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Use of Simulated Intestinal Fluids with Caco-2 Cells and Rat Ileum

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ABSTRACT In most in vitro studies of oral drug permeability, little attempt is made to reproduce the gastrointestinal lumenal environment. The aim of this study was to evaluate the compatibility of simulated intestinal fluid (SIF) solutions with Caco-2 cell monolayers and Ussing chamber-mounted rat ileum under standard permeability experiment protocols. In preliminary experiments, fasted-state simulated intestinal fluid (FaSSIF) and fed-state simulated intestinal fluid (FeSSIF) solutions based on the dissolution medium formulae of Dressman and co-workers (1998) were modified for compatibility with Caco-2 cells to produce FaS-SIF and FeSSIF "transport" solutions for use with in vitro permeability models.

For Caco-2 cells exposed to FaSSIF and FESSIF transport solutions, the transepithelial electrical resistance was maintained for over 4 h and mannitol permeability was equivalent to that in control (Hank's Balanced Salt Solution-treated) cell layers. Scanning electron microscopy revealed that microvilli generally maintained a normal distribution, although some shortening of microvilli and occasional small areas of denudation were observed. For rat ileum in the Ussing chambers, the potential difference (PD) collapsed to zero over 120 min when exposed to the FaSSIF transport solution and an even faster collapse of the PD was observed when the FeSSIF transport solution was used. Electron micrographs revealed erosion of the villi tips and substantial denudation of the microvilli after exposure of ileal tissue to FaSSIF and FeSSIF solutions, and permeability to mannitol was increased by almost two-fold.

This study indicated that FaSSIF and FeSSIF transport solutions can be used with Caco-2 monolayers to evaluate drug permeability, but rat ileum in Ussing chambers is adversely affected by these solutions. Metoprolol permeability in Caco-2 experiments was reduced by 33% using the FaSSIF and 75% using the FeSSIF compared to permeability measured using HBSS. This illustrates that using physiological solutions can influence permeability measurements.

KEYWORDS Fed state, Fasted state, In vitro, Permeability, Bile salt, Metoprolol, Ussing chamber

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INTRODUCTION

Early prediction of the absorption of drug candidates is important to the development of pharmaceutical products, making the ability of in vitro methods to provide this information extremely important to the pharmaceutical industry. The failure of in vitro results to predict adequately the in vivo performance of a drug or formulation can delay and add substantially to the cost of product development. With current in vitro drug transport models, little attempt is made to reproduce the gastrointestinal luminal environment. Pre- and post-prandial conditions in the intestine provide distinct environments and luminal conditions can affect the transport of certain drugs. However, the feasibility of using simulated intestinal fluid (SIF) rather than salt solutions for in vitro testing must be evaluated before the impact of using more physiological conditions on drug permeability can be evaluated.

The biopharmaceutics classification scheme (BCS) correlates data from in vitro tests for drug dissolution and permeation with in vivo bioavailability (Amidon et al., 1995; Lindenberg et al., 2004). The BCS categorizes drugs into four classes using two limitations to oral drug bioavailability: drug solubility and permeability. The influence of using "physiological" media in dissolution studies has been demonstrated for poorly soluble drugs (Dressman & Reppas, 2000; Kostewicz et al., 2004; Nicolaides et al., 1999; Porter & Charman, 2001). Differences in drug dissolution may result from altered drug solubility, diffusivity, or stability in SIF solutions compared to simpler buffered salt solutions. If used in drug transport studies, SIF solutions could affect drug permeability through either physicochemical effects such as altered aqueous drug concentration (Mithani et al., 1996) or through physiological/biochemical effects on drug transporters/efflux mechanisms or membrane permeabilization (Anderberg et al., 1992; Ingels et al., 2002; Schuldes et al., 2001; Meaney & O'Driscoll, 2000).

Many factors can affect drug absorption from the intestine including the presence and type of food, agitation, volume, bile salts, and pH. The two most significant components of intestinal fluid are bile salts and lipids, which facilitate the wetting of solids and the solubilization of lipophilic drugs into mixed micelles. Bile salts and lecithin are present in approximately 2–5 times greater amounts in fed-state conditions than in the fasted state. Simulated intestinal fluid (SIF) solutions developed to simulate fasted state conditions for dissolution testing have contained 3–5 mM of bile salt with lecithin incorporated in ratios of bile salt:lecithin at 4–5:1 (Porter & Charman, 2001). Simulated intestinal fluid (SIF) solutions developed to simulate fed state conditions have contained 10–15 mM of bile salt with lecithin incorporated in ratios of bile:lecithin of 2–4:1 (Porter & Charman, 2001). These SIF solutions were designed to reproduce the conditions that drug formulations may encounter in the human intestine (Charman et al., 1997). An aspect of this study was to test whether SIF solutions developed for dissolution studies are suitable for use with drug transport models.

Rat intestine mounted in Ussing chambers is a recognized model to test intestinal drug permeability in vitro, (Polentarutti et al., 1999; Ungell, 1997; Boisset et al., 2000) and in most studies, the use of a medium that maintains the tissue has been the priority. Over the last decade cell culture models of the epithelium, in particular Caco-2 cells, have become increasingly popular for assessing drug permeability. Both rat intestine and Caco-2 cell culture models can be used to predict human intestinal absorption of drug compounds (Ungell, 1997; Lennernas, 1998). Caco-2 monolayers can be used to assess paracellular transport, carrier mediated and passive transcellular drug transport, and drug efflux by P-glycoprotein (Gan & Thakker, 1997; Delie & Rubas, 1997; Artursson & Borchardt, 1997; Artursson et al., 2001). For biorelevance, it can be argued that studies assessing drug transport from the lumen of the gastrointestinal tract should be conducted in media that reproduce the conditions in the human intestine. The use of a fluid that closely mimics intestinal fluid is likely to be even more important if formulation/excipient effects are to be examined.

Human intestinal fluid contains a mixture of conjugated and unconjugated bile salts (cholic, deoxycholic, and chenodeoxycholic acids) which have different hydrophobicities, solubilizing/wetting properties, and effects on cell membranes. Permeability enhancing effects of individual bile salts have been demonstrated using rat intestine (Sinko et al., 1999; Dangi et al., 1998) and Caco-2 cell monolayers (Anderberg et al., 1992; Meaney & Driscoll, 2000), but are often associated with epithelial toxicity. The susceptibility of Caco-2 cells to damaging effects of individual bile salts will be critical to the development of SIF solutions that are compatible with the Caco-2 drug absorption model.

It has previously been shown that FeSSIF adversely affects Caco-2 cells (Ingels et al., 2002). The aim of the present study was to investigate the feasibility of modifying SIF solutions to remain physiologically relevant, but to be compatible with the two most widely used biological in vitro intestinal permeability models: Caco-2 cell monolayers and rat intestinal tissue mounted in Ussing chambers. The presence of mucus might be important for the protection of the apical surface of enterocytes from SIF-mediated toxicity, and the use of these models allows compatibility with SIF solutions of the mucus-lacking Caco-2 cell layers to be compared to the mucus-shielded intestinal tissue (Meaney & Driscoll, 1999).

MATERIALS AND METHODS

Materials

The sodium salts of bile acids [glycocholate (GC), taurocholate (TC), deoxycholate (DC), taurodeoxycholate (TDC), and cholate (C)], Hank's balanced salt solution (HBSS), and glutamine were purchased from Sigma Chemical Company, UK and used as obtained. Lecithin (Epikuron 200, 97% phosphatidyl choline) was obtained from Lucas Meyer, Germany. Sodium hydroxide, potassium chloride, potassium dihydrogen phosphate, and acetic acid were obtained from BDH Chemicals, Poole, UK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Company, Ltd. All tissue culture media and plastics were obtained from Sigma Chemical Company, Poole, UK.

Bile Salt Solutions and Simulated Intestinal Fluids

Solutions of bile salts were prepared to represent major types of bile salt for toxicity testing with Caco-2 cells. Solutions in the concentration range 0–30 mM were prepared in HBSS for use in the MTT assay. Fasted-state SIF (FaSSIF) solutions and fed-state SIF (FeSSIF) solutions were made according to the formulas of Dressman and co-workers (Dressman et al., 1998) and designated "dissolution" SIF solutions (Table 1). Modified formulas were also developed, guided by MTT test results, to be more biocompatible with Caco-2 cells and these were designated "transport" SIF solutions (Table 1). On the basis of the preliminary MTT results, the concentration of lecithin in

TABLE 1 **Composition of Salt Solutions and SIF Investigated for Compatibility with Caco-2 Cells and Rat Ileum**

Component	Hank's balanced salt solution (mM)	KBR solution (mM)	Fasted-state dissolution SIF (mM)	Fed-state dissolution SIF (mM)	Fasted-state transport SIF (mM)	Fed-state transport SIF (mM)
Calcium chloride	1.67	1.20	—	—	1.67	1.67
Fumaric acid (disodium)	—	5.40	—	—	—	—
Acetic acid	—	—	—	144.00	—	—
Magnesium sulphate	0.81	1.20	—	—	0.81	0.81
Potassium chloride	5.37	4.69	103.29	203.89	5.37	5.37
Potassium phosphate dibasic	—	0.47	22.39	—	—	—
Potassium phosphate monobasic	0.44	—	—	—	0.44	0.44
Sodium bicarbonate	0.42	16.00	—	—	0.42	0.42
Sodium chloride	136.89	108.01	—	—	136.89	136.89
Sodium phosphate dibasic	0.34	—	—	—	0.34	0.34
Sodium phosphate monobasic	—	5.38	—	—	—	—
Sodium pyruvate	—	4.90	—	—	—	—
D-Glucose	5.55	11.50	—	—	5.55	5.55
L-Glutamine	—	5.67	—	—	2.00	2.00
Lecithin	—	—	0.75	3.75	0.75	7.50
Sodium taurocholate	—	—	3.00	15.00	3.00	15.00
MES (1 M solution)	—	—	—	—	to pH	to pH
Sodium hydroxide	—	—	to pH	to pH	—	—
pH	7.3	7.4	6.5	5.0	6.5	6.0
Osmolality (mOsm/Kg H_2O)	280	312	270	635	343	336

transport FeSSIF was set at a level providing a 2:1 bile salt:lecithin molar ratio as this provided cytocompatibility at a physiologically relevant concentration. To manufacture the SIF solutions, the components were added to volumetric flasks which contained either distilled water or HBSS. The mixture was stirred continuously until all ingredients were dissolved and the solution was made up to volume. The pH was adjusted using NaOH or acetic acid for the dissolution formulas, and by 1 M solution of 2-(N-morpholino)ethane-sulfonic acid (MES) for the transport formulas. The osmolality was assessed by using a Micro-Osmometer (Vitech Scientific Ltd) and the SIF solutions were stored at −20°C and used within one month of preparation.

Cell Culture

Caco-2 cells were purchased from ATCC (Rockville, MD, USA) and were used between passage numbers 75–85. They were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal bovine serum, 1% v/v non-essential amino acids, 1% v/v L-glutamine (200 mM solution), and antibiotics (100 IU penicillin/mL and 100 μg streptomycin/mL). Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity and the medium was replaced every two to three days. Cell stocks were cultured in flasks and passaged weekly using trypsin (0.25% w/v)-EDTA (0.2% w/v) solution. For MTT assay, cells were seeded onto 96-well plates (Costar, High Wycombe, UK) at approximately 2×10^4 cells/well and incubated overnight. For permeability experiments, Caco-2 cells were seeded onto polyester Transwell® filters (Costar, UK; mean pore diameter, 0.45 μm) at a seeding density of 1×10^5 cell/mL and used to examine the effect of SIF solutions on transepithelial electrical resistance (TER) between 21–28 days.

Rat Intestine in Ussing Chambers

The intestine was removed surgically from male Sprague Dawley rats, 200–300 g in weight, and flushed with cold Krebs-bicarbonate Ringers (KBR) solution. The ileum was selected taking care to avoid Peyer's patches and the serosa was stripped away. The tissue was mounted in Ussing chambers (World Precision Instruments, Stevenage, UK) positioning 0.67 cm^2 of intestinal epithelium between mucosal and serosal chambers. Oxygenated KBR (37°C, 7 mL) was added to the mucosal and serosal chambers and the tissue was left for 30 min to equilibrate.

MTT Assay

MTT solution was used to assess the effect of bile salts and SIF solutions on the viability of the Caco-2 cells. MTT was prepared in sterile phosphate buffered saline (PBS) at a concentration of 0.5 mg/mL. The solution was filtered through a 0.22 μm filter and stored at 2–8°C. A solution of 20% w/v SDS in DMF:deionized water (1:1), pH 4.7 was used for the solubilization of formazan produced by the cleavage of MTT by mitochondrial dehydrogenase.

Toxicity was determined by replacing the culture medium with 100 μL of prewarmed bile salt solution, SIF or HBSS (control), and incubating for 2 h at 37°C. After this time, 20 μL of MTT solution was added to each well and the plates incubated for a further 2 h. Solubilizing solution (100 μL) was added to each well and the plates incubated for a further 2 h. The production of formazan was assessed spectrophotometrically at 570 nm using a SPECTRAMax 190 multiplate reader after correcting for background absorbance at 650 nm.

Electrical Measurements

Transepithelial electrical resistance (TER) of the Caco-2 cell monolayers on the Transwell® insert filters was measured using an EVOM epithelial voltohmmeter equipped with silver chloride chopstick electrodes (World Precision Instruments, Stevenage, UK). The culture medium was removed from Caco-2 monolayers, replaced by HBSS, and after 30–45 min the TER was measured (t = 0 min) at 37°C. To initiate the experiments, the apical chamber fluid was removed carefully and replaced by 0.5 mL of SIF or HBSS and the basolateral chamber fluid with 1.5 mL of HBSS. The TER was monitored over 240 min.

For Ussing chamber experiments, the fluid resistance of the system in the presence of KBR and absence of tissue was compensated using an EVC4000 Multichannel voltage/current clamp (World Precision Instruments, UK). The potential difference (PD) across rat intestine in Ussing chambers was measured continuously. Resistance was determined every 30 min by clamping the tissue at 0, ±15, and ±30 μA, noting the change in voltage and calculating the resistance using Ohms law (Polentarutti et al., 1999).

Electron Microscopy

Scanning electron microscopy (SEM) was performed after exposure to salt solution, transport FeSSIF, and FeSSIF by Caco-2 cell monolayers for 2 h and rat intestine for 90 min. After exposure to the SIF solutions, the epithelial cell monolayers in Transwells® or rat intestinal tissue in Ussing chambers were washed first with a 2% v/v solution of paraformaldehyde for 10 min, then fixed in 4% v/v paraformaldehyde and left in a refrigerator overnight. Electron microscopy was carried out using a Jeole 100 CX Mark 2 Electron Microscope. Post fixation of the cell monolayers and intestine was carried out in 1% osmium tetroxide in 0.1 M sodium cacodyate buffer followed by dehydration in acetone. The filter was cut, critically point dried, mounted, and sputter coated with gold before examination.

Solute Permeability

Caco-2 monolayers (21–28 days post seeding) were prepared by washing with HBSS followed by a 30 min recovery period. Immediately before the permeability testing, HBSS was replaced by 480 µL SIF or HBSS apically and 1.5 mL of HBSS basolaterally. For Ussing chamber experiments, rat ileum was allowed to equilibrate in oxygenated KBR for 30 min after which the fluid was replaced by 7 mL KBR in the serosal chamber and 7 mL SIF or KBR in the mucosal chamber. Permeability experiments were initiated by the addition of 20 µL of ^{14}C mannitol (250 µCi/mL in HBSS) to the apical chamber. Samples (100 µL) of basolateral solution were removed over the course of 120 min using Caco-2 cells or 90 min using intestinal tissue and replaced by 100 µL of fresh prewarmed HBSS. Experiments were conducted at 37°C. Absorptive transcellular permeability in Caco-2 cells was evaluated by measuring the permeability of ^{3}H-metoprolol, with concomitant ^{14}C mannitol permeability and pre- and post-experiment TER measurement to confirm cell layer integrity.

Samples were analyzed for ^{14}C mannitol ± ^{3}H-metoprolol following the addition of 5 mL of scintillant (Ready Protein+®, Beckman Instruments, High Wycombe, UK) by single or dual channel liquid scintillation counting using a 1209 Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland). Apparent permeability coefficients (Papp, cm s^{-1}) were calculated according to the equation

$$Papp = dQ/dt\ (1/ACo) \quad (1)$$

where dQ/dt is the gradient of the slope of flux versus time, A is the surface area of the Transwell® filter (cm^2), and C_o is the initial concentration in the apical chamber.

RESULTS

The effect of concentration of five bile salts on MTT conversion by Caco-2 cells was measured (Fig. 1). Bile salt toxicity (IC_{50}; MTT conversion <50% of control) to Caco-2 cells ranked from most toxic, DC (IC_{50} = 0.4 mM), through three bile salts with intermediate toxicity (TDC, C, GC) to the least toxic, TC (IC_{50} = 15 mM). This observation is consistent with reported erythrolytic and cytolytic effects of bile salts (Elhariri et al., 1992; Velardi et al., 1991; Coleman et al., 1980) and provided the basis for the development of SIF solutions based on the better-tolerated conjugated bile salt, TC. When TC-based dissolution FaSSIF and FeSSIF based on the formulae of Dressman and coworkers (Dressman et al., 1998) were tested for toxicity to Caco-2 cells, the FeSSIF produced an almost total abolition of dehydrogenase activity (Fig. 2) and a dramatic reduction in TER (Fig. 3a). As a consequence, modification of these fluids was undertaken to produce better tolerated transport SIF solutions (Table 1).

The dissolution FeSSIF reduced the TER of Caco-2 monolayers to the level of the filter alone within 20 min, although the dissolution FaSSIF caused less than 20% reduction of TER over 4 h (Fig. 3a). The transport FaSSIF and FeSSIF solutions were better tolerated

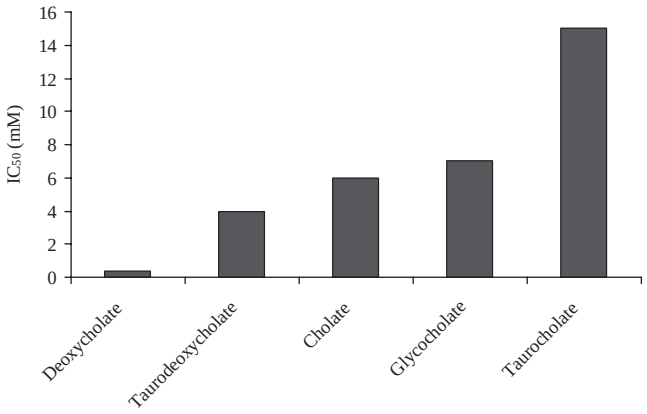

FIGURE 1 **Concentration of Bile Salts Producing a 50% Reduction in the Viability of Caco-2 Cells (IC_{50}) Compared to Control (Bile Salt-free HBSS). Viability of Caco-2 Cells Was Measured Using the MTT Test After 2 h Apical Exposure to Bile Salts at 0.1–30 mM (*n* = 8 Per Concentration).**

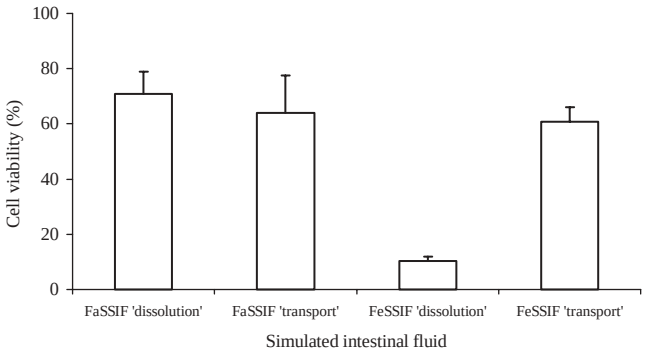

FIGURE 2 **The Effect of 2 h Exposure to SIF on the Viability of Caco-2 Cells Compared to Control (HBSS) Measured Using the MTT Assay. Data Represent Mean ± sd, *n* = 18.**

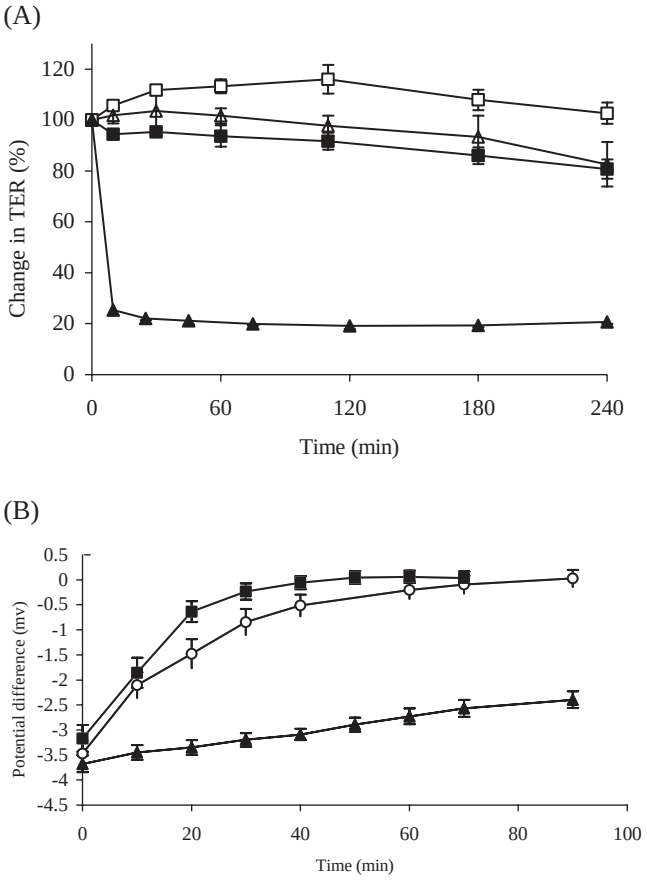

FIGURE 3 **(a) The effect of SIF on (A) the TER of Caco-2 Mo nolayers Cultured on Transwell Inserts. Key: Dissolution FaSSIF (■), transport FaSSIF (□), dissolution FeSSIF (▲), transport FeSSIF (△). Data Represent Mean ± sd, *n* = 4. (b) The PD of Rat Tissue Mounted in Ussing Chambers. Key: Krebs-bicarbonate Ringers Solution (▲), Transport FaSSIF (○), Transport FeSSIF (□). Data Represent Mean ± sd, *n* = 4.**

and TER was maintained over 4 h of exposure (Fig. 3a). Tight junctions were clearly visible by electron microscopy and, unlike monolayers exposed to dissolution FeSSIF, were maintained after exposure to the transport SIF solutions (not shown). The apical to basolateral (absorptive) mannitol transport was similar in the Caco-2 cell layers treated with the transport SIF solutions and Caco-2 cell layers exposed to HBSS (Fig. 4), indicating that the permeability barrier to small molecules was maintained. The absorptive flux of metoprolol in Caco-2 cells was reduced 33% by the use of FaSSIF and 75% by the use of FeSSIF compared to the permeability measured in HBSS (Fig. 5).

Scanning electron microscopy (SEM) of the surface of control cells revealed a uniform distribution of microvilli, which are flattened during preparation for microscopy thus revealing their length (Fig. 6a). After

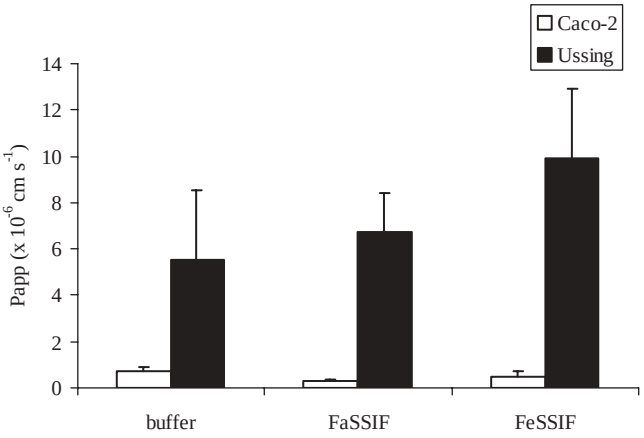

FIGURE 4 **Mannitol Permeability Across Caco-2 Cell Monolayers (*n* = 6) and Rat Intestine in Ussing Chambers (*n* = 5). Permeability Measured in the Apical to Basolateral (Absorptive) Direction After 120 min Exposure to Physiological Buffer, FaSSIF, or FeSSIF. Data Represent Mean ± sd. No Significant Difference in Permeability Was Observed with FaSSIF or FeSSIF Compared to Physiological Buffer (ANOVA; *p* > 0.05).**

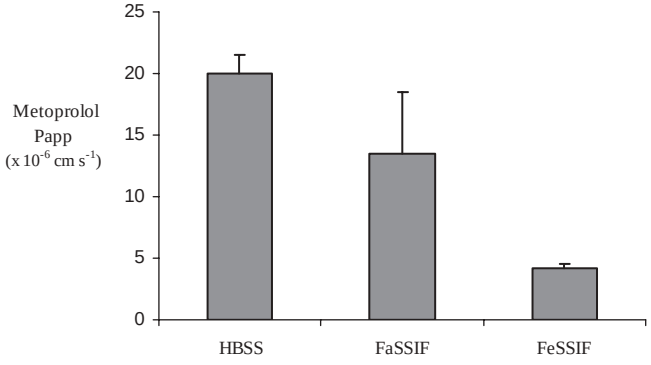

FIGURE 5 **Metoprolol Permeability Across Caco-2 Cell Monolayers (*n* = 9). Permeability Measured in the Apical to Basolateral (Absorptive) Direction After Exposure to Physiological Buffer, FaSSIF, or FeSSIF. Data Represent Mean ± sd.**

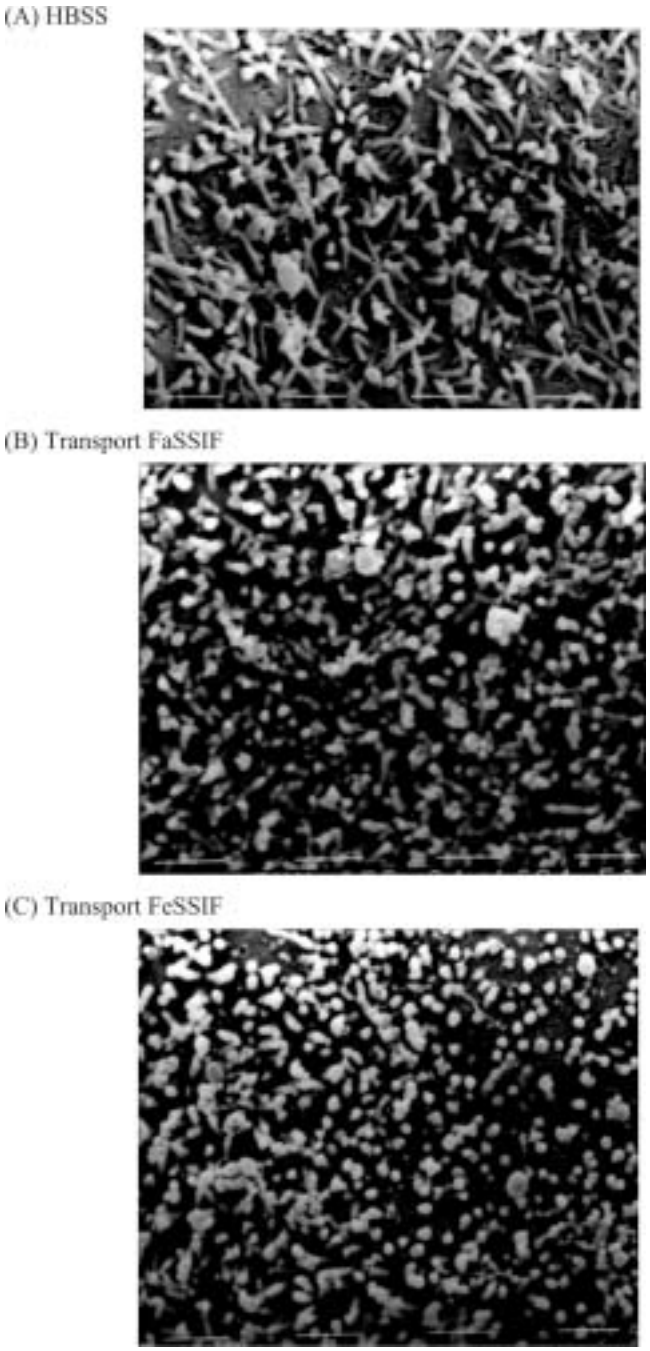

FIGURE 6 **Scanning Electron Micrographs (SEM) of Caco-2 Monolayers Exposed to (a) HBSS, (b) FaSSIF, and (C) FeSSIF for 240 min Under a Simulated Transport Experiment Protocol. Scale Bar 1 μm.**

exposure to transport FaSSIF, the distribution and appearance of microvilli was maintained, although they may be shortened slightly (Fig. 6b). After exposure to transport FeSSIF, the surface of the cells contained areas with normal appearance (bottom left quadrant; Fig. 6c) and areas where the microvilli were less dense and short (top right quadrant; Fig. 6c). These effects of the transport FeSSIF on microvilli are comparable to results obtained in previous studies of surface active agents on Caco-2 cells (Anderberg et al., 1992; Jorgensen et al., 1993; Lindmark et al., 1995).

The potential difference (PD) of the rat ileum in the Ussing chambers was in the typical range −3 to −4 mV reported for this model (Polentarutti et al., 1999) at the commencement of each experiment (Fig. 3b). When incubated with KBR, the PD was maintained for the duration of the experiment. When the transport FaSSIF was used, the PD collapsed to zero over 120 min. An even faster collapse of the PD was observed when the transport FeSSIF was used. The resistance measured over the course of the experiment was less dramatically affected with initial resistances of 40–50 Ohm cm^2 declining by 28 ± 5% and 45 ± 7% for ileal tissues exposed to transport FaSSIF (n = 4) and transport FeSSIF (n = 4), respectively. Electron micrographs revealed erosion of the villi tips and substantial denudation of the microvilli was clearly apparent after exposure to transport FaSSIF and FeSSIF solutions compared to KBR (Fig. 7). In addition, the permeability to mannitol was almost doubled when using the transport FeSSIF solutions (Fig. 4), although this increase was not significantly different from control ($P > 0.05$; Analysis of Variance, ANOVA).

DISCUSSION

The feasibility of using SIF solutions with intestinal drug transport models is dependent on the susceptibility of the epithelial layer to irritant components of SIF solutions. Bile salts are a major functional component of SIF solutions, but are also notably irritant. Preliminary studies using the MTT assay found bile salt toxicity to Caco-2 cells to conform to the recognized rank order of deoxycholate < cholate < conjugated cholate, with sensitivities similar to those reported for erythrocytes (Elhariri et al., 1992; Coleman et al., 1980). Conjugated bile salts have been widely used in dissolution media (Nicolaides et al., 1999; Porter & Charman, 2001; Vertzoni et al., 2004; Horter & Dressman, 2001), are prevalent in intestinal fluid, and are less damaging to epithelial cells (Meaney & Driscoll, 2000; Velardi et al., 1991).

The dissolution FaSSIF was reasonably well tolerated, but the dissolution FeSSIF adversely affected Caco-2 cells almost totally abolishing the ability of the cells to metabolize MTT and causing a collapse of the

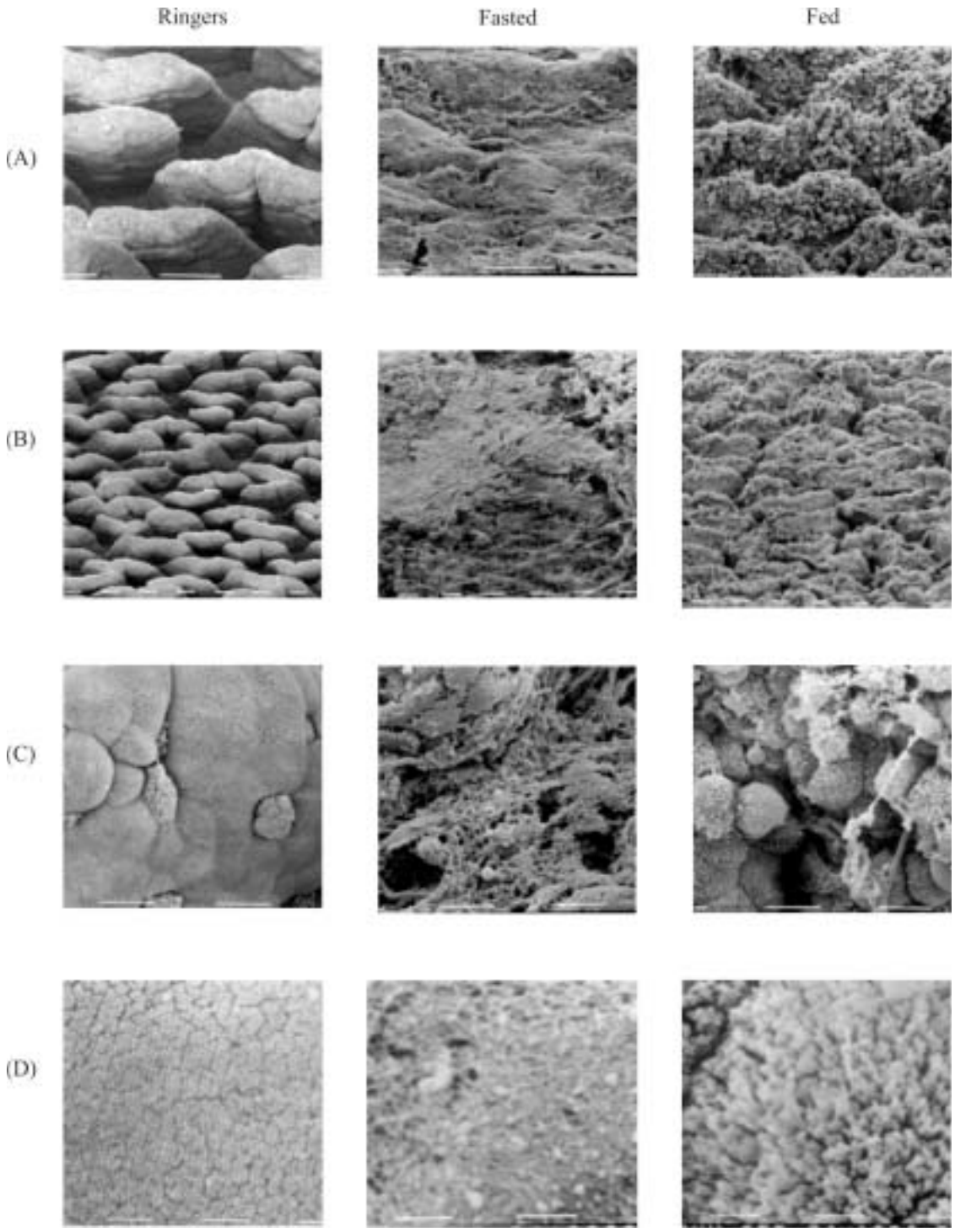

FIGURE 7 **Scanning Electron Micrographs (SEM) of the Mucosal Surface of Rat Intestinal Tissue in Ussing Chambers Exposed to Ringers Solution, FaSSIF and FeSSIF for 120 min Under a Simulated Transport Experiment Protocol. Magnification Is by Scale Bars (a) Surface of Tissue at High Magnification; Scale Bar 100 μm, (b) Surface of Tissue at Low Magnification; Scale Bar 100 μm, (c) Surface of Villi; Scale Bar 10 μm, and (d) Surface of Microvilli; Scale Bar 1 μm.**

monolayer TER. Modification of the dissolution SIF solutions with the aim of producing less irritant transport SIF solutions resulted in SIF solutions that were better tolerated by Caco-2 cell monolayers. Previous work has shown that the dissolution FaSSIF but not FeSSIF was tolerated by Caco-2 cells (Ingels et al., 2002), but no attempt has been made to modify SIF solutions to produce a Caco-2 compatible FeSSIF or to evaluate the effect of SIF solutions on intestinal tissue in Ussing chambers.

Modifications required for the transport FeSSIF included the use of HBSS rather than the less complex salt solution used for the dissolution SIF solutions, use of a 2:1 bile:lecithin ratio in comparison to the 4:1 ratio used for the other SIF solutions, and the use of less irritant pH and osmolality; pH 6 and 336 mOs-mol compared to pH 5 and 635 mOsmol for the dissolution formula. The taurocholate (TC) concentration in the FeSSIF solutions (15 mM) was at a level corresponding to the IC_{50} for TC in the MTT assay (Fig. 1), but phosphatidyl choline (PC) mitigated the toxicity as has been shown previously (Elhariri et al., 1992; Velardi et al., 1991). Taurocholate (TC) has a very low pKa, which endows the molecule with little likelihood of precipitation, change in the micellar size or toxicity with minor variations in pH (Martin et al., 1992). Compared to the fasted state, a much more variable range of conditions can be encountered postprandially. The reduction in osmolarity of the FeSSIF to the isotonic level to prevent toxicity (reduction in TER) resulted in a transport FeSSIF which still provided a realistic representation of conditions which might be encountered in vivo.

Mannitol transport and TER maintained levels that are accepted as indicative of continuous tight epithelial layers, indicating that Caco-2 monolayers maintained their barrier properties after application of the transport SIF solutions. Microscopy revealed a degree of shortening/denudation of microvilli of the Caco-2 cell, although these effects were limited and mosaically distributed. MTT conversion in the presence of transport SIF solutions was impaired compared to control, but to a lesser extent than that measured for the dissolution SIF solutions. These results show the cells maintain sufficient viability and barrier properties for transport experiments to be performed. Other studies suggest that transcellular permeability can be modified by the use of SIF solutions (Ingels et al., 2004), bile salts mixed micelles (Meaney & Driscoll, 2000), and that the use of physiological pH is important (Yamashita et al., 2000).

Rat ileum in Ussing chambers showed permeability and electrical properties similar to those previously reported (Polentarutti et al., 1999), but was more dramatically affected by the SIF solutions than the Caco-2 cells. Surprisingly, erosion of the ileal villi and microvilli was more dramatic than damage to the microvilli of the Caco-2 cells despite the presumed presence of residual protective mucus covering the surface of the ileal section. A possible explanation could be that the Ussing system is gas-stirred providing more energy (more friction) in the system than the unstirred Caco-2 cell system. Electrical parameters supported these observations with (i) a loss of potential difference in the presence of SIF solutions indicating an accelerated loss of tissue viability, and (ii) a reduction in resistance. However, the increase in permeability to mannitol when using FeSSIF was not significant compared to control conditions. In general, the progressive loss of viability that occurs over time for excised tissues was accelerated in the presence of SIF solutions, which appears to preclude the use of these fluids with Ussing chamber-mounted ileum. It should be noted that the SIF is based on HBSS, while the control experiments were performed using KBR according to the methods of Polentarutti and coworkers (Polentarutti et al., 1999); therefore, it is not possible to exclude a contribution of the HBSS to the adverse effects on the tissue.

The reduction in the permeability of metoprolol measured in FaSSIF compared to HBSS was consistent with the finding of Ingels and co-workers (Ingels et al., 2002) that passively transported compounds were either not affected or that their permeability was reduced. The use of FeSSIF in our experiments reduced the permeability to a much greater extent than the FaSIFF. The explanation for the influence of FaSSIF and FeSSIF on metoprolol permeability is likely to be that micellar solubilization by bile salt lecithin mixed micelles reduces the effective concentration of metoprolol in the donor solution, with the greater effect of FeSSIF due to the higher bile salt:lecithin concentration. The availability of both FaSSIF and FeSSIF, which are compatible with Caco-2 cells, will permit more detailed mechanistic in vitro drug transport studies.

In vitro epithelial models allow drug interaction with such factors as pre-systemic metabolism, efflux mechanisms, unstirred layers, and mucus to be studied (Charman et al., 1997; Meaney & Driscoll, 1999; Kaukonen et al., 2004; Li et al., 1996). The dependence of permeability values on factors such as the pH of transport solutions (Artursson & Borchardt, 1997; Yamashita et al., 2000) show the importance of using optimized transport models. This report indicates that drug transport studies can be performed utilizing FaSSIF and FeSSIF solutions with Caco-2 monolayers to investigate drugs permeability under more physiological

conditions than the salt solutions usually employed. The biopharmaceutics classification scheme (BCS) has driven a critical evaluation of in vitro methodology for the study of drug permeability. Simulated intestinal fluid (SIF) solutions might be useful for solubilizing poorly soluble drugs for which permeability data is difficult to obtain. The reported in vitro methods could also be applied to the development of delivery systems for difficult-to-deliver drugs by facilitating the study of biopharmaceutical processes such as metabolism/dispersion of formulations in intestinal fluid in combination with permeability measurement.

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