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Culture conditions and treatments affect Caco-2 characteristics and particle uptake

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ABSTRACT

Small intestinal microparticle uptake via a paracellular route is relevant to oral drug delivery and environmental pollution. *In vitro* investigation uses latex microparticle passage across a confluent Caco-2 cell epithelium. This paper examines the influence of culture conditions on transepithelial resistance (TER); cell dimensions from confocal microscopy; and number of particles below the epithelium. Variables investigated include level of initial TER; multiple TER measurements; involvement of medium; cell source; and pretreatment with ethanol or a range of temperatures. Data were collected after exposure to 2 μm latex particles for 5–120 min: sham groups were exposed to pretreatment but not particles. The results highlight the importance of very precise control of the experimental environment; confirm the pattern of sequential-TER increase/decrease in groups exposed only to particles and show accompanying increases in cell dimensions. Greater particle uptake was associated with ethanol-induced decreased TER, decreased cell height and increased intercellular spaces, similar to previous findings for external irradiation. Low temperatures raised TER but, despite this, cooling did not alter particle uptake. In conclusion, culture microenvironment and sham treatment are crucial considerations in studies of epithelial microparticle uptake *in vitro*.

1. Introduction

Cultured monolayers of cells, such as Caco-2 enterocytes, make a good model for studies of intestinal responses to environmental challenge: whereas they are widely used in a range of research areas, it is important to be aware of the potential impact of culture conditions and even particular assays on epithelial permeability (Hidalgo, 1996; Delie, 1998; Ranaldi et al., 2003; Matter et al., 2005; Zucco et al., 2005; Volpe, 2008), which reflects the state of the tight junctions (TJs). These could be involved in latex microparticle uptake, *in vivo* (McCullough et al., 1995), where such particles have been seen between enterocytes (Smyth et al., 2005), implying a paracellular route. The current work takes the exploration of this process further using an *in vitro* model (Moyes et al., 2007a). Intestinal microparticle uptake is relevant to encapsulated drug delivery (Ravi Kumar, 2000; Artursson et al., 2001; van der Lubben et al., 2002; Krauland and Bernkop-Schnurch, 2004), environmental toxicology (Florence et al., 1995; Florence and Hussain, 2001; Krauland and Bernkop-Schnurch, 2004; Moulder, 2004; Hodgson et al., 2005; Stather, 2007) and multi-organ dysfunction syndrome (Nieuwenhuijzen et al., 1996; Johnson and Mayers, 2001).

One important example of these variations is the exposure of the cells to standard laboratory fluids such as fresh culture medium. When testing the responses of cells *in vitro* to pharmacological agents, it is also important to understand the effect of the vehicle in which they are prepared, such as ethanol. This decreases transepithelial resistance (TER), a measurement of TJ permeability, without any apparent effect on viability, provided the concentration is not greater than 10% (v/v) (Ma et al., 1999). Temporary removal of cells from the incubator for experimental procedures could also affect TER following the associated decrease in temperature, since this is seen in canine kidney cells cultured below 37 °C (Armitage et al., 1994). Since cultured cells are regularly moved during experiments between the different temperatures of the incubator and hood, it is important to investigate the effect of such variations.

Understanding the TJ response in epithelium exposed to ethanol or to variations in temperature could provide insight into the mechanisms involved in the permeability changes seen after further environmental challenge, such as exposure to microparticles, a common feature of the interface between biological tissues and their surroundings. Paracellular permeability is unlikely to be the only factor influencing the transepithelial movement of such particles and the dimensions of the cells may also be relevant.

The current paper explores these and other influences on Caco-2 cells, testing the following hypotheses:

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Table 1
Laboratory manipulations.

Group name	Laboratory manipulations		
	First	Second	Third onwards
Sequential-TER effect	Control/TER measured for each group on day 21 of ATCC Caco-2 culture	TER repeated every 15 min for 75 min	Sequential/TER
Passage number and transient hood temperature effect		5 or 60 min particle exposure	Post-particle/TER
Control/TER level on particle effect		5 or 60 min particle exposure	Post-particle/TER
Medium-only effect		5–120 min apical exposure to culture medium	Post-medium/TER
Nutrient-deprivation effect		No medium change on day 20 prior to 5 or 60 min particle exposure pooled	Post-particle/TER

Outline of treatments and TER measurements for all laboratory manipulation groups. Treatments are outlined in full in Section 2.4.1.

1. that variation in Caco-2 cell culture conditions will affect epithelial TJ permeability, as measured by changes in TER.
2. that Caco-2 TER will be respectively decreased or increased by exposure to ethanol or lower ambient temperatures.
3. that Caco-2 cell dimensions, which may influence epithelial permeability, will be affected by the pretreatments used, such as exposure to ethanol or variations in temperature.
4. that the numbers of microparticles passing completely through a Caco-2 epithelial layer will be increased if the TJs are opened or the dimensions are altered.

2. Materials and methods

2.1. Protocol outline

The effects of variations in the environmental parameters of a Caco-2 intestinal enterocyte model were followed by recording changes in epithelial TJ permeability as measured by TER and, for some samples, changes in cell dimensions and particle uptake through the epithelium.

The TER values of American Type Cell Culture (ATCC) Caco-2 intestinal epithelial layers were measured on day 21 of culture: this value was designated 'control/TER'. These samples were then exposed to 2 μm latex particles for 5–120 min in a 37 °C incubator (Table 1) and designated the 'particle-only' group. All 21 experiments included in this paper contained particle-only samples which then collectively formed a combined particle-only group.

The effects were also recorded (Table 1) of making multiple sequential-TER measurements; of variations in passage number; of variations in the lower temperature at which the TER was measured (transient hood temperature effects); and of the level of the control/TER before the addition of particles. Variation in methods of addition of culture medium included exposure of Caco-2 cells to 1 μl of medium alone for comparison with particle addition; or to nutrient deprivation. Pretreatments included exposure to ethanol or different temperatures for 'longer term' temperature exposure (Table 2, column 2). All TER measurements and additions of fluids or particles were made in the culture hood at approximately 24 °C. Measurements of ATCC cell dimensions and particle uptake were made at the end of the experimental procedure for appropriate groups.

Differences in TER (Moyes et al., 2007a) and cell dimensions (Moyes et al., 2008) between these and cells from the European Collection of Cell Cultures (ECACC) were also compared.

2.2. ATCC Caco-2 cell culture

ATCC Caco-2 cells (HTB-37™, passage 28–39), 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®). The 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. The standard feeding regime involved replacement of the fluids in upper and lower wells with fresh culture medium (1.5 and 2.5 ml, respectively) every 2–3 days and on day 20 of culture. Data were taken from 21 experiments.

2.3. Assays and sample preparation

2.3.1. TER—measurements and data analysis

2.3.1.1. Measurements. Before cell seeding, the TER of the Transwell® insert membrane with culture medium (TER_{Insert}) was measured using a Millicell®-ERS instrument (Millipore). On day 21 of culture, the TER for the epithelium on the insert was measured (control/TER), to confirm the confluence of the cell layer. Other TER measurements included those after pretreatment (pretreatment/TER) or after particle addition (post-particle/TER). TER measurements were calculated in $\Omega\,cm^2$ to determine the TER of the epithelium alone and allow for its total surface area, using the following equation; $[TER - TER_{Insert}] \times 4.5\,cm^2$ (Wells et al., 1998). The TER meter and electrodes were tested intermittently and if necessary adjusted according to the manufacturer's instructions.

2.3.1.2. Calculation of changes in transepithelial resistance (TER). TER data were displayed in one of three ways: as raw values, percentage changes, or delta changes obtained by subtraction of two sequential measurements. Increased TER was taken to indicate tighter TJs and decreased TER looser TJs.

Percentage changes were calculated as [((pretreatment/TER − control/TER) ÷ control/TER) × 100] or as [((post-particle/TER − control/TER) ÷ control/TER) × 100]: these percentage changes were used for ease of comparison of data across many treatment groups. Delta changes were calculated as [(pretreatment/TER) − control/TER] or as [(post-particle/TER) − control/TER] and were used for detailed numerical comparisons between treatments.

The effects on groups of cells of two sequential treatments (pretreatments and particle exposure) were documented in two ways, designated 'combined' and 'time equivalence':

(i) *Combined pretreatment/particle effects*. These concerned the change in epithelial permeability in the same experimental group after both pretreatment and particle addition. The calculations used were [post-particle/TER − control/TER] for all groups.
(ii) *Pretreatment time equivalence calculations for comparison of combined and sham effects*. This accounted for the continued pretreatment effect that occurred during the particle incubation period, by the comparison of two groups with the same total experimental exposure time, one with particle addition

Table 2
Cell dimension measurements and derived percentage intercellular volume and cell–cell boundary/crosswire for the ATCC morphology-control (shaded box), particle-only and ethanol and temperature pre-treated cells.

Group	Treatment group	Cell dimensions in μm (±SEM)						Derived data	
		Cell height above insert	Height below insert	Major apical width	Minor apical width	Major basal width	Minor basal width	Percentage intercellular volume	Cell-cell boundary/ crosswire ($\times 10^6$)
Morphology-control									
A	*Morphology-control*	27.2 ± 1.7	1.9 ± 1.0	14.7 ± 0.5	8.7 ± 0.4	10.1 ± 0.4	7.1 ± 0.4	23.2	10.5
ATCC particle-only									
B	*Particle/only-5*	32.3 ± 1.1^{A}	1.4 ± 0.8	15.4 ± 0.4	11.2 ± 0.4^{A}	12.2 ± 0.6^{A}	8.5 ± 0.3^{A}	21.0	8.8
C	*Particle/only-60*	32.9 ± 1.1$^{(A)}$	2.5 ± 0.8$^{(A)}$	17.3 ± 0.6^{A}	11.6 ± 0.5^{A}	11.6 ± 0.4$^{(A)}$	8.3 ± 0.3^{B}	27.6	8.2
Ethanol exposure									
D	*Ethanol/sham-5*	23.5 ± 1.6$^{(A)}$	1.5 ± 1.0	17.1 ± 0.6^{A}	11.6 ± 0.5^{A}	9.4 ± 0.3	6.7 ± 0.3	37.5	8.2
E	*Ethanol/sham-60*	23.9 ± 1.5	2.2 ± 1.1	15.0 ± 0.5^{D}	10.7 ± 0.4^{A}	9.4 ± 0.4	6.6 ± 0.3	32.9	9.1
F	*Ethanol/particle-5*	23.0 ± 1.5$^{(A),\ B}$	1.9 ± 1.0	16.6 ± 0.5$^{A,\ B}$	10.6 ± 0.4$^{A,\ (B)}$	9.3 ± 0.3^{B}	6.5 ± 0.3^{B}	35.6	8.9
G	*Ethanol/particle-60*	24.3 ± 1.8^{C}	3.1 ± 1.3	16.3 ± 0.5$^{A,\ (E)}$	11.1 ± 0.5^{A}	9.3 ± 0.3^{C}	6.4 ± 0.3^{C}	36.5	8.7
Longer term temperature exposure Cell culture incubator: 37 °C									
H	*Incubator/sham-5*	25.6 ± 1.8	3.1 ± 1.0	14.6 ± 0.5	9.0 ± 0.4	9.3 ± 0.5	6.4 ± 0.4	29.2	10.3
A	*Incubator/sham-60*	Dimensions as described in 'Morphology-control'							
I	*Incubator/particle-5*	28.5 ± 1.8$^{(B)}$	3.1 ± 0.8	14.4 ± 0.5	9.5 ± 0.4^{B}	11.3 ± 0.5$^{(A),\ H}$	7.8 ± 0.5^{H}	18.7	10.0
J	*Incubator/particle-60*	27.3 ± 1.3^{C}	5.2 ± 0.7$^{A,\ H,\ I}$	14.9 ± 0.5^{C}	10.7 ± 0.5$^{A,\ I}$	10.7 ± 0.4	7.5 ± 0.4	26.1	9.2
Sterile culture hood: 24 °C									
K	*Hood/sham-5*	24.7 ± 1.3	5.6 ± 0.9^{A}	13.3 ± 0.4$^{(H),\ A}$	9.2 ± 0.4	9.6 ± 0.5	6.8 ± 0.3	24.8	10.5
L	*Hood/sham-60*	27.3 ± 1.7	4.0 ± 1.0	15.0 ± 0.7^{K}	9.1 ± 0.5	10.1 ± 0.5	6.7 ± 0.4	26.8	10.2
M	*Hood/particle-5*	21.0 ± 1.3$^{A,\ B,\ I,\ (K)}$	3.8 ± 1.1	15.2 ± 0.4^{K}	9.6 ± 0.4^{B}	11.2 ± 0.5$^{B,\ K}$	7.6 ± 0.4^{B}	21.9	9.7
N	*Hood/particle-60*	23.3 ± 1.9^{C}	0.7 ± 0.6$^{L,\ M}$	15.3 ± 0.5	10.3 ± 0.4$^{A,\ (L)}$	11.4 ± 0.3$^{A,\ (L)}$	8.0 ± 0.3^{L}	21.9	9.3
Ice: 0 °C									
O	*Ice/sham-5*	26.3 ± 1.6	3.7 ± 1.0	13.7 ± 0.6	9.4 ± 0.5	9.1 ± 0.4	6.1 ± 0.4$^{(A)}$	30.6	10.2
P	*Ice/sham-60*	28.5 ± 1.9	2.6 ± 1.1	14.7 ± 0.7	10.0 ± 0.6$^{(A)}$	9.9 ± 0.5	6.8 ± 0.4	28.7	9.6
Q	*Ice/particle-5*	24.9 ± 1.5^{B}	3.5 ± 1.1	13.8 ± 0.6$^{(B)}$	9.8 ± 0.6^{B}	9.1 ± 0.4$^{(A),\ B,\ I,\ M}$	6.3 ± 0.3$^{B,\ I,\ M}$	30.7	10.0
R	*Ice/particle-60*	26.4 ± 1.4^{C}	4.1 ± 0.7	15.6 ± 0.4$^{(J),\ Q}$	10.3 ± 0.4^{A}	9.9 ± 0.4$^{(A),\ C,\ N}$	6.5 ± 0.3$^{C,\ (J),\ N}$	32.5	9.2

The explanation of treatment group names is given in Sections 2.4.3 and 2.4.4. Standard error of the mean (SEM) is for $n = 27$ samples from 3 independent experiments. Apical widths for all treatment groups, are significantly larger than the equivalent basal dimensions ($p = 0.09$–0.0001) when calculated on a cell by cell basis, as described in Section 2. Further statistical differences ($p \leq 0.05$) and strong trends, designated by brackets ($p \leq 0.1$), are indicated by a superscript letter identifying the group (first column) to which that dimension differs, e.g. 'A' beside the height for the 5 min particle-only group indicates a significant difference from the morphology-control. All 'derived' values were calculated from the mean dimensions in this table, and therefore ranking rather than statistical testing was used.

and one without (sham): for example, the group pre-treated on ice for 15 min followed by particle exposure for 5 min, also on ice (ice/particle-5) was compared to a sham group treated for 20 min with the temperature change only (ice/sham-5).

2.3.2. Cell dimensions

Epithelial layers were fixed in 3% glutaraldehyde for a minimum of 20 min, mounted on to coded microscope slides with Vectashield containing propidium iodide (Vector Laboratories Ltd.) and sealed around the edges. Cell dimensions were measured by confocal microscopy, as described previously (Moyes et al., 2008), for the ATCC particle-only Caco-2 cells, ethanol and temperature pretreatment groups and the ECACC particle-only Caco-2 cells (previously published data, Moyes et al., 2008). These dimensions included cell height above and below the insert and also major and minor widths, both apical and basal ($n \geq 27$). 'Derived' dimensional data were calculated from the measurements listed above: these were percentage intercellular volume, total cell–cell boundary/single crosswire and (for ATCC only) percentage of total epithelial height below the insert.

A 'morphology-control' was used as a comparator for all pretreated and particle-only groups, since this group had only been exposed to two TER readings 75 min apart, during which time the cells remained in the 37 °C incubator: this was the sham for the relevant group exposed to particles for 60 min and its treatment was designated as incubator/sham-60.

2.3.3. Estimation of numbers of sub-membranous particles

For ATCC groups, these included 'lower well fluid' and 'wash' counts: the former were the number of particles that had passed through the epithelium and the latter, for samples collected most recently, were those collected after washing the basal side of the insert with PBS. Both samples for each well were individually centrifuged and re-suspended in PBS, which was then mounted on to coded microscope slides for counting, to give the total number of sub-membranous particles. Similar, but not identical, methods of counting lower well particles were previously used for the ECACC samples to produce the data set out in Moyes et al. (2007a, 2008).

2.4. Experimental groups

2.4.1. Routine laboratory manipulation

2.4.1.1. Sequential-TER measurements, passage number and transient temperature exposure. Following an initial control/TER on day 21, further sequential measurements were taken every 15 min for 75 min in the cell culture hood. Between assays, cells were incubated at 37 °C (Table 1). Throughout experimental procedures, records were intermittently made of hood temperature, which was always between 21 and 24 °C. Control/TER data were analysed statistically with respect to their Caco-2 passage number and the exact hood temperature to which they were transiently exposed during TER measurement. To explore possible effects of the level of control/TER on subsequent treatment, changes following particle exposure for 5 or 60 min were pooled from the five experiments with the lowest mean control/TER and the five with the highest.

2.4.1.2. Variable addition of medium. The effect of the apical addition of 1 μl of the culture medium to that already present on day 21 was explored, to compare this with the effect of adding the same volume of microparticles (Table 1; $n = 4$ independent experiments). The nutrient-deprivation regime only omitted the final medium change on day 20, all other parameters remained unchanged. Data for comparison of standard and nutrient-deprivation schedules were taken from 2 experiments (Table 1).

2.4.2. Addition of latex microparticles

Fluoresbrite® yellow-green latex particles in distilled water (Polysciences Inc.) were added to the apical medium of relevant inserts (1 μl; 2 ± 0.1 μm diameter; 5.68×10^6 particles/μl). Plates were then incubated at 37 °C for between 5 and 120 min 'particle times'.

2.4.3. Groups exposed to ethanol pretreatment

Following the measurement of an initial control/TER on day 21, the existing culture medium was replaced in full with ATCC culture medium, warmed to 37 °C, containing 10% ethanol (v/v) for 60, 65 or 120 min ($n \geq 2$ independent experiments). Those pretreated for 60 min were then exposed to particles for either 5 min (ethanol/particle-5) or 60 min (ethanol/particle-60) and compared with groups exposed to ethanol alone for 65 min (ethanol/sham-5) or 120 min (ethanol/sham-60), respectively.

TER was measured after pretreatment for all groups (pretreatment/TER) and after particle exposure (post-particle/TER).

2.4.4. Groups exposed to longer term variations in temperature

Following the measurement of an initial control/TER on day 21, the cells were exposed to one of the following three temperatures for 15, 20 or 75 min; to the normal temperature of the incubator at 37 °C; to the temperature of the cell culture hood, at approximately 24 °C; or to melting ice at 0 °C in the cell culture hood. Those pretreated for 15 min were then exposed to particles for either 5 or 60 min and compared to their 'sham' equivalent. For example, those maintained at 37 °C for 15 min, designated incubator/particle-5 or incubator/particle-60, were compared with groups exposed to temperature alone for 20 min (incubator/sham-5) or 75 min (incubator/sham-60), respectively. The incubator/sham-60 was also termed the morphology-control.

TER was measured after temperature pretreatment for all groups (pretreatment/TER) and after particle exposure (post-particle/TER) where appropriate ($n \geq$ two independent experiments). Similar protocols were used for the other two pretreatment temperatures. This produced hood/particle-5, ice/particle-5, hood/particle-60, ice/particle-60, hood/sham-5, ice/sham-5, hood/sham-60, and ice/sham-60 groups. There were also TER values for incubator/pretreatment, hood/pretreatment and ice/pretreatment, used as comparators for the relevant particle exposed TER values.

2.4.5. Groups of Caco-2 cells from different sources

Caco-2 cells from ECACC (No. 86010202, passage 30–45) were routinely cultured as for those from ATCC, but in Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids (all Gibco®). ECACC cell membranes were stained with 3.5 μg/ml FM1-43FX (Cambridge Bioscience) for 5 min. The cell dimension measurements were made in a group exposed to particles for 30 min (data previously published, $n = 8$ independent experiments, Moyes et al., 2008). Only particles in the lower well fluid were counted.

2.5. Statistical analysis

ANOVA statistical analysis was carried out on all TER and cell dimension data and Mann Whitney *U* analysis on particle counts. The results of data comparisons included in the text are either significantly different ($p \leq 0.05$) or show strong trends ($p \leq 0.1$). Linear regression analysis was used to explore the possible relationships between TER, passage number and the temperature in the hood at which the TER was measured.

Table 3
Control/TER, particle effects, passage number and culture hood temperature.

Mean control/TER ± SEM (Ω cm^2) for data pool of all untreated samples for each experiment	Delta TER change from relevant control/TER for 5 or 60 min samples		Passage number	Temperature (°C)
	5 min	60 min		
514 ± 14	143 ± 15	59 ± 18	27	21.9
359 ± 12	85 ± 9	−6 ± 3	28	23.5
413 ± 06	115 ± 6	7 ± 7	29	23.0
657 ± 07	119 ± 14	74 ± 14	29	23.0
425 ± 12	141 ± 32	93 ± 9	31	23.5
540 ± 07	98 ± 9	−9 ± 3	32	23.3
543 ± 14	138 ± 19	92 ± 7	32	22.3
357 ± 03	113 ± 10	45 ± 3	33	24.0
397 ± 04	169 ± 4	15 ± 24	34	21.0
381 ± 16*	152 ± 8	−60 ± 11	35	24.0
516 ± 11*	188 ± 15	138 ± 10	36	23.2
365 ± 09	139 ± 16	35 ± 14	36	24.0
723 ± 25	207 ± 14	120 ± 18	36	24.0
328 ± 09	75 ± 10	31 ± 8	38	23.5
532 ± 23	144 ± 5	50 ± 8	38	24.8
416 ± 07	174 ± 22	2 ± 10	39	22.0
387 ± 09	125 ± 14	55 ± 13	39	22.7
395 ± 10*	244 ± 12	182 ± 9	39	23.5
541 ± 22	156 ± 17	−3 ± 12	39	24.4
351 ± 60	167 ± 15	126 ± 51	42	23.5
540 ± 12	182 ± 22	84 ± 19	42	24.5
457 ± 23	Particle-only mean control/TER for all 21 experiments			
535 ± 20	Morphology-control mean control/TER from data subsets of '*' above experiments			

The mean control/TER, taken on day 21 of Caco-2 culture, and subsequent post-particle/TER data are displayed, for all experiments included in this paper, in order of passage number, regardless of the date at which the experiment took place. Delta TER change is calculated from the individual control/TER values, for either the 5 or 60 min particle exposure inserts, on a sample-by-sample basis. While their control/TER values are included in the whole particle-only pool for the experiment (column 1) they may vary from the calculated mean.

2.6. Ranking of derived values for ATCC cell dimensions

No statistical analysis was possible for data that were calculated from a single mean value, such as the derived dimensions described above (Section 2.3.2): comparisons were made of these by ranking the values.

2.7. Establishment of criteria for quality control and sample selection

Only wells with control/TER on day 21 of at least 70 Ω higher than that of TER_{insert} were considered confluent. Experiments were also only included if the relevant particle-only groups showed a marked TER increase, implying TJ closing, 5 min after microparticle addition. After noting the possible causes of errors in the pilot data, steps were taken to avoid these. For example, during experiments, two individuals were involved in the recording and checking of TER measurements. In order to identify signs of cell death, the epithelial layers were assessed for damage. Samples exhibiting holes were removed and their related data discounted.

3. Results

All results were for ATCC Caco-2 cells, apart from previously published data from ECACC Caco-2 cells (Moyes et al., 2007a, 2008). Confocal microscopy showed that cells from all ATCC groups had no multilayering above the insert membrane, but some cellular material within and below its pores, detailed below as sub-insert epithelium. For every ATCC group, the columnar nature of the epithelium was confirmed by height being significantly greater than apical dimensions ($p = 0.0001$; Table 2): the latter were significantly greater than the basal measurements ($p = 0.0001$), demonstrating the presence of a basal intercellular space. The apex and base of the cells were polygonal, with one width measurement always significantly greater than the other ($p = 0.0001$; Table 2).

3.1. ATCC controls

3.1.1. Control/TER values

The mean control/TER value, measured on day 21 of Caco-2 culture for 21 independent experiments, was 457 ± 23 Ω cm^2 and varied across experiments with a range of 328–723 Ω cm^2 (Table 3). The TER quality control procedures led to fewer than 5% of the total samples collected being discarded. The mean control/TER value for the morphology-control groups was 535 ± 20 Ω cm^2, above the mean but within the range.

3.1.2. Morphology-control group dimensions

The morphology-control group cells were 27.2 μm high above the insert, 14.7 μm by 8.7 μm wide apically and 10.1 μm by 7.1 μm basally (Table 2). The length of apical cell-cell boundary per cross-wire of 10.5×10^6 was one of the highest for groups unexposed to particles. This group was also among the lowest of the rankings, both for intercellular space deep to the apical junctions between adjacent cells (23.2% of epithelial volume) and for sub-insert epithelium (6.5% of total epithelial height).

3.2. Exposure of ATCC cells to routine laboratory manipulations, medium or particles

3.2.1. Routine laboratory manipulation effects on TER

Linear regression statistical analysis suggested that, whereas a higher passage number decreased the TER from the initial control/TER value ($p = 0.0001$, Table 3), transient exposure to the lower temperature of the culture hood during the latter measurement had no apparent effect: these were not obvious when graphical techniques were used (data not shown). When control/TER was measured at hood temperature (23.5 °C for both experiments) then the samples returned to the incubator, there was a significant delta increase when the TER was measured again 15 min later (incubator/pretreatment group, $p = 0.0001$; Fig. 1a): this increase was maintained on sequential measurements every 15 min for 75 min.

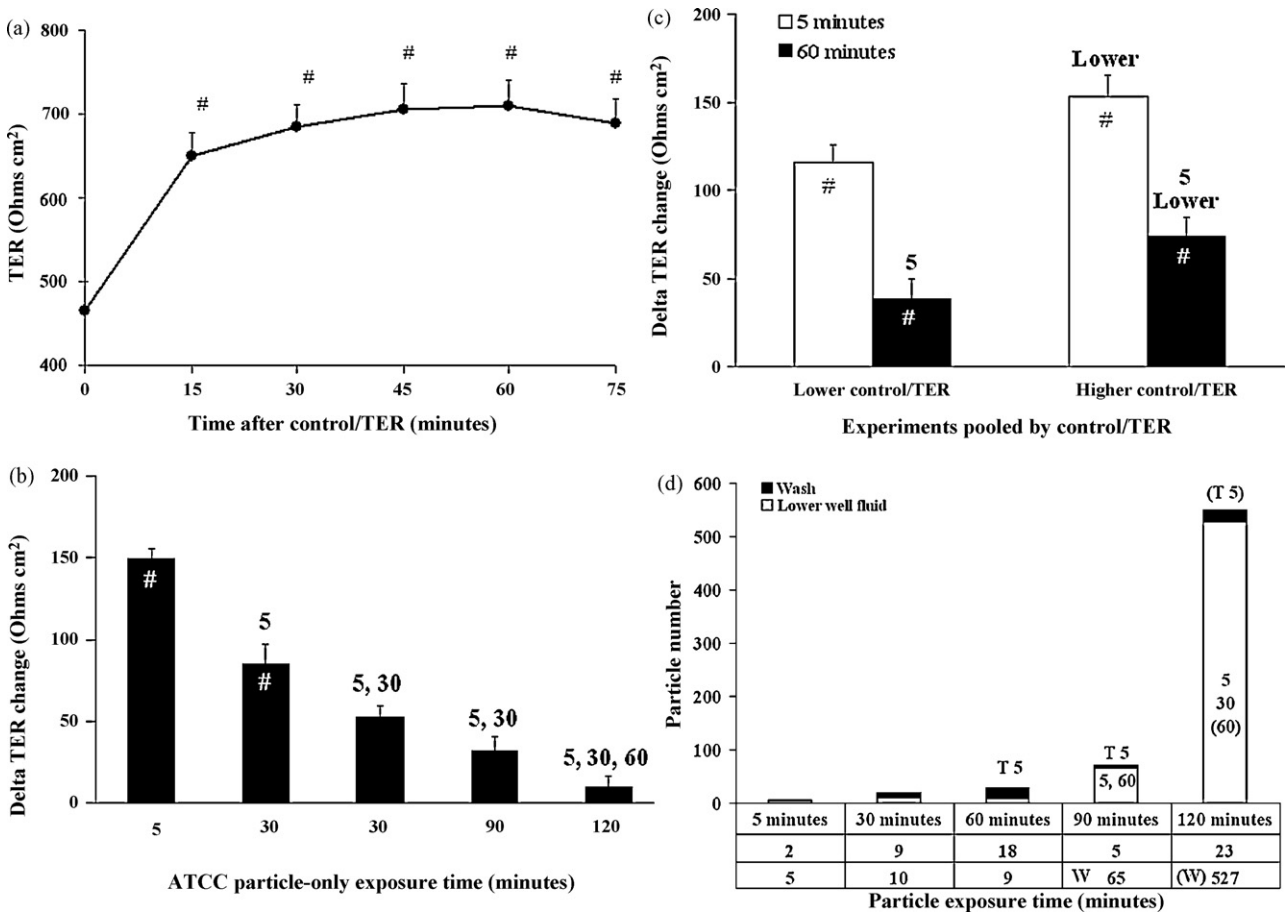

Fig. 1. (a) Second and subsequent TER measurements are raised. Mean ± SEM of control/TER for the ATCC sequential-TER effect group is shown at 0 min time-point and after sequential 37 °C incubations and re-measurement. '*' shows significant increases from control/TER ($p = 0.0001$). Data are from 12 inserts over 2 experiments. (b) Particle exposure increase/decrease TER response. Cells from all particle-only groups, for all 21 ATCC experiments, were exposed to particles for 5–120 min. Significant differences ($p \leq 0.05$) are as follows; '#' indicates a significant difference from control/TER, '5', '30' and '60' a difference from the 5, 30 or 60 min particle exposure time. Data are from 12 to 97 inserts over 2 to 21 experiments. (c) Control/TER level affects the amplitude of the ATCC increase/decrease particle response. Significant differences are as outlined in (b) and 'Lower' above the individual histogram bars signifies a significant difference to changes for 5 and 60 min particle exposure times seen for cells with lower control/TER values. Data are from 20 to 22 inserts over 5 experiments. (d) ATCC particle-only group particle counts increase with exposure time. Statistical differences ($p \leq 0.05$) and strong trends ($p \leq 0.1$), indicated by brackets, are as follows; 'T 5' above a histogram bar signifies a difference from 5 min total sub-membranous particle counts, '5', '30' and '60' located within the bars show a difference from the lower well fluid particle counts at those time-points and 'W' a difference between lower well fluid and wash numbers. Data are from 6 to 21 inserts over 2 to 5 experiments.

For the nutrient-deprivation groups the mean control/TER value was $481 \pm 18\ \Omega\,cm^2$, higher than the control/TER value for the standard-fed groups of $385 \pm 7\ \Omega\,cm^2$ ($p = 0.0001$). The range of the TER values for the nutrient-deprived groups was 201–906 $\Omega\,cm^2$ which was also larger than that for the standard-fed groups, namely 231–517 $\Omega\,cm^2$.

3.2.2. Particle addition effects on TER

The ATCC particle-only group showed a large significant increase in TER delta after a 5 min particle exposure to 2 μm particles ($p = 0.0001$, Fig. 1b), followed by a significant relative TER decrease with increasing exposure time ($p = 0.0001$–0.02 for 30–120 min, respectively). This TER 'increase/decrease' pattern was typical of all data subsets and represented a TJ closing and reopening. When 1 μl of medium alone, as a microparticle control, was added to standard-fed ATCC Caco-2 cells, the TER was increased at all time-points by comparison with the control/TER ($p = 0.01$–0.001, data not shown).

TER increased to a greater extent 5 or 60 min after particle exposure in epithelial layers with a higher initial control/TER ($p = 0.05$ for 5 min and 0.02 for 60 min; Table 3; Fig. 1c), although both showed the same increase/decrease pattern of responses. Nutrient deprivation delayed the maximum response to microparticle addition from 5 to 30 min (data not shown) and increased the variability of the responses when compared to the relevant particle-only group (data not shown).

3.2.3. Particle addition effects on cell dimensions and particle uptake

For all groups, confocal microscope Z-stack images showed that particles were either on the epithelial surface or within the epithelial layer, although typically resolution was not sufficient to determine whether particles were within or between cells. However, as damaged epithelial samples were excluded, particles were not merely passing through discontinuities (data not shown). By comparison with the morphology-control group, particle-only cells 5 and 60 min after particle addition were taller ($p = 0.08$–0.03), larger apically ($p = 0.05$–0.0001) and basally ($p = 0.08$–0.0005) and had less cell–cell boundary at the later time-point (Table 2). The small amount of sub-insert epithelium in the morphology-control group (6.5% of total epithelial height) was little changed after particle addition (4.1 and 7.1% for 5 and 60 min, respectively).

The total number of sub-membranous particles increased with particle exposure time (Fig. 1d), with significantly more in the lower well than in the wash fluid ($p = 0.008$–0.1) at 90 and 120 min.

Table 4
Ethanol pretreatment (yellow dashed boxes) and particle exposure (green bold boxes) effects on TER.

Treatment	Experimental timeline (minutes from control/TER) with TER values (Ω·cm²)					Delta changes in TER (Ω·cm²)	
	Control/TER 0mins	5	60	65	120	Ethanol effect cf control/TER	Particle effect cf pretreatment/ TER
ATCC particle-only							
Particle/only-5	391 ±12	596 ±18					213 ±17
Particle/only-60			500 ± 30				102 ±41
Ethanol exposure							
Ethanol/sham-5	321 ± 28			251 ±20		# -70 ±23	
Ethanol/sham-60	368 ±18				132 ±14	sham-5 # pretreat -236 ±13	
Ethanol/particle-5	345 ± 13		255 ±16	309 ±19		# -89 ±19	80 ±8
Ethanol/particle-60					151 ±10		-131 ±15

Mean TER ± standard error of the mean (SEM) from 3 independent experiments, before and after treatment with 10% ethanol in culture medium and particle exposure. All cells were maintained in the incubator for the duration of the experimental treatment, with the exception of TER measurements which were carried out in the sterile cell culture hood and typically took around 2.5 min. Delta changes in TER were calculated on a sample-by-sample basis and therefore may differ from direct subtraction of pooled mean control/TER and pretreatment/TER values. Significant differences ($p \leq 0.05$) are as follows; '#' is a change from control/TER and 'pretreat' and 'sham-5' a change from the 60 min pretreatment effect or the ethanol/sham-5 group. Time equivalence comparisons were calculated for ethanol/particle-5 as the [particle effect (blue zigzag box) – ethanol effect (blue zigzag box)]. The same calculation was carried out for the ethanol/particle-60 group using data in the grey triple-outlined boxes.

3.3. Exposure of ATCC Caco-2 cells to pretreatment and/or particles

3.3.1. Ethanol and/or particles

3.3.1.1. Effect of sham treatment. The TER was significantly lower ($p = 0.0001$; Table 4) than the control/TER after exposure to 10% ethanol, for 60 min (ethanol/pretreatment), 65 min (ethanol/sham-5) and 120 min (ethanol/sham-60), with the longest incubation time increasing the effect ($p = 0.0001$). Ethanol exposure for 65 min in the ethanol/sham-5 group tended to shorten the cells and made them significantly wider apically ($p = 0.006$, Table 2): it also led to more intercellular space and less cell-cell boundary. After 120 min treatment in the ethanol/sham-60 group, the cells were still significantly wider apically ($p = 0.006$, Table 2), but to a lesser extent than in the earlier group ($p = 0.006$, Table 2).

3.3.1.2. Combined effect of ethanol and particles. For the combined effect at the end of treatment with ethanol followed by particle exposure, there was no increase from control/TER in the ethanol/particle-5 group, unlike the ATCC particle-only group ($p = 0.0001$; Table 4; Fig. 2a): instead the ethanol/particle-60 group had a significant TER delta decrease ($p = 0.0001$). The cells in both groups were significantly shorter, narrower basally and had more intercellular space than the corresponding particle-only group (Table 2).

3.3.1.3. Time equivalence comparison. The time equivalence comparison of ethanol/particle with their sham groups, calculated from Table 4, showed that ethanol pretreatment markedly diminished the post-particle TER increase seen with the particle-only group at both 5 and 60 min ($p = 0.01$).

Cells exposed to particles and/or the extended pretreatment with ethanol generally had similar heights and widths (Table 2). The total number of particles passing through the epithelium for samples pre-treated with ethanol and then exposed to particles was significantly higher than for the ATCC particle-only group at both 5 and 60 min time-points (Fig. 2b).

3.3.2. Longer term temperature variation and/or particles

3.3.2.1. Effect of sham treatment. As previously mentioned for cells maintained in the incubator (Section 3.2.1; Fig. 1a), a second TER reading was higher than one taken 15 min earlier (control/TER). This also occurred for the incubator/sham-5 value after 20 min ($p = 0.0001$, Table 5), whereas after 75 min the incubator/sham-60 value returned to control/TER level. The corresponding changes from control/TER for hood/pretreatment and ice/pretreatment were more marked ($p = 0.0001$ for both, Table 5). There was a near linear correlation between time and temperature-induced percentage TER change from control/TER, calculated from Table 5. When compared to the morphology-control (incubator/sham-60), the incubator/sham-5 group had similar dimensions, but cooler temperatures produced fewer changes than ethanol, although there were some differences in apical and basal widths (Table 2). However, the amount of sub-insert epithelium appeared higher for the hood/sham-5 group (18.5% of the total epithelial height) than for other sham groups.

3.3.2.2. Combined effect of temperature and particles. The combined TER effect of both incubator pretreatment and particle exposure at the end of the experiment (Table 5), was similar to that of the particle-only cells, namely the typical increase/decrease TER response. Hood or ice pretreatment, however, led to a higher 5 min increase ($p = 0.0001$ for both), with an even further increase after 60 min compared to its particle-only group or the incubator pretreatment ($p = 0.0001$ for both).

For cell dimensions in groups with combined treatments, the ice/particle-5 and ice/particle-60 cells (Table 2) were significantly shorter above the insert and narrower than those in the particle-only group, but only the shorter exposure time increased the cell–cell boundary. Pretreatment at incubator or hood

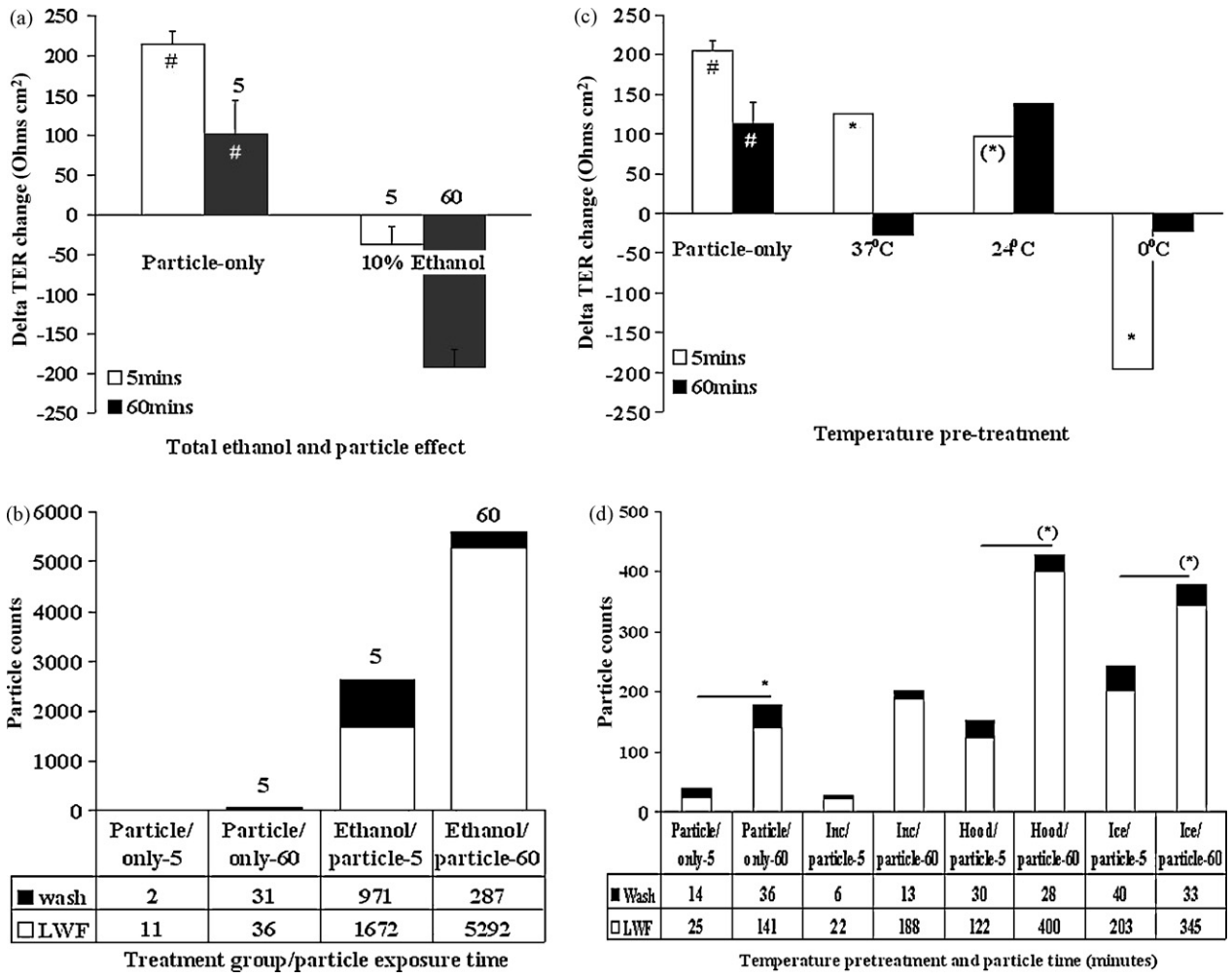

Fig. 2. (a) Combined ethanol/particle treatment lowers TER, cf. to the particle-only group. Zero line data are control/TER. Significant differences ($p \leq 0.05$) are illustrated as '#' for a change from control/TER and '5' or '60' a change from the ATCC particle-only group at those particle exposure times. Data are from 7 to 9 inserts over 3 experiments. (b) Ethanol/particle groups allow more particles through than particle-only groups. Significant differences are given as in (a). 'LWF' is lower well fluid. Data are from 6 inserts over 2 experiments. (c) Temperature/particle vs. temperature/sham (time equivalence) shows an unusual initial TER decrease for ice. Zero line data are time equivalence sham values. Pretreatment time equivalence calculation took into account the continued temperature effect occurring during the particle incubation period. Significant differences ($p \leq 0.05$) or strong trends ($p \leq 0.1$), indicated by brackets, are illustrated as '*' for a change from its sham group. (d) Sub-membranous particle counts are unchanged by low temperatures. LWF is lower well fluid. Statistical differences are given as significant differences ($p \leq 0.05$) and strong trends ($p \leq 0.1$).

temperatures brought fewer changes to cell shape. However, 5 min particle exposure appeared to increase the amount of epithelium below the insert from 4.2% of the total epithelial height for the particle-only group to 15.5 and 12.3%, respectively, for hood and ice groups.

3.3.2.3. Time equivalence comparison. Time equivalence comparisons of temperature/particle groups with their sham groups (Table 5; Fig. 2c) showed that long-term cell cooling increasingly altered the 5 min particle effect from a TER increase for incubator and hood groups to a TER decrease for ice. However, the 60 min particle effect was not significantly different from the control/TER level, irrespective of the direction or extent of its earlier change. Both ice/particle groups had similar dimensions to the corresponding ice/sham cells (Table 2). The groups exposed to particles after hood and incubator temperature pretreatments showed some significant increases in dimensions by comparison to their sham groups, but were less high only for the hood/particle-5 group, whereas the incubator/particle-5 group had less intercellular volume than its incubator/sham-5 group. The incubator/particle-60 group had less cell–cell boundary than its incubator/sham-60 group but a higher proportion of epithelium below the insert (16.1% of the total epithelial height compared to 6.5%, respectively). The hood/particle-60 group had a very low proportion of epithelium below the insert (3%) by comparison with the hood/sham-60 (12.8%).

The number of particles that passed into the sub-membranous compartment was usually significantly greater at 60 than at 5 min, but there was no change from the corresponding ATCC particle-only group for any of the temperature pretreatment groups or times (Fig. 2d).

3.4. Comparison of Caco-2 cells from different sources

Key results for ATCC particle-only groups for TER and cell dimensions respectively have been summarised in Tables 2, 4 and 5. For ECACC cells, the data on TER, particle count and cell dimensions have been previously published (Moyes et al., 2007a, 2008) and were included here only for comparison with those of the ATCC particle-only group.

The mean control/TER value for ECACC Caco-2 cells was $993 \pm 36\,\Omega\,cm^2$, with a range of 302–3041 $\Omega\,cm^2$ (Moyes et al., 2007a). This was significantly higher than the ATCC cells ($p = 0.002$, Section 3.1.1). Although cells from both sources started from very

Table 5
Temperature pretreatment (yellow dashed boxes) and particle exposure (green bold boxes) effects on TER.

Treatment	Experimental timeline (minutes from control/TER) with TER values (Ω·cm²)						Delta changes in TER (Ω·cm²)	
	Control/TER 0mins	5	15	20	60	75	Temperature effect cf control/TER	Particle effect cf pretreatment/ TER
ATCC particle-only								
Particle/only-5	410 ±11	615 ±16						# 205 ±13
Particle/only-60					524 ±24			113 ±28
Cell culture incubator: 37°C								
Incubator/sham-5	472 ±38			574 ±43			# 102 ±9	
Incubator/sham-60	535 ±20					578 ±24	44 ±7	
Incubator/particle-5	515 ±17		639 ±16	723 ±40			# 125 ±7	† 99 ±18
Incubator/particle-60						548 ±35		† -105 ±22
Sterile culture hood: 24°C								
Hood/sham-5	466 ±57			808 ±72			# inc 343 ±17	
Hood/sham-60	527 ±42					956 ±73	# inc 429 ±32	
Hood/particle-5	422 ±18		798 ±41	835 ±39			# inc 375 ±38	63 ±15
Hood/particle-60						1017 ±83		† 193 ±21
Ice: 0°C								
Ice/sham-5	471 ±69			1358 ±119			inc # hood 15m 887 ±52	
Ice/sham-60	513 ±48					1493 ±111	inc # hood pretreat 980 ±65	
Ice/particle-5	419 ±17		1069 ±32	1085 ±60			inc # hood 650 ±19	72 ±24
Ice/particle-60						1406 ±47		† 280 ±28

Mean TER ± standard error of the mean (SEM) from 2–3 independent experiments, before and after temperature treatment and particle exposure. Longer term hood temperature groups exposed cells to this lower temperature for considerably longer than for a standard TER measurement which was typically around 2.5 min. Delta changes in TER were calculated on a sample-by-sample basis and therefore may differ from direct subtraction of pooled mean control/TER and pretreatment/TER values. Significant differences ($p \leq 0.05$) are as follows; '#' shows a change from control/TER, '†' a change from pretreatment/TER, 'inc' and 'hood' a change from the incubator or hood temperature pretreatment at the same exposure time and 'pretreat' a change from the 15 min pretreatment effect. Time equivalence comparisons were calculated for all temperature groups, i.e. incubator/particle-5 as the [particle effect (blue zigzag box) – incubator/sham effect (blue zigzag box)]. The same calculation was carried out for the incubator/particle-60 group using data in the grey triple-outlined boxes.

different control/TER levels, both showed an increase/decrease TER response. However, by 60 min the ECACC cells showed a delta decrease, where the TER dropped below the original control/TER, making it significantly different from the ATCC particle/only-60 group ($p = 0.007$). At later time-points the range of TER change was larger in ECACC Caco-2 cells (Moyes et al., 2007a).

Confocal microscopy of ECACC Caco-2 cells showed that, 30 min after particle administrations, they were similar to their ATCC comparators with respect to lack of multilayering and the positioning of cellular material (Fig. 3). Cell dimensions for ATCC particle-only cells after 5 and 60 min were similar (Table 2) and the height and apical dimensions of the ECACC cells were smaller than those for the ATCC particle-only group at both time-points ($p = 0.004$–0.0001; Fig. 3). By comparison to all ATCC groups the ECACC percentage intercellular space was very low (4.7%), whereas the cell–cell/crosswire boundary (10.4×10^6) was high.

A complete comparison of ECACC and ATCC sub-membranous particle numbers was not possible because of the lack of wash particle samples in the former treatment group, since the methodology was still being developed.

4. Discussion

The current paper explores further the effects on Caco-2 cells of variations in an experimental protocol, in this case microparticle addition (Moyes et al., 2007a, 2008). The discussion is in two sections: comments on the model itself and effects of pretreatment on particle uptake.

The effects of laboratory manipulation on TER described here confirm the variations across laboratories, with passage number and temperature (Delie and Rubas, 1997; Sambuy et al., 2005; Hughes et al., 2007; Volpe, 2008). The level of TER could also be

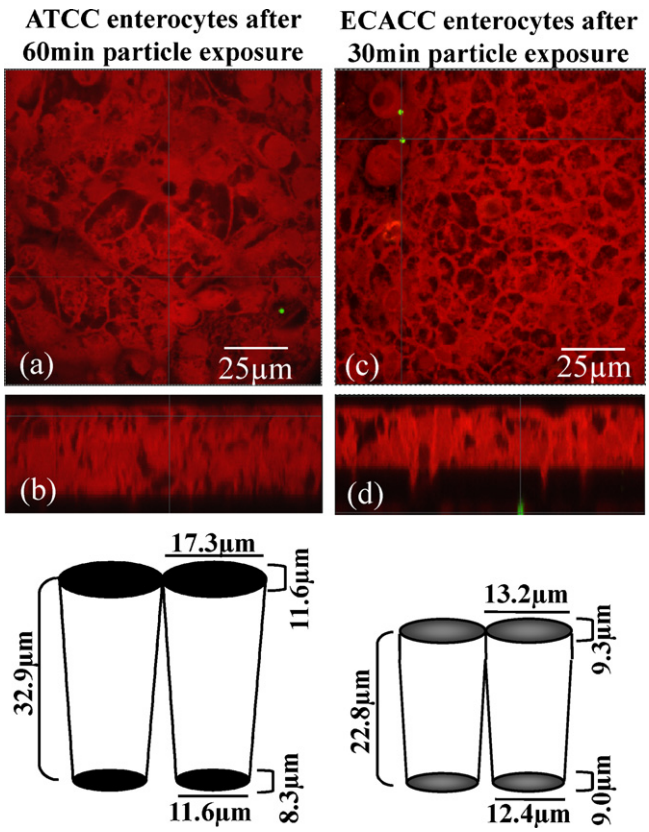

Fig. 3. Particle-laden ECACC cells are smaller than ATCC cells. Representative confocal images to illustrate ATCC and ECACC Caco-2 cells stained red with propidium iodide or FM1-43 FX, respectively. The ATCC particle-only group was exposed to particles for 60 min (a and b) and the ECACC model exposed for 30 min (c and d). (a and c) Intercellular junctions can be seen as polygonal outlines in this transverse plane and particles are visible within the epithelium. (d) A particle is visible below the epithelium and insert membrane. The Z plane views (b and d) are compiled from a minimum of 57 sequential images 0.85 μm apart. Schematics of ATCC cell dimensions were based on data from Table 2, which also shows statistical differences, whereas those for ECACC were taken from Moyes et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

related to the influence of the medium (Ranaldi et al., 2003), since the current data confirm that addition of a volume of medium similar to that of the particle suspension increases the TER (Moyes et al., 2007a). ATCC monolayers have a lower control/TER value than ECACC monolayers, in keeping with reported variations in cells from different sources (Delie and Rubas, 1997). The smaller ECACC apical width may contribute to their higher control/TER, as this would imply more cells/unit area and therefore more TJ regions.

The fact that TER is so affected by these factors dictates the use of standard laboratory and recording techniques and the exclusion of samples whose data do not meet defined criteria, such as those with low control/TER values or those lacking the increase/decrease pattern response to particle addition. Furthermore, the impact of temperature and sequential-TER recording suggests that the number of TER measurements should be minimised, their timing planned, and their values considered critically. The results show that variations in Caco-2 cell culture conditions affect epithelial TJ permeability as measured by TER, thus validating the first hypothesis of this study.

Control/TER values from readings for confluent ATCC Caco-2 cells are within the range of literature values (Delie and Rubas, 1997) and provide an adequate comparator for other treatment groups. They also influence the extent of the particle-induced TER change, but do not alter the increase/decrease response pattern. However, of the 50% increase in TER recorded 5 min after particle exposure, up to half may be due to the 'cooling and sequential-TER effect'. The lack of agreement between this particle-induced increase/decrease pattern and the unchanged TER reported by McClean et al. (1998) is probably due to differences in the type of particle and electrophysiological technique. The current results show that Caco-2 cell source affects epithelial TJ permeability as measured by TER, which further addresses the first hypothesis. This increase/decrease pattern confirms earlier results on ECACC cells (Moyes et al., 2007a, 2008), although the ATCC increase is higher and the subsequent decrease is less marked.

The height of the columnar epithelium of the morphology-control group is 27.2 μm, within the literature range of 17.5–29.6 μm for Caco-2 cells *in vitro* (Hidalgo et al., 1989; van't Hof and van Meer, 1990; Nicklin et al., 1992) and of 25–29.6 μm for *in vivo* human enterocytes (Delie and Rubas, 1997). The apical dimensions of 14.7 μm × 8.7 μm are smaller than the 18.95 μm × 11.78 μm quoted by des Rieux et al. (2005) and the 36.3 μm × 19.8 μm of cells on cover-slips (de Carvalho et al., 2006). The fact that these dimensions are so near to literature values and that only a very few samples are rejected on the basis of the presence of holes in the epithelial layer, implies that the viability of the sheet is not compromised. However, this could be explored further with the use of tests specifically designed to identify apoptosis or necrosis (Galluzzi et al., 2009).

The increase in cell size on addition of particles is a new finding. Dimensional changes could be caused by the presence of particles, by TJ changes producing cellular effects separate from their gate/fence roles (Matter et al., 2005) or by changes in the cytoskeleton via its association with TJ proteins (Madara, 1987; Aijaz et al., 2006). The changes in cell dimensions may take longer than TER to return to normal: these differences in timing could be explored further by identifying the changes in TJ and adhesion proteins using immunocytochemistry. This technique displays the network of proteins associated with these apicolateral membrane specialisations and how they are disrupted after some treatments (Ma et al., 1999; de Carvalho et al., 2006; Musch et al., 2006). The more detailed information given here on sub-insert epithelial dimensions are similar to those for ECACC cells (Moyes et al., 2007a). This small amount of sub-insert material shows that the particle-induced height increase above the insert is not just due to a redistribution of cellular volume through the pores.

With respect to uptake through to the lower well for particle-only groups, analysis of ratios of particle numbers shows that the rapid early uptake in the first 5 min is not maintained; there are 4–5 times as many particles at 60 min as at 5 min, but if the process are strictly time-dependent the ratio would be 12/1. Although uptake of latex microparticles is similar in the Caco-2 model and in the *in vivo*, *in situ* rodent small intestine (Moyes et al., 2007a), the continuing increase in particle numbers at 60 min *in vitro* cannot be directly comparable to changes *in vivo*, since there is no onward movement as there is in intestinal tissue (Hodges et al., 1995; Smyth et al., 2008).

The second major point for discussion relates to the effects of pretreatments, which can be used to predict how the particle-only response might be affected by them. The ethanol-induced changes, which include looser TJs, apical spreading and larger intercellular spaces, all predict more particle uptake. The small amount of sub-insert epithelium confirms that the total epithelial height is genuinely thinned by the ethanol and not redistributed through the pores. The TJ loosening is possibly due to separation of the ZO-1 protein from the cellular junctions, forming intercellular gaps (Ma et al., 1999). Although this is, as mentioned above, not likely to be associated with major cell loss, application of cell viability techniques to samples from all groups would allow further information on this to be collected. The apical spreading and lower epithelium

could be due to ethanol-induced cytoskeletal changes, such as disruption of perijunctional actin and myosin filaments through activation of myosin light chain kinase (Ma et al., 1999), dysfunction of NF-kappaB input to the F-actin assembly (Banan et al., 2007) and changes in arrangement of microtubules (Joseph et al., 2008). These tie into the roles of TJs in wider aspects of cell behaviour (Aijaz et al., 2006). Chronic ethanol intake affects rodent intestinal absorption (Kaur et al., 1993), with toxic compounds crossing into deeper tissues (Bjarnason et al., 1984). The ethanol pretreatment effect on TER is relevant to these *in vivo* considerations. It is also relevant with respect to its use as a vehicle, for example for reproductive hormones, where the lower concentrations needed to dissolve estrodiol and progesterone still decrease TER of Caco-2 cells after exposure for 21 days (Moyes et al., 2007b).

Maintenance of the cells at different temperatures produces a range of outcomes, as already reported for physiologically relevant temperatures above 37 °C, suggesting a TJ opening (Dokladny et al., 2006). However, exposing cells to lower temperatures, as seen here, produces TER and dimensional changes which may counteract each other. These higher TERs confirm those for Caco-2 cells moved from the incubator to a cooler sham treatment room (Moyes et al., 2008) and are in line with the response of chilled canine kidney (MDCK) cells, attributed to the changing ionic conductivity of the culture medium (Armitage et al., 1994). The TER increase, predisposing to less particle uptake, is counteracted by a trend to smaller basal dimensions: the reduced uptake predicted by the high TER will only take place if it is the only important parameter.

In summary, the second hypothesis is supported, since Caco-2 TERs are respectively decreased or increased by exposure to ethanol or lower ambient temperatures. However, the third hypothesis is only partially supported, since Caco-2 cell dimensions, which may influence permeability, are affected by ethanol but less so by variations in temperature. When the combined effects of pretreatment and particles are considered, the above predictions can be compared against actual particle numbers.

As predicted, ethanol-induced total particle uptake is significantly larger than that of particle-only groups, being nearly 200 times higher after 5 min. However the ethanol-induced increase shows less clearly than the particle-only groups the pattern of a high immediate rate diminishing with time, implying that the opened TJs and associated dimensional changes allow such an early increase in uptake that it cannot continue to rise. The ethanol-induced TJ opening is more important in the combined response than any effect of particle addition because the combined ethanol/particle TER is not different from the sham value at both time-points, implying that ethanol opens TJs substantially and the added particles merely use this already opened route. This TJ opening, with possible disruption of ZO-1 protein and perijunctional actin and myosin (Ma et al., 1999) probably contributes to the greatly increased number of sub-membranous particles: further work using immunocytochemical techniques would be useful here.

All the changes in cell dimensions with ethanol treatment also contribute to the increase in particle uptake and there are few differences between pretreatment/particle groups and the corresponding sham values, confirming the conclusions from the TER results that ethanol is more important than particle addition in producing the final combined effect.

With respect to other treatments that increase particle uptake, the ethanol-induced increase in lower well particle numbers is in the same range as that for irradiated ECACC Caco-2 cells, despite the fact that pretreatment and particle times differ (Moyes et al., 2008). The dimensional changes are also broadly similar, although there are some differences in width and intercellular space. The possible mechanistic differences could be explored further by investigation of the changes in junctional proteins.

The situation for temperature/particle-related changes is less straightforward than that for ethanol, since the high TER caused by cooling is not matched by clear evidence of low particle uptake, although the ice-treated group does have the smallest increase in particle numbers from 5 to 60 min of all of the groups. For cell dimensions, apical and basal parameters appear to act on particle uptake in opposite directions, apart from the hood/particle-60 group, where both would reduce uptake. The one parameter consistently facilitating increased uptake and therefore counterbalancing the increased TER is cell height, where there is no change after pretreatment at lower temperatures, but a continuing decrease in all cooled particle groups. It may be relevant that pretreatment times used here and taken from the literature are different for temperature (Armitage et al., 1994) and ethanol (Ma et al., 1999): the shorter pretreatment time for temperature groups possibly does not allow dimensional changes to develop fully and reduced uptake could be produced by longer cooling. For now, the prediction that fewer particles would be taken up at lower temperatures is not met, implying that TER is not the sole important factor: the final outcome is a balance between decreased particle uptake due to higher TER and increased uptake due to the lower height of the epithelium.

The results for pretreatment with ethanol and temperature variation provide some insight into the changes after particle addition. From the TER increase associated with low temperature, it could be deduced that the initial increase after particle addition might be related to the transient cooling it produces. On the other hand, the subsequent TER particle-induced decrease and associated increase particle uptake may have a TJ-related mechanism similar to that produced by ethanol.

Control of particle uptake is therefore multi-factorial, with TER, epithelial height and intercellular spaces all important, thus addressing and supporting the last hypothesis. The demonstration of the significance of laboratory microenvironment, including the sequential-TER measurements and temperature effect, also highlights the importance of taking such factors into account when estimating the true effects of an experimental treatment such as the exposure to ethanol or external irradiation.

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