells in the cell monolayer integrity studies[a]

	Mannitol		Propranolol	
	No *L. lactis*	*L. lactis* added	No *L. lactis*	*L. lactis* added
Caco-2 monolayer	99.88 (1.14)	97.3 (1.1)	94.0 (1.6)	92.1 (0.9)
No Caco-2 monolayer	101.2 (1.9)	98.8 (1.5)	96.8 (2.4)	93.3 (1.8)

[a] % Recovery, calculated by the sum of the total radioactivity in the apical and basal media at the end of the study against the total radioactivity given at time zero. Values are expressed as mean (S.D.) of nine wells.

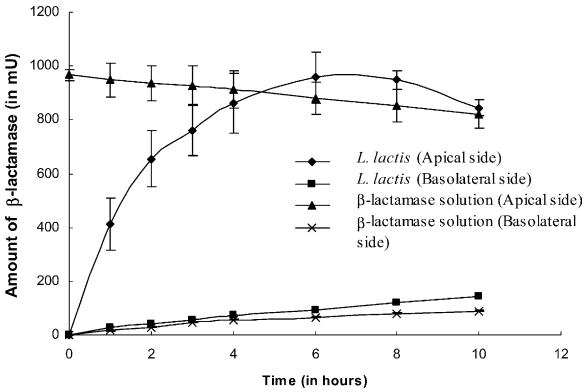

Fig. 5. Transport of β-lactamase through the Caco-2 monolayer. β-Lactamase solution (around 1000 mU/well) in *s*-DMEM without antibiotics or *L. lactis* suspension (~4×10^7 bacterial cells/well) in *s*-DMEM without antibiotics was added to the apical side of each well. The plates were briefly centrifuged and then incubated at normal cell culture conditions. Samples were taken both from the apical side (50 μl) and basal side (200 μl) of the transwell for the assay of β-lactamase by fluorometry (mean ± S.D., $n = 9$).

delivered by *L. lactis* or the free solution form, respectively. The delivery by *L. lactis* was almost the double of that by the solution form ($P < 0.01$), demonstrating the high efficiency by this living delivery system. This enhanced delivery is probably due to the concentrate of β-lactamase on the absorption surface (the monolayer surface) and the opening of the tight junction between the Caco-2 cells by *L. lactis*.

3.6. TEER value

At the end of this study, tumor cell viability was also analyzed and was found to be the same as shown in Fig. 2. The transepithelial electric resistance (TEER) was monitored throughout the transportation studies and is shown in Fig. 6. The results indicate again that the monolayer integrity did not compromise for the whole period of the study. It was seen that the TEER value increased to a large extent when *L. lactis* was added to the cell culture media. This increase might be due to adhesion of bacteria to Caco-2 cell monolayer, which hindered flow of current through the monolayer. No clear correlation can be drawn between the absorption and TEER value. Our previous studies have shown that TEER values cannot be used alone to ensure the integrity of the monolayer (Mukherjee et al., 2004). Thus, mannitol and propranolol were also used for the same purpose.

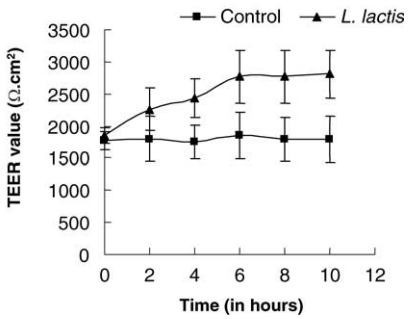

Fig. 6. Transepithelial electric resistance of the Caco-2 cell monolayer in the transwells during the β-lactamase transport study (mean ± S.D., $n = 9$).

4. Conclusion

The present studies have demonstrated that probiotics such as *L. lactis* when transformed by special plasmid can be a living source for protein drugs. In vitro studies have shown that *L. lactis* can significantly increase the transportation of β-lactamase through Caco-2 monolayer and almost double the transportation rate as compared to the solution form. This increase is probably due to the concentrate of β-lactamase by *L. lactis* on the monolayer (absorption surface) and the opening of the tight junction of Caco-2 monolayer by *L. lactis*. The present results also have proven that it is feasible to use cell culture technique to evaluate the drug delivery by normal flora. *L. lactis* do not have any appreciable affect on the viability of the Caco-2 cell monolayer within a sufficient period of time.

Acknowledgement

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