International Journal of Pharmaceutics 407 (2011) 21–30

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Roles of pre-treatment time and junctional proteins in Caco-2 cell microparticle uptake

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ARTICLE INFO

Article history:
Received 6 September 2010
Accepted 29 December 2010
Available online 8 January 2011

Keywords:
Caco-2
Latex microparticle uptake
Ethanol
Low temperature
Transepithelial resistance (TER)
Occludin
ZO-1
E-cadherin

ABSTRACT

Microparticle uptake in the small intestine is relevant to both the delivery of pharmaceutics and exposure to environmental pollutants. The Caco-2 enterocyte model is a useful tool to study the parameters that affect epithelial microparticle permeability and the mechanisms controlling them. The current study used this model to explore further the different effects of 10% ethanol v/v or ice on transepithelial resistance (TER), microparticle uptake and immunofluorescent labelling of intercellular junctions. The same exposure times for both treatments were used, rather than those shown in the literature to produce demonstrable changes induced by each. The effects of both pre-treatments were greater after 60 min than after 15 min. Ethanol pre-treatment for 60 min decreased TER, increased particle uptake and was associated with a disorganisation of tight and adhering junctional proteins. Pre-treatment with ice for 60 min however, increased TER, decreased particle uptake and was associated with concentration of intercellular junctional proteins in a more constrained manner. These findings on the effects of pre-treatment with ethanol or ice for 60 min suggest that the extent of uptake is influenced by changes in the distribution of intercellular junctional proteins.

1. Introduction

Caco-2 cell monolayers provide a popular model of small intestinal epithelium (Delie and Rubas, 1997) due to their morphological and physiological similarities to the cells in the *in vivo* situation. They are a useful tool for the study of microparticle uptake, relevant to drug delivery and environmental challenge (Ravi Kumar, 2000; Florence and Hussain, 2001; Krauland and Bernkop-Schnurch, 2004; Hodgson et al., 2005; Stather, 2007). The current paper stems directly from two recent publications from the group.

The effects on the Caco-2 model of exposure to ethanol and lower temperatures used were previously reported (Moyes et al., 2010a). However, the times chosen for these 'pre-treatment' effects were those that, from literature reports, should have given identifiable effects (Armitage et al., 1994; Ma et al., 1999). This meant that ethanol was given 60 min to produce its tight junction (TJ) opening effect, as shown by lower transepithelial resistance (TER): it also increased microparticle uptake. On the other hand, rapid cooling of the culture by standing it on ice for 15 min, while closing TJs, did not decrease the number of particles taken up. Since this 15-min exposure was so much shorter than the 60 min used for the ethanol, it seemed appropriate to explore whether chilling for the longer time would affect uptake.

The results of the immunofluorescent labelling of intercellular junctional proteins were also linked (Moyes et al., 2010b) with the group's earlier *in vivo* transmission electron microscopy work showing particles within intercellular spaces (Smyth et al., 2005). Labelling for tight junction proteins occludin and ZO-1 confirmed their apical position and outlined the regular polygonal nature of the untreated Caco-2 cells, while labelling for E-cadherin, actin and nuclei provided more information on the structure of the epithelium. Despite the rigorous nature of the immunofluorescence protocol, many of the fluorescent latex microparticles remained: more that two thirds of these were located at or near intercellular junctions (Moyes et al., 2010b).

The current paper therefore tests the hypothesis that increasing pre-treatment time on ice, in addition to increasing transepithelial resistance (TER), decreases Caco-2 particle uptake: this would indicate that a 60-min pre-treatment time is appropriate for the comparison of the effects of ethanol and low temperature. It also explores whether the different responses in TER and microparticle uptake produced by pre-treatment with ethanol or ice are matched by different changes in junctional proteins.

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doi:10.1016/j.ijpharm.2010.12.033

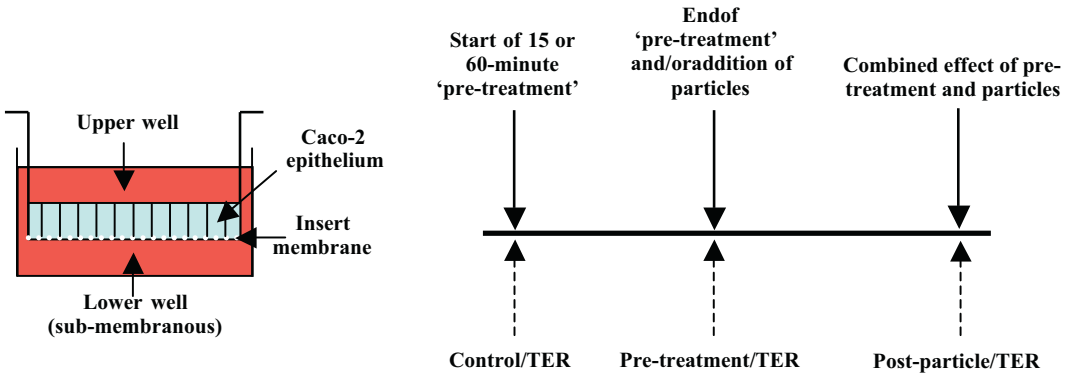

Fig. 1. *Schematic of the Caco-2 model and timeline of experimental procedures.* Caco-2 cells were cultured on insert membranes with 3 μm pores over a 6-well receiver plate. Fluorescent latex microparticles 2 μm in diameter were added to the upper well: the numbers counted in the lower well at the end of the experiment indicated the extent of uptake across the epithelium. After the post-particle/TER is recorded (right-hand arrows), sample collection for particle uptake and immunofluorescent labelling are carried out.

2. Materials and methods

2.1. Protocol outline

The effects on epithelial permeability and microparticle uptake of Caco-2 epithelium were studied after pre-treatment with 10% ethanol v/v at 37 °C or ice for either 15 or 60 min: the TER measurement after pre-treatment was called 'pre-treatment/TER' and after combined pre-treatment and particle exposure was called 'post-particle/TER' (Fig. 1). Fluoresbrite® yellow-green latex microparticles in distilled water (Polysciences Inc.; 1 μl; 2 ± 0.1 μm diameter; 5.68×10^6 particles/μl) were added to the upper well of some groups and left for 5 or 60 min. Group names reflected both the extent of pre-treatment and whether or not particles were added (e.g. untreated; particle-only/5; ethanol-15/particle-5; ice-60/particle-60). As 60-min pre-treatment times produced the greater changes to both permeability and uptake, immunofluorescence labelling of junctional proteins, actin and nuclei was carried out on Caco-2 cells at this time point both with and without exposure for a further 60 min to particles and the continuing effects of the relevant pre-treatment.

2.2. Caco-2 cell culture

ATCC Caco-2 cells (HTB-37™, passages 24–42), were cultured in a 37 °C incubator with a 5% CO_2 atmosphere in ATCC-formulated Eagle's Minimum Essential Medium supplemented with 10% foetal bovine serum (FBS) Gold (PAA Laboratories) and 1% penicillin/streptomycin (Gibco®). Cells were seeded at a density of 1×10^6 cells per 6-well polyester Transwell® insert (3 μm pores, Corning Costar) and were cultured to confluence over 21 days.

2.3. Assays and sample preparation

2.3.1. TER measurements and data analysis

Before cell seeding, the TER of the Transwell® insert membrane with culture medium (TER_{Insert}) was measured by use of a Millicell®-ERS instrument (Millipore). On day 21 of culture, the TER for the epithelium on the insert was measured before any treatment (control/TER), after pre-treatment (pre-treatment/TER) and after particle exposure (particle/TER). Proportional (%) TER changes following pre-treatment, subsequent particle addition or their combined effects were calculated as previously described (Moyes et al., 2010b).

2.3.2. Immunofluorescence

Immunofluorescence for proteins at tight and adhering junctions and also for actin was carried out, as previously described (Moyes et al., 2010b), for particle-only groups and for 60-min pre-treatment groups. After pre-treatment and exposure to particles for the predetermined length of time, the Caco-2 epithelium was fixed in 4% paraformaldehyde in PBS. Sodium citrate buffer was used for antigen retrieval (Shi et al., 1993) and cells permeabilised in 0.1% Triton X-100 for 15 min before blocking in 3% bovine serum albumin for 30 min. Primary antibodies, incubated for 2 h, were rabbit anti-occludin (Invitrogen 71-1500, 1 ng/μl), rabbit anti-ZO-1 (Invitrogen 61-7300, 25 ng/μl) or mouse anti-E-cadherin (Abcam ab1416, 500 ng/μl). The cells were washed, incubated for 1 h with biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG (Vector Laboratories, 15 ng/μl) and then exposed to a mixture of fluorescein Avidin-D (Vector Laboratories, 20 ng/μl) and TRITC-labelled phalloidin-tetramethylrhodamine B for F-actin (Sigma 77418, 20 ng/μl), in PBS for 45 min. After washing, the samples were mounted using Mowiol mounting medium containing DAPI. Negative controls, omitting either the primary antibody or the actin stain, were also carried out.

All images were taken using a Zeiss LSM510 confocal microscope and analysed with LSM Image Browser software for the distribution of the junctional proteins and cytoplasmic f-actin. For junctional protein distribution, staining was analysed in a minimum of 6 sites of 3 samples from 2 to 3 experiments. Low power z-stack images (20×) were taken to record the uniform staining and high power z-stacks (63×, sometimes with 2× digital zoom) for charting the relationships between the particles, the junctions and other parts of the cell. For cytoplasmic actin distribution, 18 cells were analysed from 9 samples over 2–3 experiments per treatment group.

The parameters of the junctional proteins (Table 1) included: (a) Apical position. This is in the *z*-plane, at the level of the upper edge of the protein, measuring down from the apex of the cell; (b) Extent downwards. This is in the *z*-plane, parallel to the long axis of the cell and is the distance from the upper to the lower border of the junctional protein; (c) Lateral width. This is in the *x*–*y* plane and represents the site where two cells meet. This is marked by the presence of the junctional protein.

These parameters for belt-like/linear, punctuate or spherical structures/multicellular junctions (MCJ) were measured with reference to the cell dimensions. The cell height was measured in the *z*-plane from the first to the last appearance of actin. For E-cadherin, measurements in Table 1 refer either to the belt-like

Table 1
Effects of pre-treatment for 60 min with ethanol or ice on Caco-2 junctional parameters. Parameters relating to junctional location and dimensions were taken from 18 cells per treatment group, while those concerning the particle/cell relationship were from 18–25 confocal images per treatment group. Definitions for the parameters included in this table are in Section 2.3.2. E-cadherin labelling was separated into that representing either a zonula adherens (ZA) or a desmosome/macula adherens (MA). Statistical differences ($p \leq 0.05$) are: 'OCC' and 'ZO-1' from within the same treatment group, 'CACO' for a change from the equivalent untreated Caco-2 group; 'ETOH' from the equivalent ethanol 60 group; 'BELT' comparing multicellular junction (MCJ) and belt formations of the same junctional protein.

	Caco-2 cells without pre-treatment			Ethanol 60 minutes			Ice 60 minutes		
	Occludin	ZO-1	E-cadherin	Occludin	ZO-1	E-cadherin	Occludin	ZO-1	E-cadherin
Cell height	23.0 ± 0.6			21.0 ± 0^{CACO}			21.0 ± 0^{CACO}		
Apical position of junctional protein	0.2 ± 0.08	0.3 ± 0.09	0.4 ± 0.14	0.4 ± 0.1	0.7 ± 0.1^{CACO}	1.0 ± 0.14^{CACO}	$0.7 \pm 0.1^{6\,CACO}$	$0.6 \pm 0.1^{2\,CACO}$	$0.1 \pm 0.0^{6\,CACO\ ETOH}$
Extent downwards of junctional protein	2.0 ± 0.07	1.8 ± 0.13	ZA: 1.5 ± 0.17^{OCC} MA: 15.0 ± 0.59	6.3 ± 0.9 CACO	2.6 ± 0.2^{CACO}	ZA: 2.5 ± 0.6^{CACO} MA: 12.4 ± 1.5	1.6 ± 0.11^{CACO}	$1.3 \pm 0.0^{6\,CACO}$	ZA: $1.2 \pm 0.08^{OCC\ ZO\text{-}1}$ MA: 10.5 ± 0.14^{CACO}
Lateral width of junctional protein	0.5 ± 0.01	0.5 ± 0.04	ZA: 0.5 ± 0.02 MA: 0.8 ± 0.07	1.2 ± 0.2 CACO	1.1 ± 0.1^{CACO}	ZA: $1.0 \pm 0.1^{2\,CACO}$ MA: 1.1 ± 0.14	$0.3 \pm 0.0^{3\,CACO}$ ETOH	$0.4 \pm 0.0^{3\,CACO}$ ETOH	ZA: $0.4 \pm 0.03^{ZO\text{-}1\ ETOH\ CACO}$ MA: 1.0 ± 0.12
Apical position of MCJ	0 ± 0	0.2 ± 0.10	–	–	–	–	0.4 ± 0.2	0.8 ± 0.41	–
Extent downwards of MCJ	2.8 ± 0.2^{BELT}	2.7 ± 0.30^{BELT}	–	–	–	–	2.4 ± 0.4^{BELT}	2.4 ± 0.26	–
Lateral width of MCJ	1.0 ± 0.4	1.2 ± 0.30	–	–	–	–	0.8 ± 0.03^{BELT}	1.4 ± 0.17^{BELT}	–
Remaining particles/sample	1.1 (18 samples)			2.8 (25 samples)			1.6 (25 samples)		
Particles on junctions (%)	48.7 ± 12.7			51.9 ± 11.1			54.9 ± 12.8		
Particles near junctions (%)	46.2 ± 13.2			26.4 ± 8.9			43.4 ± 12.5		
Particles not near junctions (%)	0.4 ± 0.4			15.3 ± 9.3^{CACO}			0.7 ± 0.7^{ETOH}		

zonula adherens (ZA) or the deeper presence of punctuate staining representing the presence of desmosomes/maculae adherentes (MA).

To relate the position of the remaining particles to the localisation of tight and adhering junctions 60 min after particle exposure, 18–25 confocal images from at least 3 experiments per group were analysed and particles categorised as on, near or distant from intercellular junction: those categorised as 'near' were within 2 μm of a junction.

2.3.3. Estimation of numbers of sub-membranous particles

Sub-membranous particle counts were carried out as previously described: briefly, particles in the lower well medium and those washed off the underside of the insert membrane were counted by UV microscopy on a sample-by-sample basis (Moyes et al., 2010b).

2.4. Pre-treatment groups

2.4.1. Ethanol pre-treatment

The existing medium was replaced by ATCC culture medium previously warmed to 37 °C and containing 10% ethanol v/v ($n = 11$ inserts over four independent experiments). This remained in place during incubation at 37 °C for 15 or 60 min. Some groups were then exposed to particles for either 5 or 60 min (ethanol-15/particle-5, ethanol-15/particle-60, ethanol-60/particle-5, ethanol-60/particle-60).

2.4.2. Temperature pre-treatment

The 6-well culture plates with their inserts were placed on ice for 15 or 60 min in the cell culture hood ($n = 11$ inserts over of four independent experiments). Some cultures were then exposed to particles for either 5 or 60 min (ice-15/particle-5, ice-15/particle-60, ice-60/particle-5, ice-60/particle-60).

2.5. Statistical analysis

Following the Levene's test showing homogeneity of variance, group ANOVA and Student's *t*-test statistical analyses were carried out on the transepithelial resistance, particle numbers and junctional dimensions and location, as measured. For the particle/cell relationship data, as the Levene's test did not show homogeneity of variance the non-parametric Kruskal–Wallis ANOVA and Mann–Whitney *U* tests were carried out. Quantitative results were included in the text if they were significantly different ($p \leq 0.05$) with respect to the values of reference groups.

3. Results

3.1. Untreated Caco-2 epithelium

Untreated Caco-2 cells on day 21 had a control/TER value of $442 \pm 6\,\Omega\,cm^2$. Actin staining was found apically as a horizontal 'sheet' along the lateral walls of the cells and also co-localising with the basally located nuclei. This resulted in an actin-free supranuclear region in most cells. The nuclei were all oval, with their long axes parallel to those of the columnar cells, they were almost without exception located basally.

Immunofluorescent labelling of junctional proteins (Fig. 2, left hand column, Table 1) outlined predominantly regular polygonal cells, in a columnar sheet: a few cells were more pleomorphic in shape. Occludin and ZO-1 labelling were continuous around the lateral wall of the cells and located near the apex of the cell at the level of the TJ: both formed a sharply defined, discrete belt extending little further into the cell. At the point where several cells met at a multicellular junction (MCJ) there was a larger accumulation of TJ proteins, appearing at the same depth as the main belt-like outlines

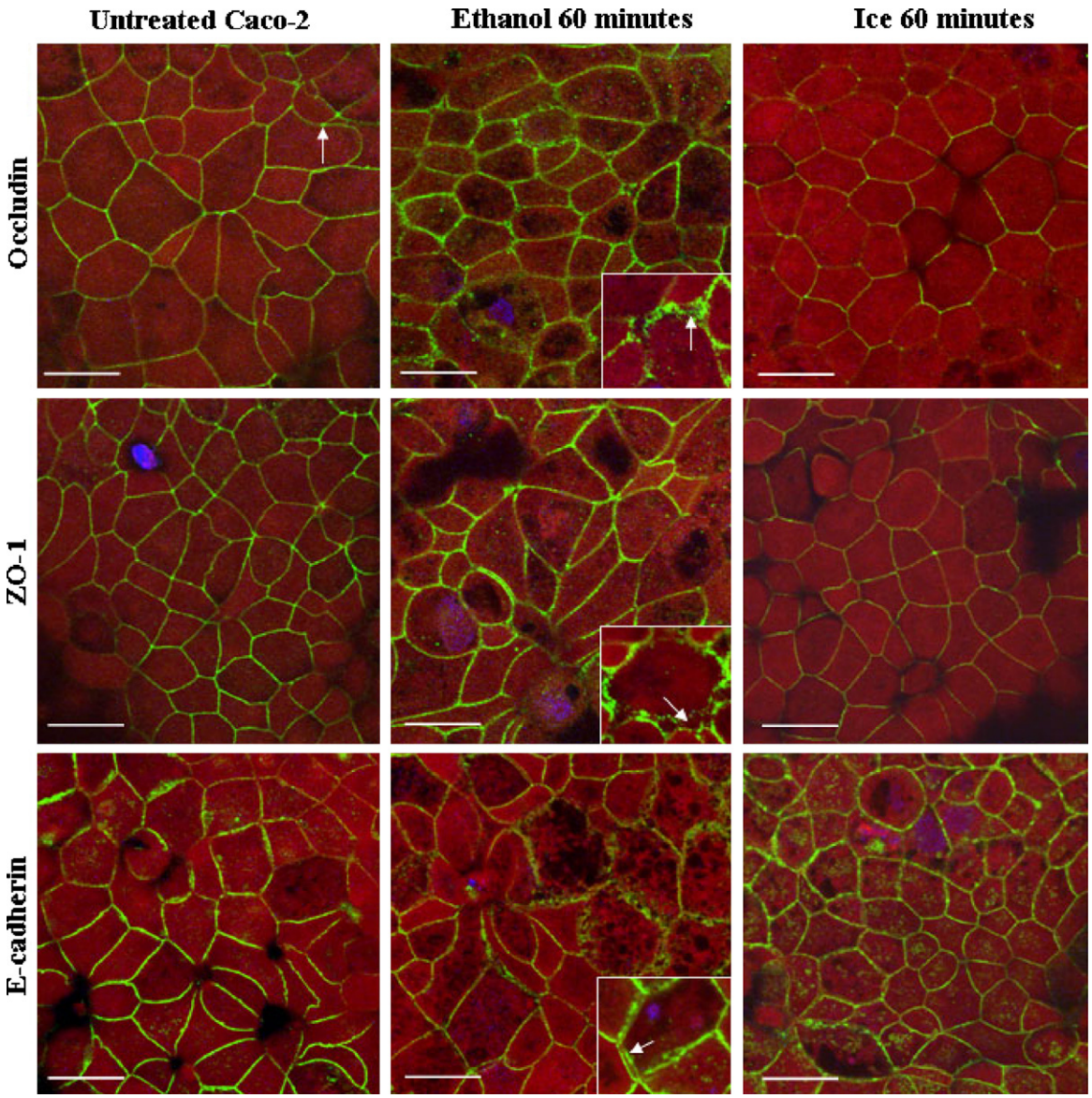

Fig. 2. *Effects of pre-treatments on Caco-2 cell junctional proteins and morphology*. Untreated Caco-2 cells (left column) and those pre-treated with ethanol (middle column) or ice (right column) were labelled for occludin (top), ZO-1 (middle) or E-cadherin (bottom) junctional proteins (FITC), actin (TRITC) and a nuclear counter-stain (DAPI). All confocal images were taken at 63× and the scale bars indicate 20 μm. Inserts in the middle, ethanol pre-treatment column highlight representative structures of interest (arrows, 126×), with that for occludin showing the diffuse nature of this intercellular junction, ZO-1 irregular separation of the junctional labelling and E-cadherin the double lines between adjacent cells. Darker areas are not indicative of epithelial discontinuities but represent either variations in height of the cells or the supranuclear actin-free region. The bright, spherical aggregations of junctional protein are best illustrated in the top left image by the arrow. (The reader is referred to the web version of the article to see these images in colour.)

of the cells. These sharply defined, spherical aggregations were also brighter and extended deeper into the cell than the junctional belt with which they were associated (Table 1, $p = 0.0001$).

E-cadherin also formed a continuous sharply defined apicolateral belt around each cell, at the level of the zonula adherens (Table 1). Below this point on the lateral wall the labelling became more diffuse and punctuate, possibly representing desmosomes: this continued down to the base of the cell.

3.2. Effects of pre-treatment alone

Pre-treatment, with 10% ethanol for 15 min, increased TER when compared to control/TER ($p = 0.0001$, Fig. 3A), whereas 60-min pre-treatment caused a TER decrease ($p = 0.0001$). Many cells lacked the actin-free supranuclear region seen in untreated Caco-2 cells. Finally, the nuclei were more varied in shape, apico-basal location and relationship to the long axes of the cells.

Immunofluorescent labelling of junctional proteins (Fig. 2, middle column, Table 1), carried out only after a 60-min pre-treatment, outlined a columnar but more disorganised epithelium, with more pleomorphic cells. Occludin and ZO-1 labelling were still both discrete apicolaterally, but by comparison with the untreated Caco-2 cells the labelling extended further downwards ($p = 0.0001$) and was wider laterally ($p = 0.0001$). Furthermore, the occludin distribution was more diffuse, while ZO-1 localisation remained sharp. The highly stained multicellular junctions seen in the untreated Caco-2 cells were not a marked feature after ethanol pre-treatment. Instead, more diffuse, less brightly stained regions could be identified as possible sites of cell separation (Fig. 2, middle column, ZO-1, insert). The most striking change caused by ethanol pre-treatment

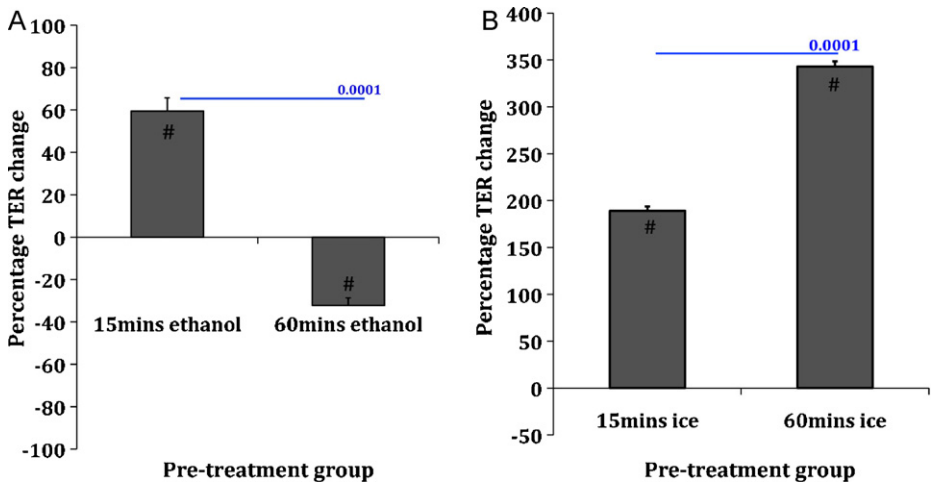

Fig. 3. *Effect of pre-treatment exposure time on Caco-2 cell transepithelial resistance (TER).* Caco-2 cells were incubated with 10% ethanol v/v or placed on ice for either 15 or 60 min. Histograms illustrate the percentage TER change following ethanol (A) and ice (B) pre-treatments after both time points. Significant differences ($p \leq 0.05$) are indicated by '#' for a change from control/TER and by interconnecting lines for differences between treatment times.

for 60 min was the presence of double lines of junctional protein, with some showing a marked separation between them where actin was not visible in the intervening space. This was most often seen with ZO-1 labelling and was the most extreme form of the widening of the intercellular junctional regions visible for all three proteins.

Double lines were also seen with E-cadherin labelling (Fig. 2, middle column, E-cadherin insert). Furthermore, the labelling at the level of the zonula adherens was less sharp than was the case for the untreated Caco-2 cells: it was situated less apically ($p = 0.01$), extended further downwards ($p = 0.0001$) and was wider laterally ($p = 0.0001$). The deeper, possibly desmosome-associated E-cadherin staining was less punctuate.

For Caco-2 cells exposed to ice, TER increased with pre-treatment time ($p = 0.0001$, Fig. 3B). Actin staining was similar to that seen in the untreated Caco-2 cells.

After pre-treatment, for 60 min, immunofluorescent labelling of junctional proteins (Fig. 2, right hand column, Table 1) outlined a regular epithelium of predominantly polygonal cells similar to the pattern of untreated controls. Occludin and ZO-1 labelling were both continuous apicolaterally: by comparison with the untreated Caco-2 cells these proteins were found further from the cell apex ($p = 0.05$ and 0.04 respectively) but extended less far down the lateral border of the cell ($p = 0.001$ and 0.003 respectively) and formed a less wide intercellular belt ($p = 0.0001$ and 0.003 respectively). E-cadherin labelling was also discrete at the level of the zonula adherens, but was more diffuse at the level of the desmosomes.

3.3. Particle-only group

After exposure to particles for 5 min the TER for the Caco-2 particle-only group increased ($p = 0.0001$, Fig. 4A), while after 60 min the increase was significantly diminished ($p = 0.0001$). The number of sub-membranous particles increased with exposure time ($p = 0.0001$, Fig. 4B). Confocal microscopy of the labelled samples which had been exposed to particles for 60 min showed that a substantial number remained associated with the epithelium (Fig. 4C, Table 1).

3.4. Combined effects of ethanol and particles on Caco-2 epithelium

For the ethanol-15/particle-5 group the large combined TER increase was little greater than that caused by the ethanol pre-treatment alone, implying little particle-induced effect: on the other hand, for the ethanol-15/particle-60 group there was a marked particle-induced TER decrease ($p = 0.0001$, Fig. 5A). However, the situation for both the 60-min ethanol pre-treatment groups is different, with the ethanol-60/particle-5 group's small combined TER decrease masking a particle-induced increase, whereas TER changes for the ethanol-60/particle-60 group are decreased from the relevant comparator ($p = 0.001$–0.0001). Sub-membranous particle numbers for the 15-min ethanol pre-treatment groups were similar to those for the particle-only groups, increasing with exposure time from 5 to 60 min ($p = 0.04$, Fig. 5B). However, those for the 60-min pre-treatment groups were markedly higher than the particle-only groups at both time points ($p = 0.008$ and 0.0001).

Confocal microscopy of the labelled ethanol-60/particle-60 samples showed many of the changes seen after pre-treatment alone. In addition, while a substantial number still remained associated with the epithelium, fewer of these, particularly those near junctions, were associated with junctions than seen with the particle-only/60 group, although the percentage remained high (Fig. 6, left hand column, Table 1).

3.5. Combined effect of low temperature and particles on Caco-2 epithelium

For both ice-15/particle-5 and/particle 60 groups the larger combined TER increase is attributable to both the earlier pre-treatment and particle addition ($p = 0.0001$ and 0.02 respectively), with the longer particle exposure producing a more marked effect ($p = 0.0001$): on the other hand, for the ice-60/particle-5 group the particle effect is that of a TER decrease ($p = 0.02$, Fig. 7A). Sub-membranous particle numbers for the 15-min ice pre-treatment group were similar to those for the particle-only groups, increasing with particle exposure time ($p = 0.03$, Fig. 7B). However, those for the 60-min pre-treatment group were markedly lower for than the particle-only groups ($p = 0.02$).

Confocal microscopy of the labelled ice-60/particle-60 samples showed many similarities to the features of the groups exposed to ice alone. With respect to the relationship between the cells and particles, a substantial number was still associated with the epithelium, the distribution was similar to that of the particle-only/60 group and the overall percentage associated at or near junctions remained high (Fig. 6, right hand column, Table 1).

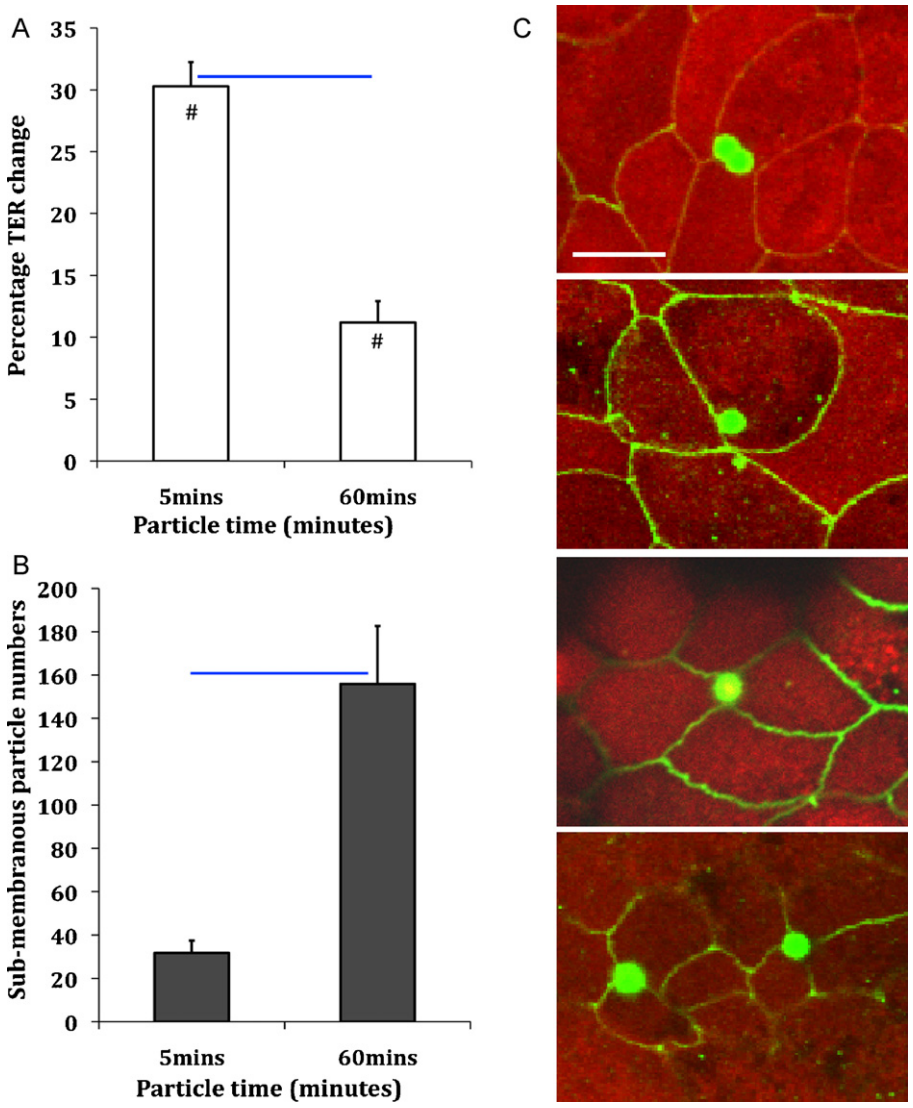

Fig. 4. *Effects of particle exposure time on transepithelial resistance (TER), sub-membranous counts and particle/cell interactions for the particle-only group of Caco-2 cells.* Caco-2 cells were exposed to particles for 5 or 60 min. Epithelial permeability was measured by TER before and after this treatment (A) and particle uptake by the number of sub-membranous particles (B). Significant differences ($p \leq 0.05$) are indicated by '#' for a change from control/TER and by interconnecting lines for differences between treatment times. Confocal images of the different relationships between microparticles (fluorescent spheres) and junctional proteins (FITC-labelled lines) is also illustrated (C), with cells TRITC-labelled for actin. Scale bar is 10 μm and applies to all four images. The second top confocal image illustrated one particle categorised as 'near' a junction, while the others have a particle, or particles, on a junction or at a multicellular junction (MCJ).

4. Discussion

Some routine protocol points require comment before any conclusions can be drawn about the mechanisms involved in the interaction between the Caco-2 cell epithelial sheet and the microparticles added apically to it. The assays used here have all been used before and the current control data compared well with previous results. The TER measurements for untreated Caco-2 cells were of a similar range to those reported previously (Delie and Rubas, 1997). The only minor protocol variation with respect to TER was the use of percentage values to report the changes seen, now the method of choice. The technique for counting particles which have passed through the epithelium into the lower well is also now well established and the values here for the particle-alone group at both time points were well within range (Moyes et al., 2007). Finally, the immunofluorescent labelling approach has been widely used by other groups (de Carvalho et al., 2006; Musch et al., 2006) and the current images of untreated Caco-2 intercellular junctions, actin localisation and nuclear parameters were all similar to those reported recently, as was the high proportion of particles associated with the junctions (Moyes et al., 2010b). It is surprising, given the relative invasiveness of the techniques for production of the immunolabelled samples, that so many particles remained associated with the Caco-2 cell junctions. This suggests that the latter could play an important part in the attachment of a particle to the apical epithelial surface. This and other aspects of the changes seen in an epithelial monolayer after particle addition require further investigation using the detailed analysis of junctional protein location applied here.

There are two remaining protocol points which are central to the discussion of the current data. The first is the fact that inclusion of groups covering two time-points for both ethanol and ice pre-treatment and also for particle exposure led to the exclusion, on the grounds of practicality, of the time-equivalence 'sham' groups which had been so useful in the earlier paper (Moyes et al., 2010a) for the separation of the impact of particle addition from the ongo-

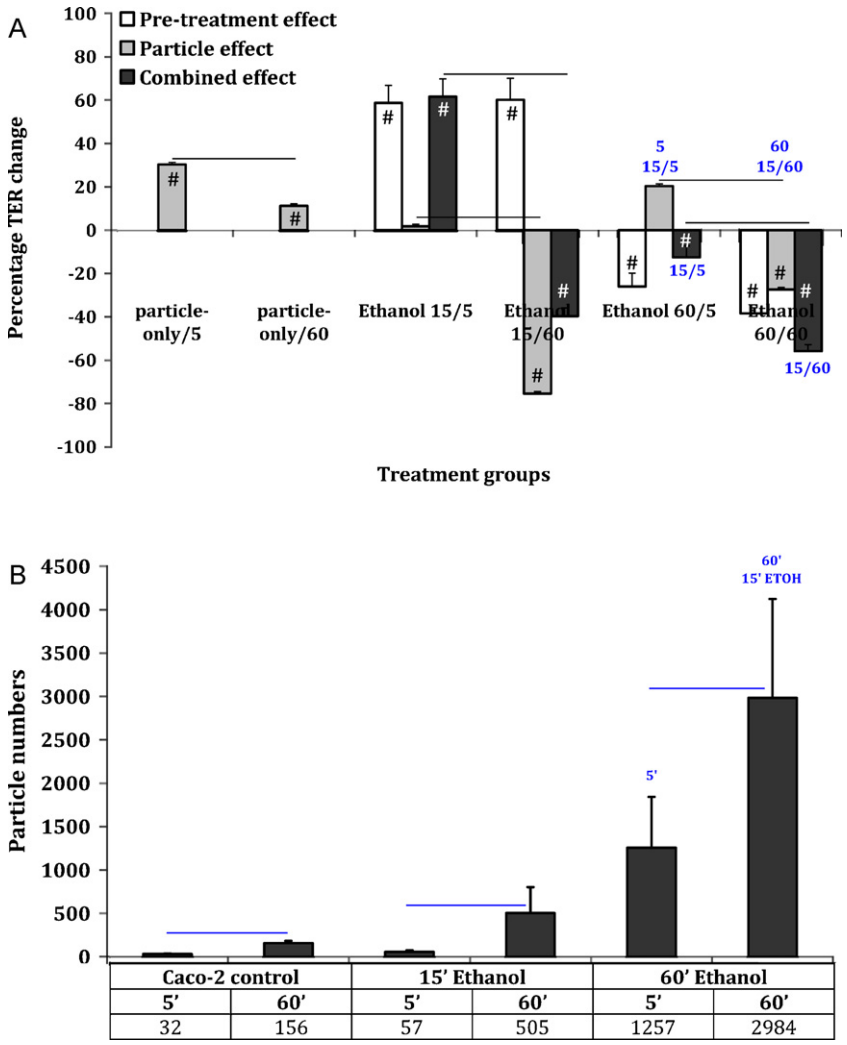

Fig. 5. *Effects of ethanol pre-treatment and particle exposure on Caco-2 cell TER and sub-membranous counts*. After incubation with 10% ethanol v/v for either 15 or 60 min Caco-2 cells were then exposed to microparticles for 5 or 60 min. Histograms illustrate the percentage TER change after particle exposure (A) and sub-membranous particle counts (B). Significant differences ($p \leq 0.05$) are indicated by: '#' for a change from control/TER; interconnecting lines for differences between comparable treatments; '5' or '60' above a histogram bar for a change from the particle-only group 5 or 60 min after particle addition; '15/5' and '15/60' for a difference from the relevant ethanol group; and '15' ETOH' for comparison with the 15-min ethanol pre-treatment group at the same particle time.

ing effect of the pre-treatment. As a result, the data displays for the current paper could not be identical to those in the earlier paper, making direct comparison less straightforward than it might have been. However, the TER and particle uptake levels for the appropriate time points agreed tolerably well when the current and previous data were compared.

The second remaining protocol point is fundamental to the discussion of the mechanisms involved in the development of changes in Caco-2 epithelium after pre-treatment and/or particle addition. This point stems from the variation in the literature in the time used to produce clearly identifiable changes in TER after exposure to ethanol, where 60 min was used (Ma et al., 1999) as opposed to temperature variation, where 15 min was sufficient (Armitage et al., 1994). This led to the authors' recent report (Moyes et al., 2010a) that, while exposure for 60 min to ethanol decreased TER thus opening tight junctions (TJs) at the same time as the epithelium allowed more latex particles to pass through, cooling on ice for 15 min had the opposite effect only on TER and did not produce less particle uptake than untreated Caco-2 cells. This latter situation appeared to confirm the supposition by Armitage et al. (1994) that the increased epithelial TER was not associated with changes in the TJs themselves but was instead related to physical alterations in the medium consequent on its being chilled. Both these findings raised doubts about particle uptake occurring through epithelial sheets of intestinal villi, despite acceptance in the literature that this is one of the likely routes (Florence, 1997) and the sightings of latex particles in the intercellular spaces of villous epithelium *in vivo* (Smyth et al., 2005).

The data reported in the current paper showed, however, that, if sufficient time be given for a greater effect of the low temperature to be felt, the TJ closure was indeed accompanied by a reduction in particle uptake. This addresses the hypothesis set out at the end of Section 1 and confirms that a 60-min pre-treatment period does allow proper comparison of the effects of ethanol and cooling on ice. It also suggests that the TJ route is important in microparticle uptake, since there appears to be an inverse relationship between particle uptake and changes in TER. However, the latter on its own can only point to the involvement of the TJs, for example in the increased uptake after ethanol: direct information on the tight junction proteins is also needed. The current data showed that the ethanol-affected Caco-2 cells have disrupted junctions, both tight and adhering, leaving a clear route for particles to pass. Indeed the epithelium as a whole was less organised and included some of the pleomorphic cells that occurred when Caco-2 cells were exposed to

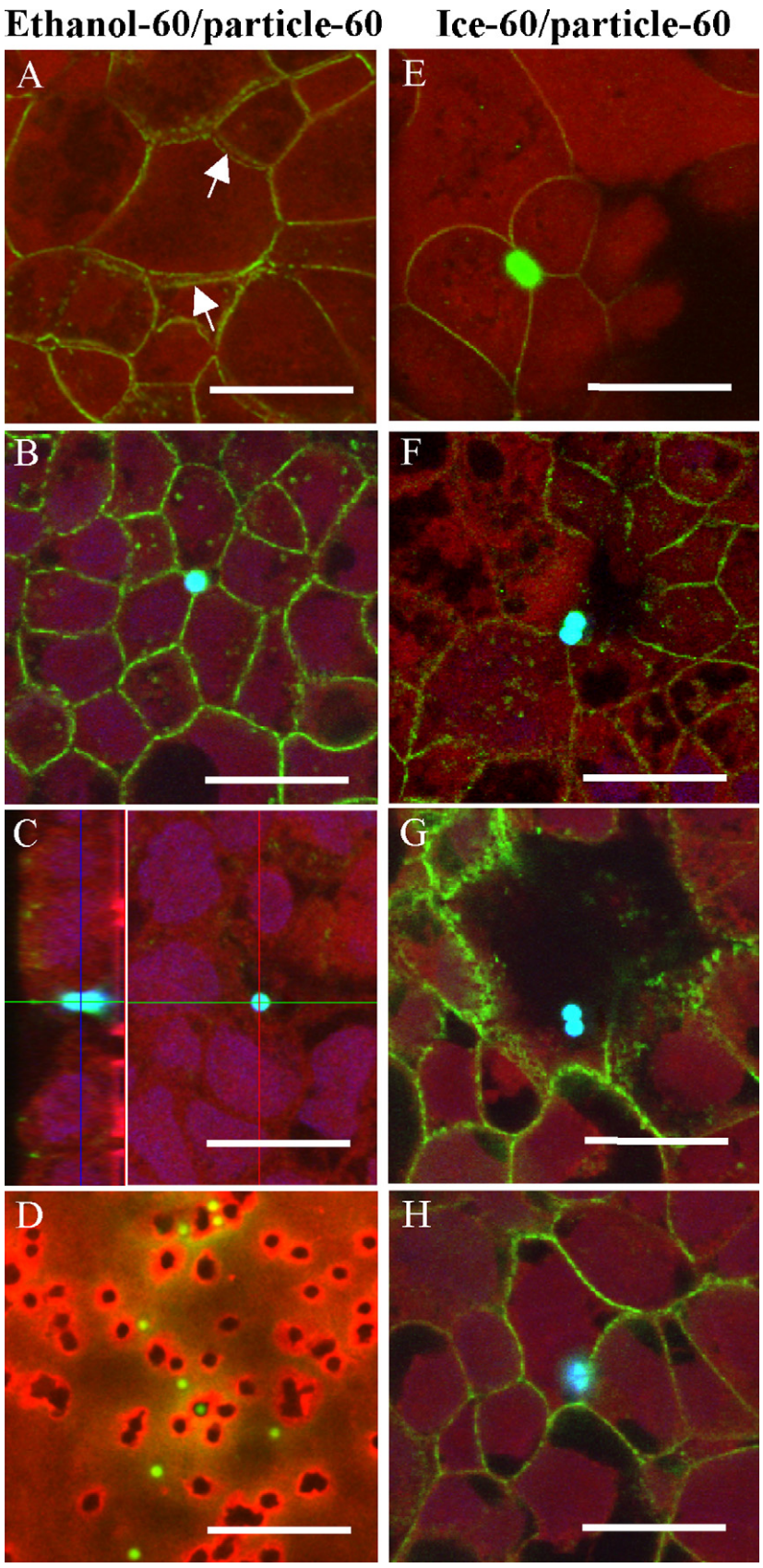

Fig. 6.

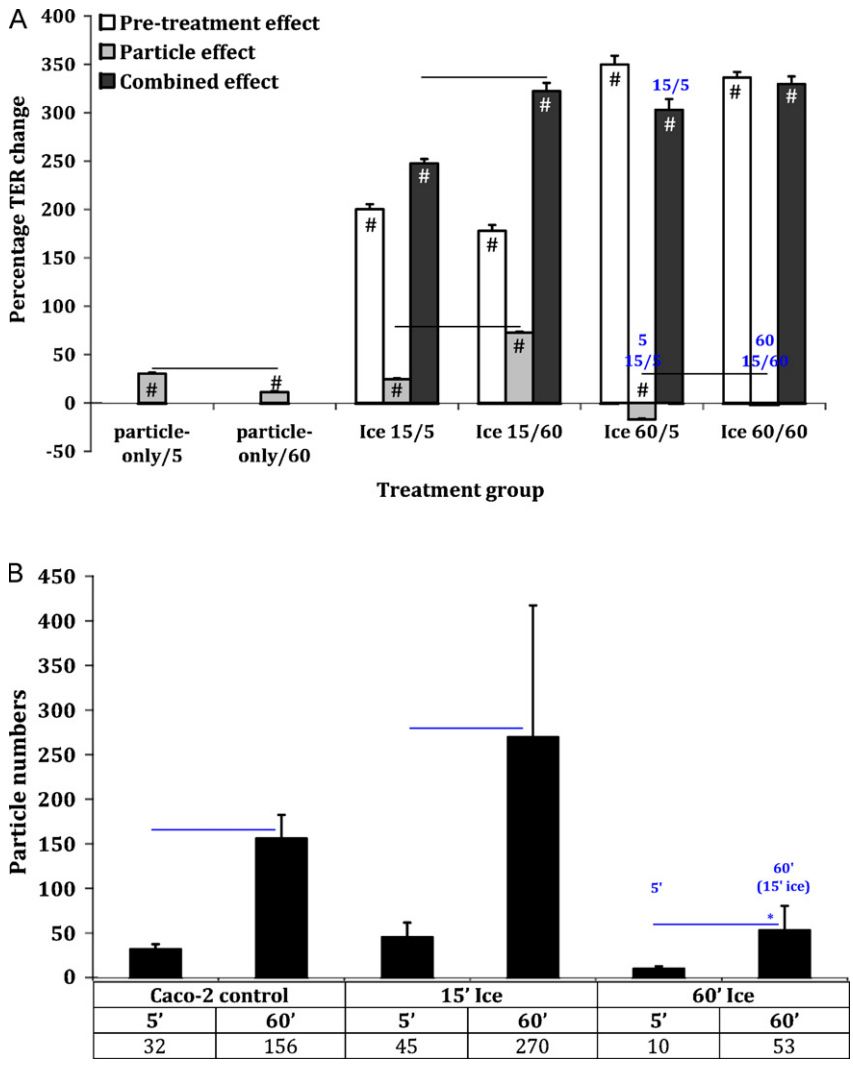

Fig. 7. *Effects of ice pre-treatment and particle exposure on Caco-2 cell TER and sub-membranous counts.* After remaining on ice for either 15 or 60 min Caco-2 cells were then exposed to microparticles for 5 or 60 min. Histograms illustrate the percentage TER change following particle exposure (A) and sub-membranous particle counts (B). Significant differences ($p \leq 0.05$) are indicated by: '#' for a change from control/TER; interconnecting lines for differences between comparable treatments; '5' or '60' above a histogram bar for a change from the particle-only group 5 or 60 min after particle addition; '15/5' and '15/60' for a difference from the relevant ice group; and '15' ice' for comparison with the 15-min ice pre-treatment group at the same particle time.

medium from macrophages, where cytokine-related effects were associated with lower TER values increased particle uptake and disorganised junctional protein staining (Moyes et al., 2010b). The more detailed analysis of the changes in the junctions reported in the current paper provided details of the way in which proteins were rearranged, with some dispersal of the usual clear apical belt of TJ protein, clearer signs of the double junctional lines which could reflect the pulling apart of adjacent cells and disintegration of the usual clear junctional protein localisation at multicellular junctions. The adhering junctions must also be less efficient at holding the epithelial cells together, given the movement of the E-cadherin belt deeper into the epithelium. It is, however, interesting that the effects discussed above required 60 min to develop while there was an initial TER increase after 15 min ethanol pre-treatment. As a similar initial TJ closure has also been seen following such short-term treatments as sequential TER measurements (Moyes et al., 2007), it could be considered as a precautionary, protective response by the cells.

The picture was very different for the ice-induced changes. The higher TER measurements after exposure for 60 min could indeed relate to closure of TJs rather than merely to changes in the chilled medium, since the TJ proteins were concentrated in a more constrained space, both in terms of extent downwards and lateral width, possibly reflecting greater efficiency in their gate or fence functions. The more apical expression of the adhering junction proteins could contribute to a strengthening of the epithelial sheet and

Fig. 6. *Effects of ethanol or ice pre-treatment and particle exposure on the Caco-2 cell model.* Confocal images highlight different findings after Caco-2 cells have been exposed to either ethanol (A–D) or ice (E–H) pre-treatment and a subsequent 60-min particle exposure. Caco-2 cells are labelled for junctional proteins (FITC), actin (TRITC) and nuclei (DAPI) and microparticles appear as large green/blue spheres. Scale bars are all 20 μm long. Ethanol-60/particle-60 (A) illustrates the presence of double lines (arrows) of E-cadherin between adjacent cells; (B) a particle located on an E-cadherin labelled multicellular junction (MCJ); (C) a particle located deep within the epithelium, with the basal orthogonal view illustrating its relationship with the surrounding cells and its position just above the insert membrane (ZO-1); (D) particles located within the pores of the insert membrane; ice-60/particle-60 (E) two particles located on a ZO-1 labelled junction; (F) two particles located on an E-cadherin labelled MCJ; (G) two particles seemingly located far from a junction higher up the *z*-plane: however, several optical slices further down, it is obvious that they are in contact with an oblique E-cadherin junction (H). (The reader is referred to the web version of the article to see these images in colour.)

therefore a greater resistance to particle uptake. However it must be remembered that decreasing temperature inhibits metabolic activity and may alter the balance between the active and passive processes involved in the relationship between particles and epithelium (Roger et al., 2009).

The conclusions for the *in vitro* situation of the Caco-2/microparticle model are that exposure to both ethanol and ice for 1 h can be useful as comparators for other experimental schedules as they are investigated for changes to TER, microparticle uptake and junctional proteins. For example, the factors involved in the TJ opening and increased particle uptake after external radiation (de Carvalho et al., 2006; Moyes et al., 2008) could be explored further by comparing such data with those from samples exposed as additional 'control groups' to ethanol or to a range of temperatures, including chilling on ice. The *in vivo* applications are less obvious, although the Caco-2 model does produce uptake numbers similar to those from an in *vivo in situ* rodent model (Moyes et al., 2007). However, the data on ethanol-induced TJ opening and disorganisation confirmed not only the results of other *in vitro* studies (Amin et al., 2009) but also the clinical findings on increased intestinal permeability in alcoholic patients (Bjarnason et al., 1984). It is clearly not helpful to consume alcohol along with exposure to microparticles, even if they were being administered with a pharmaceutical purpose. The possible applications of the ice protocol lie in its likely usefulness not as a treatment to reduce particle uptake after accidental exposure but more as a model to explore mechanisms of uptake and how to counteract its effects.

The overall conclusions are firstly, that if time and finances allow, dose–response and time-course studies are useful during protocol construction, rather than relying on inferences from the data of other groups. It can also be deduced that ethanol and ice if both applied for sufficient time have opposite effects on tight junctions and particle uptake. In mechanistic terms, the junctional specialisations, both tight and adhering, appear to have a role in attachment of the particles and in their onward movement through the epithelial monolayer, a role which is dependent on the relevant proteins continuing to be correctly positioned and not dispersed from their original sites.

Acknowledgments

We are grateful to the Department of Health's Radiation Protection Research Program for financial support. The authors would also like to thank the following people; Professor Dame K. E. Davies for the use of cell culture facilities; Drs C. Norbury and K. Gibson for advice on the actin immunofluorescence labelling; the Department of Statistics for advice on data analysis; Dr J. Runions and Professor C. Hawes at Oxford Brookes University for their advice and access to the Zeiss LSM510 confocal microscope; and colleagues in the Department of Physiology, Anatomy and Genetics for access to the Zeiss LSM710 confocal microscope.

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