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Featured Article

Disruption of Epithelial Tight Junctions by Yeast Enhances the Paracellular Delivery of a Model Protein

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Purpose. The aim of this study was to investigate the effect of heat-killed yeast cells on the integrity of epithelial tight junctions *in vitro*.
Methods. Changes in barrier potential of Caco-2 cell monolayers were assessed by transepithelial electrical resistance (TEER) measurements and by an increasing permeability to a marker protein, horse–radish peroxidase (HRP). Visualisation of tight junction disruption was carried out directly through electron microscopy and indirectly through fluorescence confocal microscopy and immunoblotting of the tight junction-associated proteins zonula occludens ZO-1, occludin and actin.
Results. Yeast cells opened tight junctions in a reversible dose- and time-dependent manner, as shown by a decrease in TEER and an increase in HRP permeability. These changes to barrier potential were shown not to be due to cytotoxic effects but due to modulation of the tight junctions. ZO-1, actin and occludin proteins were demonstrated to be involved in yeast-induced tight junction opening through the use of confocal microscopy and western blotting. Electron microscopy confirmed a direct opening of tight junctions after application of yeast.
Conclusion. Yeast modulated epithelial tight junctions in a reversible manner by contraction of the actin cytoskeleton and shift of ZO-1 and occludin tight junction proteins from the membrane to cytoskeletal areas of the cell.

KEY WORDS: TEER; tight junctions; yeast; penetration enhancement; Caco-2.

INTRODUCTION

Delivery of drugs in vivo through simple epithelial cell layers, such as the intestine after oral administration or via the nose following a nasal spray application, is an attractive route of drug delivery in terms of patient convenience compared to the parenteral route. Epithelial cells form an effective boundary between the external environment and the blood circulation, through the formation of junctional seals in between neighbouring cells. The most apical of these junctions is the tight junction, a complex multiprotein structure, which is responsible for the restricted movement of small molecules, ions and nutrients via the paracellular route (1,2). Tight junction-associated proteins span the cell from membrane to cytosolic regions, and as well as interacting with each other, they interact with the actin cytoskeleton (3). Occludin (4), a transmembrane protein, provides both structural integrity and regulation of the barrier function (2). Occludin forms a complex with the cytosolic scaffolding protein zonula occludens ZO-1 (5), a protein also involved in the regulation of tight junction permeability (1). The C-terminal portion of ZO-1 in turn interacts with F-actin of the cytoskeleton (6) and previous work has suggested that actin can also affect tight junction integrity (7). Actin is present both as a belt around the periphery of the cell and as stress fibres or filaments which extend from the tight junction (8). It is these filaments that bind directly to ZO-1 (6), thus forming a direct connection from the paracellular space, through the membrane, to the actin–myosin ring (9).

In order to increase the absorption of poorly permeable drugs, tight junctions can be modulated through the use of penetration enhancers. These must be able to open tight junctions in a safe, reversible and timely manner. Complete recovery of the barrier function is desirable, so that unwanted foreign particles or toxins do not enter and also so that upon repeated application, cells do not become damaged. Previously published penetration enhancers include surfactants, bile salts, and fatty acids (10–12), which have been shown to disrupt tight junction proteins allowing drug transport through the paracellular pathway. However, some penetration enhancers have been found to be toxic *in vitro* and/or *in vivo*, with the improved drug permeability observed being a

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ABBREVIATIONS: DMEM, dulbecco's modified eagle medium; ECL, enhanced chemiluminescence reagent; EDTA, disodium eth ylenediaminetetraacetate; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; HRP, horse–radish peroxidase; P_{app}, apparent permeability coefficient; PBS, phosphate buffered saline; PKC, protein kinase C; SEM, scanning electron microscopy; S.E.M., standard error of the mean; TEER, trans-epithelial electrical resistance; TEM, transmission electron microscopy; ZO-1, zonula occludens-1.

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result of mucosal damage (13,14). Therefore safe, preferably non-toxic penetration enhancers are more suitable.

Yeasts, such as *Saccharomyces cerevisiae,* commonly known as Bakers' yeast, have been used in the food and drink industry for many years (15). However, more recently their potential in the pharmaceutical industry has been realised. Yeast, in particular yeast cell walls, have been shown to provide a good natural coating to various pharmaceuticals (15–17), where acidified yeast cell walls were coated onto tablets containing a model drug, resulting in a slow, controlled release profile of the drug. Chitosan, a deacetylated form of chitin, present within fungal cell walls and crustacean cells, has been shown to disrupt epithelial tight junctions (18). Chitin is present in small amounts throughout the yeast cell wall, but about 90% of it is found at the cell surface in and around bud scars, after the mother cell and daughter cell split from one another, making up to 2% of the yeast dry weight (19). It is unclear at present whether yeast cell wall chitin/chitosan could be responsible for tight junction opening.

It was the aim of the work described in this paper to investigate the effect of heat-killed yeast cells on tight junction integrity and to examine whether yeast can act as a penetration enhancer for proteins, using a model protein, horseradish peroxidase (HRP). Caco-2 cell monolayers were used for all experimental work, as this is a well characterised and frequently used cell line in order to study paracellular permeability *in vitro,* with many characteristics of simple epithelium *in vivo* such as brush borders and fully formed cellular junctions (20,21). When allowed to proliferate and grow to confluence, the Caco-2 cell monolayer becomes polarized, forming intercellular junctions, which restrict the movement of small electrolytes between the apical and basolateral sides of the cell monolayer. This restricted movement results in the generation of a trans-epithelial electrical resistance (TEER) which can be measured (22) and is therefore a simple test of tight junction integrity. The effect of yeast on tight junction integrity was therefore measured by observing a decrease in TEER and a subsequent increase in permeation of HRP. Further evidence of increased epithelial permeability after the application of yeast cells was also investigated, using both electron microscopy on an ultrastructural level and fluorescence confocal microscopy and immunoblotting of the tight junction-associated proteins ZO-1, occludin and F-actin on a molecular level.

MATERIALS AND METHODS

Materials

S. cerevisiae yeast was obtained from Aventine Renewable Energy Inc. (Pekin, IL, USA) as a dry powder. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Life Technologies, Gibco BRL, UK. Rabbit anti-ZO-1 was purchased from Invitrogen (California, USA) and Goat anti-occludin was purchased from Santa Cruz Biotechnology Inc (California, USA). Foetal calf serum (FCS) was obtained from Biowest (Nuaillé, France), as was the enhanced chemiluminescence reagent (ECL). All other chemicals, materials and secondary antibodies were purchased from Sigma–Aldrich (Poole, Dorset, UK) unless stated otherwise.

Methods

Preparation of Yeast Suspensions

Dry, powdered yeast (50 g) was hydrated in water (100 ml) and mixed at 300 rpm for 5 h at 40°C. The yeast suspension was then centrifuged at 4000 rpm at 10°C for 20 min, before spray drying (inlet temperature 180°C) using a Buchi mini spray–dryer B-290 (Flawil, Switzerland). The dried, sterile and non-viable (data not shown) yeast cells were, on the day of experimentation, diluted to produce yeast suspensions of 0% (control) to 2% (w/v) in 'cell culture medium', which consisted of DMEM, supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids and 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Cell Culture

Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and were seeded at 1×10^5/ml in 75 cm^2 tissue culture flasks and supported in cell culture medium. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and the medium was changed every two days. Cells from passages 47–60 were used in all experiments.

Trans-Epithelial Electrical Resistance (TEER) and HRP Permeability Experiments

Caco-2 cells were seeded into the apical chamber of polycarbonate inserts with 0.4 μm pores and 1.1 cm^2 growth area (Costar, Cambridge, Massachusetts, USA) at a density of 1×10^5 in 1 ml of cell culture medium. The basal chamber was filled with 1.5 ml of cell culture medium and cells were incubated at 37°C (5% CO_2). The cell culture medium was changed every two days and the TEER was measured across the monolayer using Millicell ERS apparatus (Millipore, USA) and "chopstick electrodes". The resistance typically reached a plateau after 30 days with a value of about 800 ohms, indicating that a continuous cell monolayer with junctions was fully formed. Blank chambers where no cells were present determined the background resistance level and this value was subtracted from all experimental values.

TEER and permeability experiments involved the addition of pre-warmed (37°C) yeast suspensions (0%–2% w/v) with 0.1 mg/ml HRP (final concentration) to the apical compartment and TEER measurements were taken at various intervals. The presence of HRP in the basal compartment after 60 min was then determined by a colourimetric assay (22). Briefly, the basal test sample was added to a substrate solution (50 mM sodium phosphate, 542 μM *o*-dianisidine dihydrochloride, 0.003% (v/v) hydrogen peroxide) and incubated at 37°C for 20 min. The colour generated was then read using a plate-reading spectrophotometer at 405 nm.

To check that cell monolayer permeability could be fully restored, yeast suspensions were removed. The cell monolayers were washed three times in PBS and fresh cell culture medium was added to both the apical and basal compartments. The TEER was then monitored over a further 48 h to assess barrier recovery. HRP permeability across the cell monolayer, as a marker for tight junction integrity, was also measured after 24 h of cell recovery. A basal sample was taken after

1 h of HRP (0.1 mg/ml) application and the concentration of protein permeated was calculated using the above colourimetric assay. All TEER values were expressed as the % of original (baseline) TEER. Permeability coefficients of HRP were calculated using Eq. 1.

$$P_{app.} = \frac{(dQ/dt)}{A\,x\,C_0} \quad (1)$$

Where dQ/dt is the flux of HRP across the monolayer (μg/ml transported/s), A (cm^2) is the surface area of the inserts and C_0 (μg/ml) is the initial HRP concentration in the apical compartment.

Assessment of Cell Viability

Following the treatment of Caco-2 cell monolayers with yeast suspensions, an assessment of cell viability was carried out. Cells were released using trypsin-EDTA solution (0.5 mg/ml and 0.2 mg/ml, respectively). Cell culture medium containing FCS, was then added to quench trypsin activity and cells were removed from the membrane surface using a cell scraper. Trypan blue (0.4% solution) was then diluted 1:8 in the resulting cell suspension. Cells were counted in a haemocytometer and viability (i.e., exclusion of trypan blue) was expressed as a percentage of the total number of cells present.

Fluorescence Studies

Caco-2 cells were seeded onto coverslips at 5000 cells/coverslip and grown for at least 30 days at 37°C (5% CO_2). Yeast suspensions (2% or 0% w/v) were applied to cell monolayers for 1 h. Some yeast-treated cell monolayers were washed in PBS and allowed to recover for 24 h in fresh cell culture medium. All cells were then fixed in 10% formalin, followed by permeabilisation in 0.5% (v/v) Triton X-100. Cells were then processed as below for either F-actin, ZO-1 or occludin fluorescence.

F-Actin Fluorescence

Cytoskeletal F-actin was visualised through incubation of cells with 50 μg/ml fluorescein isothiocyanate (FITC)-phalloidin diluted in PBS for 1 h.

ZO-1 and Occludin Immunofluorescence

Following permeabilisation, cells were incubated with 5% (w/v) milk powder in PBS for 1 h. After washing three times in PBS, ZO-1 antibody or occludin antibody were applied to cells at 1:1000 or 1:100, respectively, in blocking solution (2.5% w/v milk powder in PBS) for 1 h. Cells were again washed in PBS before the addition of FITC-conjugated goat anti-rabbit IgG antibody at 1:1000 or FITC-conjugated rabbit anti-goat IgG antibody at 1:400 (respectively) in blocking solution for 1 h.

All coverslips were then mounted onto microscope slides using fluoromount-G and tight junction protein fluorescence was visualised using a Zeiss LSM 510 confocal microscope, excitation 494 nm and emission 528 nm. Lamp intensity was kept to a minimum to allow comparison of fluorescent intensity between images.

Preparation of Cell Fractions

Caco-2 cells were grown to confluence on 25 cm^2 tissue culture flasks and were subsequently treated with 0% (control) or 2% (w/v) yeast suspensions for 1 h or 24 h. Some Caco-2 cells were washed three times in PBS and allowed to recover for 24 h in fresh cell culture medium. Cells were fractionated using the technique as described in Smith *et al.* (23), resulting in membrane, cytosolic and Triton-insoluble cell fractions. Cell fraction proteins were then separated using 4% (for ZO-1) or 10% (for occludin) SDS-PAGE according to the standard Laemmli protocol (24). Proteins were transferred onto nitrocellulose membranes and incubated with ZO-1 or occludin primary antibodies at 1:1000 or 1:500 (respectively) for 1 h. Secondary antibodies of HRP-conjugated goat anti-rabbit IgG antibody (1:10,000) or HRP-conjugated rabbit anti-goat IgG antibody (1:5000) for ZO-1 and occludin protein visualisation, respectively, were then applied for a further 1 h. Bands were visualised using Enhanced chemiluminescence (ECL) reagent and captured digitally using a Fujifilm LAS1000 Intelligent Dark Box. Densitometry analysis was carried out on all visualised bands using Aida software. Bands were quantified as a percentage of the control.

Electron Microscopy

Caco-2 cells were seeded onto microscope coverslips at 5000 cells/coverslip in cell culture medium. After 30 days, 0% or 2% (w/v) yeast suspensions in cell culture medium were applied for 1 h. Some Caco-2 cells were allowed to recover in fresh cell culture medium for 24 h, following yeast removal. Samples were then fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH6.9) overnight. Samples were then post-fixed in 1% osmium tetroxide for 4 h and then further processed for either scanning electron microscopy (SEM) or transmission electron microscopy (TEM).

SEM Sample Preparation

Post-fixed tissue was dehydrated in an ascending series of ethanol and critical-point dried in 100% CO_2. Specimens were mounted onto microscope studs and coated with a thin layer of gold using a Polaron sputter coater under a low pressure argon atmosphere. Visualisation was carried out using a CamScan series III SEM (Cam Scan ltd, Waterbeach, Cambridgeshire, UK).

TEM Sample Preparation

Dehydration of the sample occurred using an ascending ethanol series of 20, 40, 60, 80, 100 and 100% (v/v), with each step taking 30 min. The sample was then embedded in plastic Spur resin. Propylene oxide/Araldite solutions (25%:75% v/v) were then applied to the sample overnight. Neat, fresh Araldite was then applied for a further 4 h, before the sample was transferred to an embedding mould with fresh Araldite and allowed to polymerise overnight at 60°C. Sections (1 μm) were made using a microtome, stained using 1% osmium tetroxide and visualised using a Philips CM10 electron microscope (Philips, Guildford, Surrey).

Statistical Analysis

All data are presented as mean ± S.E.M. and all results are a composite of at least three separate experiments. Data were analysed using the unpaired 2-tailed *t* test, where differences between groups were considered to be significant at $p < 0.05$.

RESULTS

Yeast Decreases the TEER of Caco-2 Cell Monolayers

After treatment of Caco-2 cell monolayers with heat-killed yeast suspensions a time- and dose-dependent decrease in TEER was observed over 1 h (Fig. 1). After just 5 min, application of 2% (w/v) yeast caused a drop in TEER to 73.7% and after 1 h the TEER decreased to 43.8% of the original. At 1 h the 0.5 and 0.05% (w/v) yeast suspensions decreased the TEER of cell monolayers to 61.8 and 72.6% of the original, respectively. The TEER of control (no yeast) treated cell monolayers remained above 89% of the baseline. Changes in TEER after treatment with 2 and 0.5% (w/v) yeast were significantly different from control after 1 h, where $p = 3.29 \times 10^{-5}$ and 1.16×10^{-4}, respectively.

Cell Monolayers Recover After Yeast Removal

To test the ability of cell monolayers to revert to their original impermeable state, the TEER was monitored over a 48 h period after removal of yeast suspensions and application of fresh cell culture medium (Fig. 2). A rapid increase in TEER was observed within 1 h of removal of yeast suspensions and a plateau in resistance developed after 24 h. At 48 h, all cell monolayers had returned to near baseline levels, with TEER values of 94.3, 96.0 and 98.9% after application and subsequent removal of 2, 0.5 and 0.05% (w/v) yeast suspensions, respectively. This indicated that the barrier potential had fully recovered, due to no significant difference from control (0% yeast).

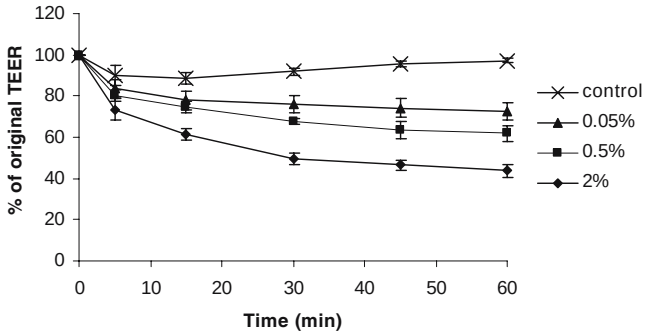

Fig. 1. Dose-dependent decrease in TEER of Caco-2 cell monolayers following the application of yeast cells. Caco-2 cell monolayers were treated with 0% (control), 0.05, 0.5 or 2% (w/v) yeast suspensions in cell culture medium for 1 h and the % change in TEER from the original baseline was noted ($n \geq 7$ ± S.E.M).

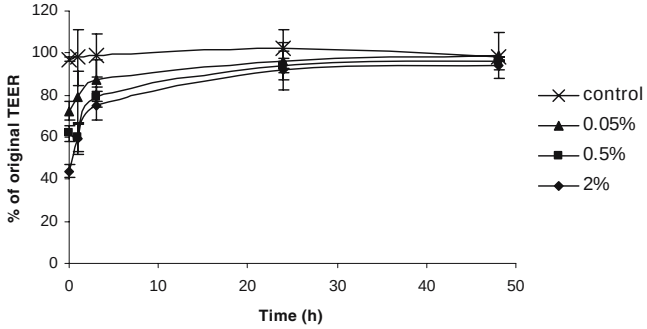

Fig. 2. Dose-dependent recovery of Caco-2 TEER after removal of yeast cells. After a 1 h application to Caco-2 monolayers, 0% (control), 0.05, 0.5 or 2% (w/v) yeast suspensions were removed and epithelial cells were allowed to recover for 48 h in fresh cell culture medium, where the % of original TEER was monitored ($n \geq 7$ ± S.E.M).

Effect of the Application and Removal of Yeast on the Transport of Marker Protein HRP

To demonstrate that a decrease in TEER is related to an increase in monolayer permeability, HRP, a 40 kDa protein, was applied to Caco-2 monolayers along with the yeast and the enzyme activity was measured on the basal side after 1 h. In control monolayers the apparent permeability coefficient (P_{app}) of HRP was 0.18×10^{-6} cm/s (Table I). Application of yeast suspensions resulted in a dose-dependent increase in HRP transported with coefficients of 1.82×10^{-6} cm/s and 1.19×10^{-6} cm/s following 2 and 0.5% (w/v) yeast treatment, respectively. These data were significantly different to control ($p = 3.21 \times 10^{-3}$ and 1.34×10^{-3} after 2 and 0.5% w/v yeast treatment, respectively). Overall, a 10-fold increase in HRP permeation after 1 h was observed following 2% (w/v) yeast application to cell monolayers, compared to control.

Table I. Apparent Permeability (P_{app}) of HRP Across Caco-2 Monolayers and Cell Viability After the Application and Subsequent Removal of Yeast

Treatment (w/v)	P_{app} (10^{-6} cm/s)	% Viable Caco-2 cells
0% Yeast (control)	0.18 ± 0.013	98.32 ± 1.07
0.05% Yeast	0.24 ± 0.015	97.08 ± 1.13
0.5% Yeast	1.19 ± 0.035	93.92 ± 1.93
2% Yeast	1.82 ± 0.030	92.28 ± 2.89
Recovery after 0.05% yeast removed	0.21 ± 0.030	98.12 ± 0.68
Recovery after 0.5% yeast removed	0.19 ± 0.045	96.95 ± 1.91
Recovery after 2% yeast removed	0.24 ± 0.023	97.22 ± 2.39

Yeast suspensions were applied to Caco-2 monolayers along with HRP for 1 h. P_{app} of HRP was calculated after appearance of enzyme activity in the basal compartment. Cells were allowed to recover after removal of test solutions for 24 h, after which, HRP was again added for 1 h and the P_{app} was calculated. Exclusion of trypan blue from cells after the application of yeast suspensions for 1 h provided a measurement of cell viability, calculated as a % of the total number of cells present. ($n \geq 6$ ± S.E.M. * = $p < 0.05$).

After 1 h, the yeast suspensions were removed and Caco-2 cells were allowed to recover for 24 h in fresh cell culture medium. HRP was again applied and presence of the protein in the basal layer was assayed after a further 1 h. Following this recovery period, the barrier function appeared fully re-established and no significant difference in HRP P_{app} was observed (Table I).

The viability of yeast-treated Caco-2 cells also showed no significant difference to control following a trypan blue exclusion assay, indicating that the decrease in TEER and increase in permeability was not due to a loss of viable cells (Table I).

Yeast-Induced Changes in the Localisation and Staining of Tight Junction Proteins

The tight junction associated proteins F-actin, ZO-1 and occludin were observed in control and yeast-treated cell monolayers to investigate their involvement in the change in barrier potential. In control cells (Fig. 3a, g and j) F-actin, ZO-1 and occludin occurred at cell–cell contacts forming a distinct intact ring around the periphery of individual cells. After treatment with 2% (w/v) yeast suspensions for 1 h (Fig. 3b), actin staining around the apical part of the cell was less intense with some areas of discontinuous staining being present (as indicated by arrow). There was also an increase in cytoplasmic staining (as shown by arrow–head), implying disruption of cell–cell contacts. After the removal of yeast and following a 24 h recovery period, cell integrity seemed to be re-established with staining intact around the cell periphery, although some perinuclear staining was still visible (Fig. 3c). Basolateral actin filament localisation was also investigated, present in control (untreated) cells as long arranged filaments (Fig. 3d). In 2% (w/v) yeast-treated cell monolayers, the filaments appeared shorter and disorganised (Fig. 3e). Organisation and an increased length of filaments reappeared in cells following yeast removal (Fig. 3f). The application of 2% (w/v) yeast also caused an increase of occludin and ZO-1 fluorescence within the cell interior (Fig. 3h and k). After cell recovery, staining was again observed at the cell periphery and ruffled borders at cell–cell contacts was present, suggesting formation of new tight junctions (Fig. 3i and l).

Yeast Treatment Results in Movement of ZO-1 and Occludin Proteins from the Cell Membrane to Cytoskeletal Fractions

Caco-2 cell monolayers were treated with 0% (control) or 2% (w/v) yeast suspensions for 1 h or 24 h, after which some cells were allowed to recover in yeast-free medium. Cells were then lysed and separated into fractions, of either membrane, cytosolic, or Triton-insoluble (cytoskeletal) fractions. Immunoblotting of these fractions with ZO-1 or occludin antibodies allowed an understanding of the localisation of these proteins after yeast treatment, which could help to further elucidate the mode of yeast action on epithelial tight junctions. Within control treated cells (0% yeast), ZO-1 and occludin appeared mainly in membrane fractions, with some present in the cytosolic and sparing amounts in the Triton-insoluble cell fractions (Fig. 4). Following treatment with yeast for 1 h, a significant shift in ZO-1 from the cytosolic and membrane fractions occurred, to the Triton-insoluble fraction, with a loss of 34 and 82% from the former two fractions, to an increase of 157% in the latter (where $p = 0.042$, 5.83×10^{-4} and 0.029, respectively), implying a movement in tight junction proteins into the cytoskeleton. This shift was also observed with occludin, with a decrease of 32 and 65% of the protein from the cytosolic and membrane fractions of the cell, and an increase of 168% in the Triton-insoluble fraction ($p = 0.015$, 0.002, 0.005, respectively). After 24 h of yeast application, it was observed that although no significant change in cytosolic ZO-1 and occludin content had occurred compared to control ($p = 0.24$ and 0.36, respectively,) there was a clear and significant shift from the membrane to Triton-insoluble cell fractions, with a decrease of 71% ZO-1 and 50% occludin in the former fraction and an increase of 127% ZO-1 and 152% occludin in the latter fraction. After allowing yeast treated cells to recover for 24 h, it appeared that cells resembled those of control, with ZO-1 and occludin shifting back from Triton-insoluble fractions, to cytosolic and membrane bound fractions of the cell, where no significant difference to control was observed.

Electron Microscopy Revealed Changes in Caco-2 Cell Monolayer Junctions after the Application of Yeast

In order to observe changes in monolayer integrity at a higher level of resolution, cells treated with yeast were visualised by SEM and TEM. SEM was used to visualise the surface of Caco-2 monolayers after the application of yeast cells. Control cells appeared as smooth and intact cell sheets (Fig. 5a and c) whereas cell monolayers treated with 2% (w/v) yeast for 1 h revealed obvious gaps at cell–cell contacts (arrowheads), suggesting the opening of junctions (Fig. 5b and d). It appeared that yeast cells did not necessarily need to be at the point of contact between neighbouring cells in order to open tight junctions (arrow indicates yeast cells).

TEM was used to reveal the ultrastructure of Caco-2 cells treated with 0% (control) or 2% (w/v) yeast for 1 h. Control cells showed a continuous apical membrane with distinct microvilli (Fig. 6a). Electron-dense material between neighbouring cells, near the brush border implied the presence of an intact tight junction (arrow). After treatment with yeast for 10 min, yeast had adhered to the apical Caco-2 cell surface (Fig. 6b) and after 1 h of yeast treatment, a widening of tight junctions occurred (Fig. 6c arrowhead). After removal of yeast suspensions and subsequent recovery in fresh cell culture medium, there was no longer a gap between neighbouring cells and therefore the tight junction appeared intact (Fig. 6d), although some curling of microvilli was apparent.

DISCUSSION

There have been many previous studies which have investigated the effects of using penetration enhancers to disrupt epithelial integrity (reviewed in 25). However, none has considered yeast as a penetration enhancer. The present results confirm that yeast can be used to increase epithelial cell monolayer permeability by disruption of tight junctions.

Yeast had a dose- and time-dependent effect in reducing Caco-2 TEER (Fig. 1) and therefore tight junction integrity,

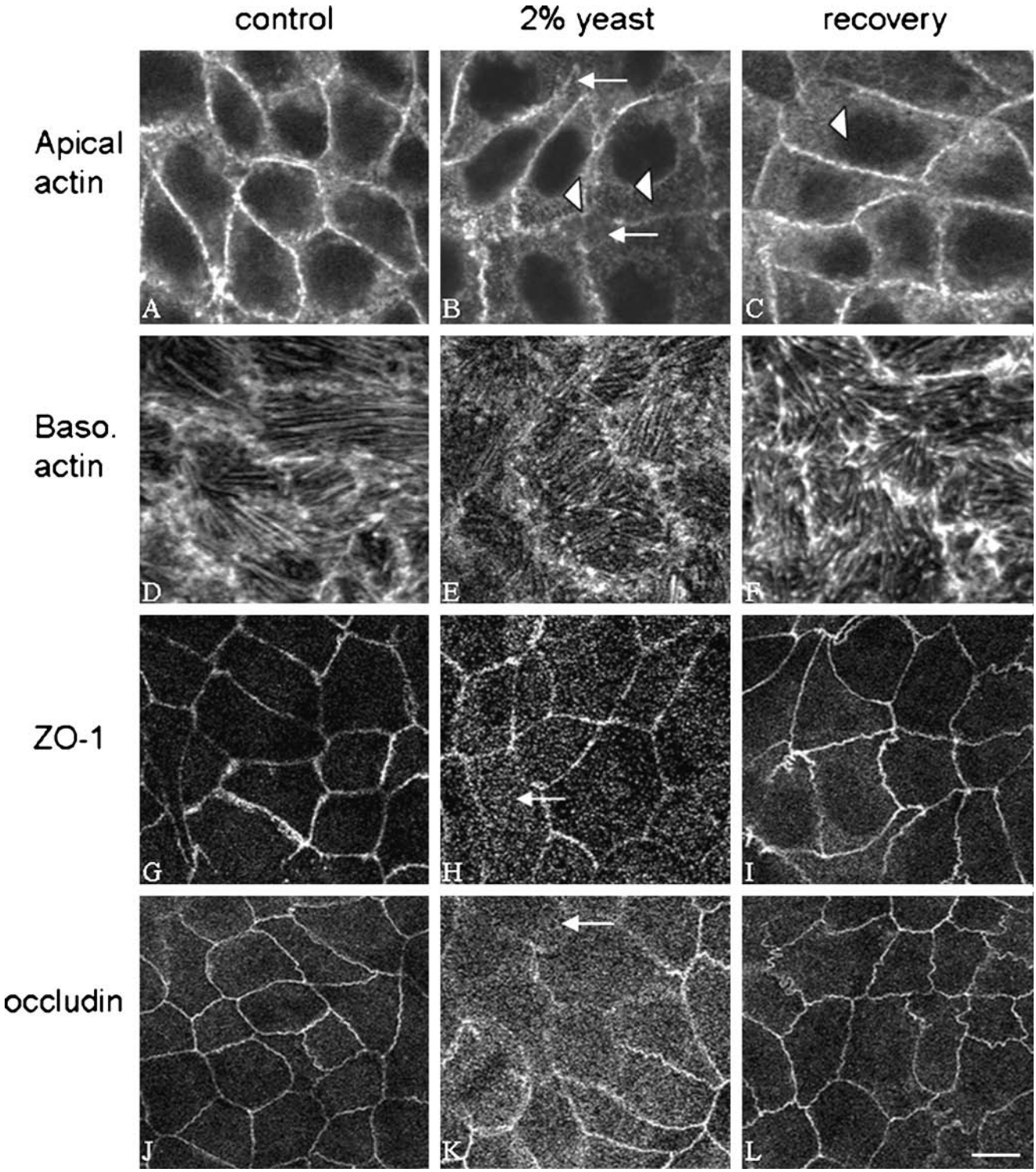

Fig. 3. Changes in the distribution and staining of tight junction associated proteins after the application of yeast cells to a Caco-2 monolayer. Control (A, D, G, J) or 2% (w/v) yeast suspensions (B, E, H, K) were applied to confluent cell monolayers for 1 h. For recovery of the barrier function, (C, F, I, L) cell monolayers were washed and allowed to recover in cell culture medium for a further 24 h. Cells were then washed and incubated with either FITC-phalloidin (for F-actin visualisation) or ZO-1 or occludin primary antibodies and appropriate fluorescently-labelled secondary antibodies before visualisation using a confocal microscope. Discontinuous staining (arrows) and an increase in cytoplasmic staining (arrowheads) was observed ($n \geq 4$. line = 10 μm).

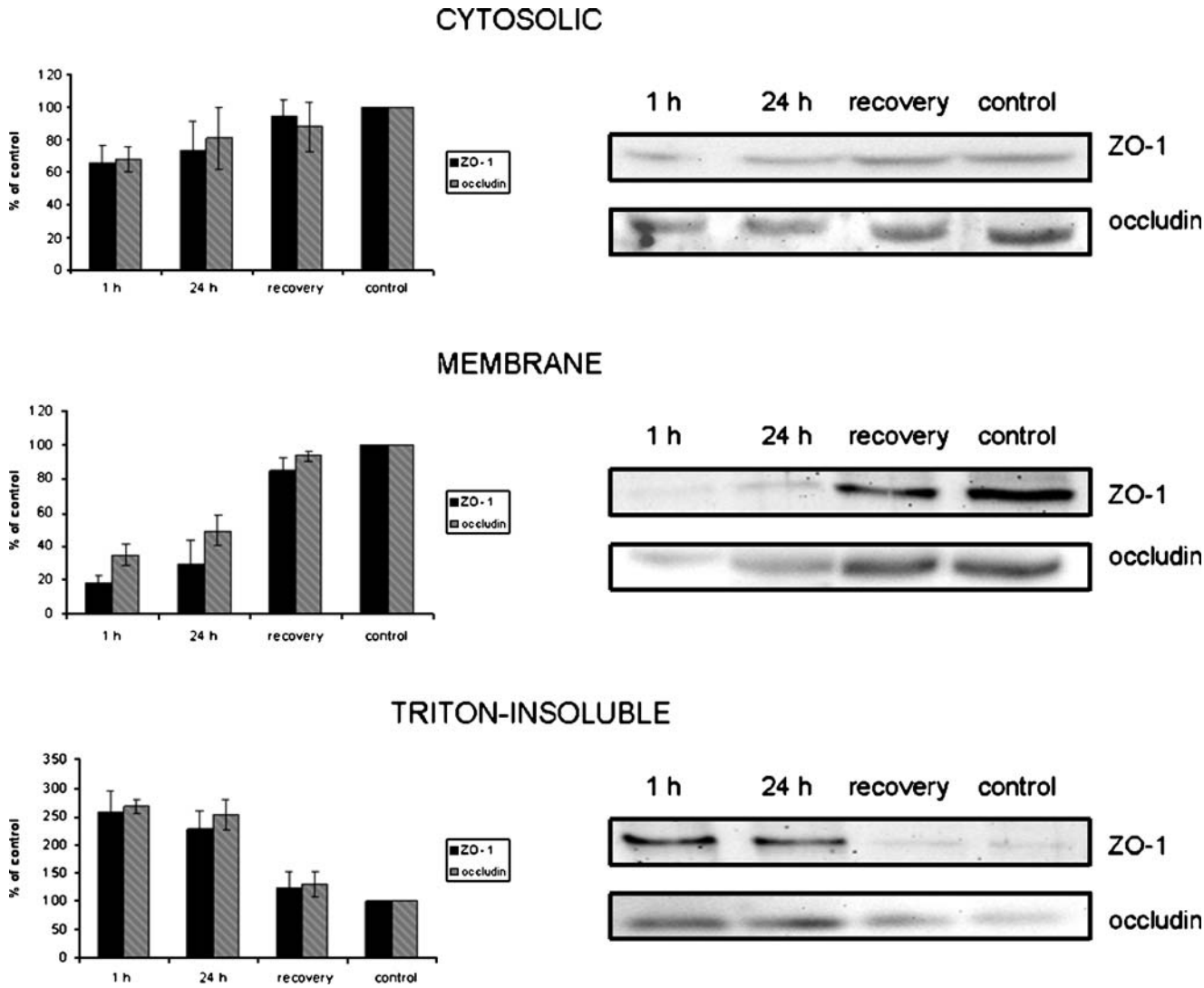

Fig. 4. Redistribution of ZO-1 and occludin tight junction proteins following treatment of Caco-2 cells with yeast. Confluent Caco-2 cells were treated with 0% (control) or 2% (w/v) yeast in cell culture medium for 1 h or 24 h. Following this, some cells were allowed to recover for 24 h in fresh cell culture medium. All cells were then fractionated into cytosolic, membrane and Triton-insoluble fractions (cytoskeletal) and immunoblotted for ZO-1 or occludin protein. Blots and densitometry studies showed that treatment with yeast caused a shift from the cytosolic and membrane-bound cell fractions to the Triton-insoluble compartment of the cell. $n = 4 \pm$ S.E.M.

which was further demonstrated by an increase in permeation of the protein, HRP (Table I). The effects observed after the application of yeast cells were shown to be due to opening of tight junctions rather than as a result of yeast-induced cell toxicity because no significant difference in cell viability was observed (Table I) as measured through the exclusion of trypan blue. These results therefore imply that the cell membrane was left intact following application of yeast. Due to the restoration of the cell barrier after yeast removal, as observed by an increase in TEER to baseline levels (Fig. 2), no epithelial damage or toxicity appears to have been sustained. Previous studies using a variety of penetration enhancers have demonstrated varying degrees of tight junction disruption. For example, *N*-trimethyl chitosan chloride (TMC) a quaternized chitosan derivative, was applied to Caco-2 cells for 1 h at 0.25% (w/v), and reduced TEER to 45 (26) and 70% (27) of baseline values, respectively. These penetration enhancers resulted in P_{app} ratios for mannitol that were comparable to those observed for HRP in this paper, despite the 220-fold difference in molecular weight (^{14}C mannitol 182.2 Da compared with HRP 40 kDa). Yeast as a penetration enhancer therefore shows obvious potential. Larger decreases in TEER to about 20% of baseline values were noted after the application of just 0.05% (w/v) of dodecylmaltoside (DDM) to Caco-2 cell monolayers in 1 h, with a P_{app} transport ratio of mannitol of 7.8×10^{-6} cm/s (28). However, after 12 h of recovery of epithelial cells, TEER was only at about 30% of the original, suggesting toxicity to Caco-2 cells could have been involved in the lower TEER levels. Recovery after yeast application is therefore rapid in comparison.

Failure of the barrier function to recover after treatment of an *in vivo* epithelium could lead to potential problems with infection or other toxic materials being absorbed. It is therefore paramount that penetration enhancers not only effectively and rapidly increase the permeability to poorly absorbed drugs at sub-toxic levels but also subsequently allow the full recovery of barrier function. In this study it was

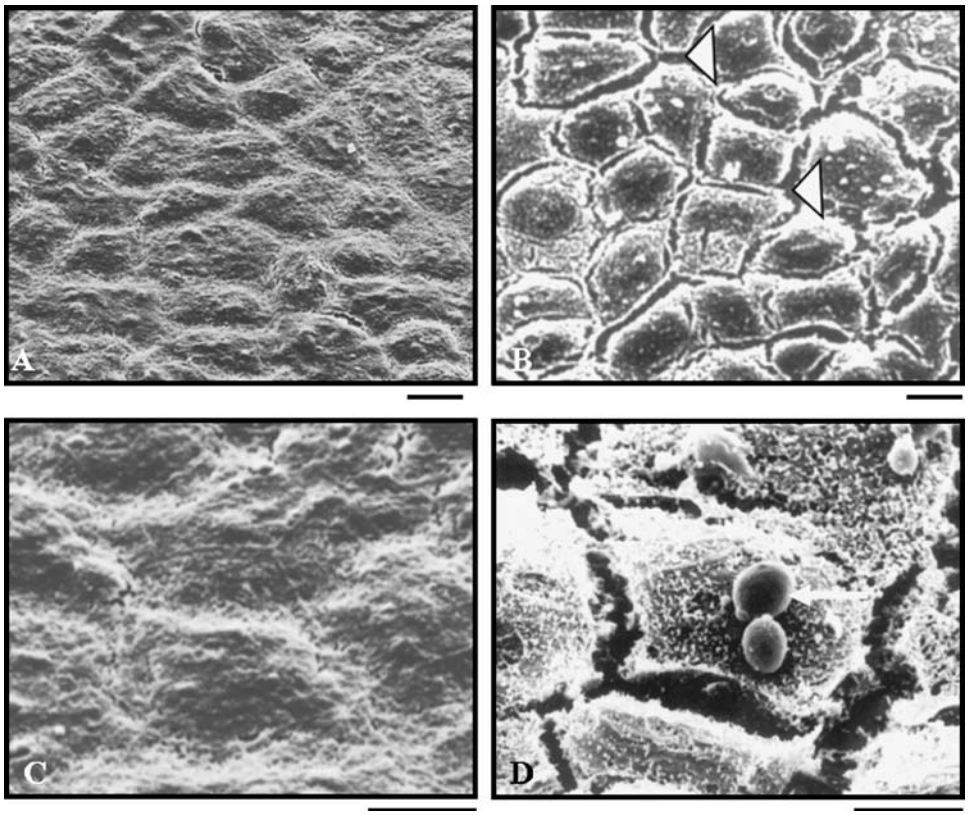

Fig. 5. Superficial changes observed after application of yeast suspensions to cell monolayers through SEM. Caco-2 cells were grown to confluence on microscope coverslips and treated with 0% (control) or 2% (w/v) yeast suspensions for 1 h. Following fixing and processing for SEM visualisation, control cells at low (A) and high (C) magnifications, showed intact and continuous cell sheets. After treatment with yeast suspensions, the cell sheet became cracked (B arrowheads), with obvious gaps present at cell borders when viewed at higher magnification (D). Bar = 10 μm, arrow = yeast cell.

observed that 24 h after the removal of yeast from cell monolayers, barrier function was fully re-established as indicated by a plateau in TEER (Fig. 2) and a reduction in HRP transport across the cell monolayer (Table I) to values not significantly different to the control. However, TEER did not reach 100% of baseline levels, as was the case after the application of chitosan and labrasol-containing microemulsions (23,29). This may have been due to the ease of small electrolytes being able to pass through newly formed tight junctions more readily than through mature ones. Mucoadhesive interactions of yeast cells with epithelial cells caused difficulties in totally removing yeast cells by washing without damaging the Caco-2 cell surface. Therefore it seems more likely that these explanations are the reason for absence of recovery to baseline levels rather than toxicity issues. Thus killed yeast cells are on the whole non toxic.

The effect of yeast on the proteins ZO-1 and occludin was investigated in order to elucidate the mode of action of yeast on epithelial tight junctions. Immunofluorescent staining with ZO-1 and occludin antibodies revealed a decrease in fluorescent intensity from some membrane-associated areas of the cells as well as an increase in protein presence within the cytoplasm in yeast treated cell monolayers compared to control (Fig. 3). This observation of discontinuous staining at cell–cell junctions or an increase in intracellular diffuse ZO-1 fluorescence was also noted after the application of labrasol containing microemulsions (29) and chitosan (23,30). The immunofluoresecence results concurred with those obtained after immunoblotting of yeast-treated cell fractions, with both ZO-1 and occludin proteins (Fig. 4). Following yeast treatment, a significant shift of both ZO-1 and occludin was observed from the cytosolic and membrane bound regions of the cell, to the Triton-insoluble fraction (cytoskeletal), demonstrating a change in tight junction integrity. After thorough removal of yeast from the cell monolayer by washing, ZO-1 and occludin proteins fully returned to control conditions and therefore tight junction integrity was resumed, as confirmed by the rise in TEER and the impermeability to HRP. Similar translocation of the tight junction proteins, from membrane to cytoskeleton fractions occurs during PKC activation, therefore it is possible that yeast-mediated tight junction opening involves the signal transduction pathway, as was previously reported as the mechanism of chitosan action (31). However this is not as yet clear and it is possible that other mechanisms are involved.

Tight junction proteins are believed to be closely associated with the actin cytoskeleton (32) therefore it was important to investigate whether changes observed in tight junction localisation after the addition of yeast would correlate with F-actin localisation (Fig. 3). This indeed appeared to be the case. In control cells (0% yeast), F-actin was present largely at cell–cell contacts but after yeast application, an increase in cytoplasmic actin staining was observed. This could suggest a retraction of the actin belt. The appearance of stress fibres

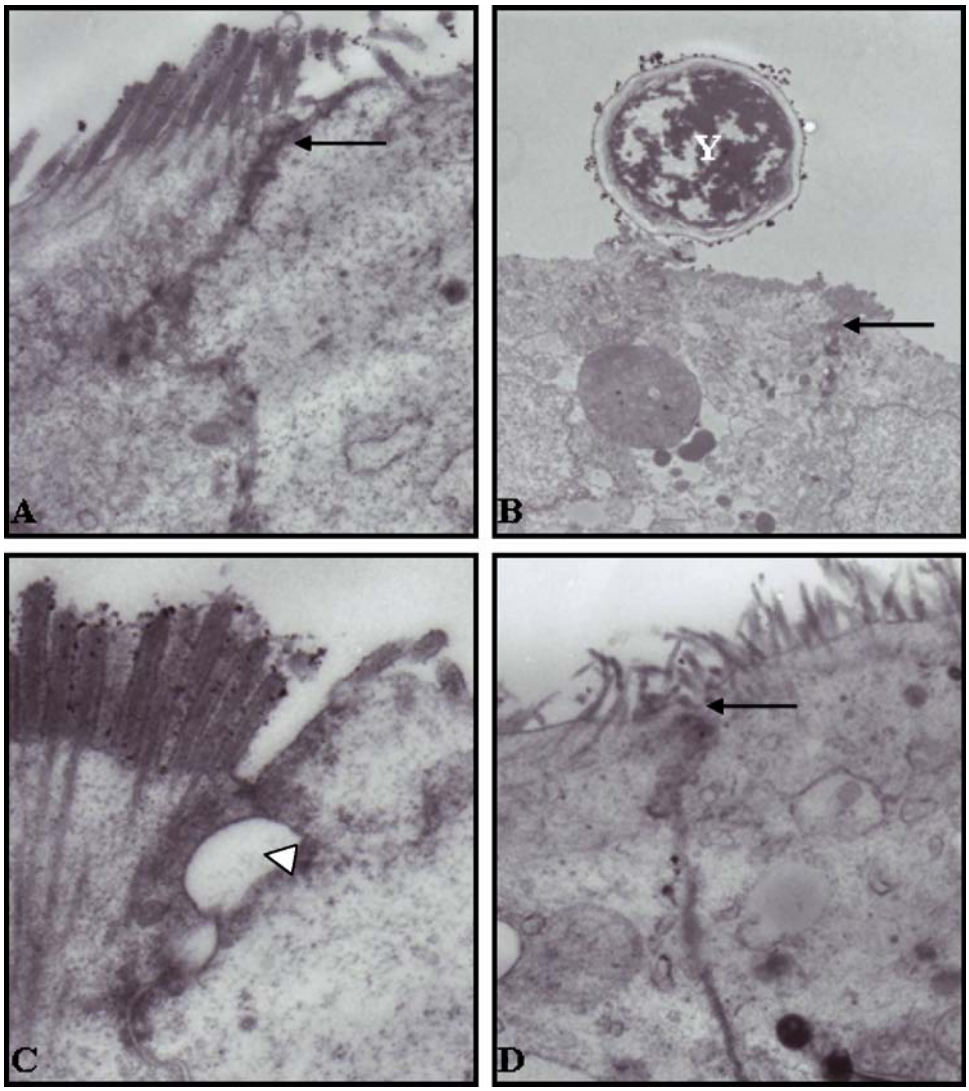

Fig. 6. Ultrastructural changes to Caco-2 cells following the application of yeast, observed by transmission electron microscopy. Caco-2 cells were grown to confluence on microscope coverslips and treated with 0% (control) or 2% yeast suspensions for 1 h. Some cells were then allowed to recover for 24 h in fresh cell culture medium. Following fixing and processing for TEM, intact tight junctions (electron dense area indicated by arrow) were observed in control cells (A). After yeast treatment for 10 min (B), a yeast cell was observed adhered to the Caco-2 cell surface and after 1 h, a gap appeared between neighbouring cells (arrowhead). Following removal and recovery of cells, the tight junction reformed (D). Y = yeast.

seemed to change slightly after the application of yeast, which concurred with observations following the addition of chitosan (31), with long laterally arranged fibres present in the control and shorter disorganised fibres present after treatment. An increase in fluorescent staining of F-actin within the cytoplasm was also noted after the addition of protamine (33). It could be concluded therefore, that the rearrangement of the cytoskeleton was the precursor to tight junction disruption.

Electron microscopy was used to directly show the opening of cell junctions after the application of yeast to previously confluent cell monolayers. Scanning electron micrographs showed a superficial change to monolayer integrity, where a tightly packed pavement of cells in the control became a cracked cell sheet with obvious gaps present at cell–cell borders after the application of yeast (Fig. 5). To observe ultrastructural changes to the cells, transmission electron micrographs were taken. Fully formed and tight junctions were observed in control cells whereas a widening of junctions between neighbouring cells after yeast treatment occurred (Fig. 6). The electron-dense area still present at the cell periphery after yeast treatment suggested that tight junctions had not been damaged. Side-effects of yeast treatment seemed to be the appearance of large vacuoles in the cytoplasm and modification of the brush border. Indeed both these observations have been reported after the application of chitosan (30) and vanadium compounds (34). After the application of positively charged labrosol-containing microemulsions, the microvilli became curled in appearance and also the gap between tight junctions became apparent (29). This apparent damaging effect to the microvilli perhaps explains why the recovery of the cell monolayer was slow. However, protecting mucus present *in vivo* on the apical sur-

face of cells would certainly decrease this effect (35). Clearly this may also impair the effectiveness of particulate penetration enhancers such as yeast.

CONCLUSION

In conclusion, yeast cells can be used to increase the permeability of Caco-2 cell monolayers as shown by a decrease in TEER, thus allowing active ingredients to be transported by the paracellular route, in a non-toxic and reversible manner. The mode of action of cell disruption by yeast would appear to be by translocation of ZO-1 and occludin tight junction associated proteins from the membrane to cytoskeletal cell areas, which could involve PKC activation. Further work needs to be carried out in this novel area so that this process can be elucidated and thus optimised. These preliminary data suggest that yeast could be used as a penetration enhancer of drugs, to facilitate delivery across the mucosa.

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