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Single versus repeated exposure to human polarized intestinal epithelial monolayers for *in vitro* protein hazard characterization

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ABSTRACT

Recent studies suggest human-derived intestinal epithelial cell (IEC) lines cultured as polarized monolayers on permeable Transwell® filters are effective at differentiating between hazardous and non-hazardous proteins following a single exposure. In this study, IEC polarized monolayers were subjected to hazardous or non-hazardous proteins in nine exposures over 30 days and compared to a single exposure of the same protein. The objective was to evaluate whether repeated exposures to a protein differently alter barrier integrity or compromise cell viability compared to single exposures. Proteins tested included *Clostridium difficile* toxin A, Streptolysin O, Wheat Germ Agglutinin, *Phaseolus vulgaris* Hemagglutinin-E, bovine serum albumin, porcine serum albumin, and fibronectin. Evidence of diminished barrier integrity and/or cell viability following exposure to hazardous proteins was more pronounced in magnitude when IECs were subjected to multiple rather than single exposures. In some cases, an effect on IEC monolayers was observed only with repeated exposures. In general, IEC responses to non-hazardous proteins following either single or repeated exposures were minimal. Results from these studies support the utility of using cultured human IEC polarized monolayers to differentiate between hazardous and non-hazardous proteins and suggest that repeated exposures may reveal a greater magnitude of response when compared to single exposures.

1. Introduction

Evaluation of the safety of proteins expressed in genetically modified (GM) crops is required by numerous regulatory agencies throughout the world. To date, safety testing has relied on a tiered weight of evidence approach that incorporates hazard identification (Tier I) and hazard characterization (Tier II; Delaney et al., 2008a). Hazard identification studies include several individual components that can be completed without isolation of the actual protein. These include information about the source of the gene (history of safe use) and bioinformatics analysis to determine if significant sequence similarities to known allergens or protein toxins exist. Other components of protein hazard identification typically require isolation and characterization of small quantities of the protein. These components include determination of the mode of action, susceptibility to degradation upon exposure to digestive enzymes *in vitro*, and stability upon exposure to heat. Conceptually, if a hazard was not identified following these robust analyses then further testing requiring larger amounts of protein (i.e. Tier II – hazard characterization) would not be necessary. This tiered approach has been supported with evidence from many proteins that did not demonstrate proof of hazard from Tier I testing and which subsequently did not exhibit adverse effects in acute, and in some cases, repeated dose animal toxicology studies (Delaney et al., 2008b; Mathesius et al., 2009; Juberg et al., 2009; Xu et al., 2009; Cao et al., 2010, 2012; Stagg et al., 2012; Wang et al., 2016). Despite these results, some regulatory agencies continue to require hazard characterization, i.e. *in vivo* acute or repeated dose toxicity studies in mice or rats, irrespective of outcomes following Tier I analysis.

While the Tier II toxicology protocols for protein hazard characterization were historically established *in vivo* using mice and rats, there has been a globally-supported effort to replace, reduce, and refine experimental approaches involving live animals where scientifically feasible (Russell and Burch, 1959; EFSA, 2009; European Commission, 2010; European Commission, 2018; Krewski et al., 2010; NRC, 2007a;

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NRC, 2007b; US-EPA, 2016). Application of an *in vitro* method employing human intestinal epithelial cell lines (IEC) to generate polarized intestinal barrier monolayers for evaluating acute toxicity potential of proteins has some advantages over *in vivo* methods. The IEC *in vitro* method would not only reduce or eliminate the number of animals required for protein safety assessment, it would also establish a human-based experimental platform that requires significantly less test substance. Furthermore, such a system has the potential to provide additional information regarding the safety of unknown proteins. This is important in the case of some proteins expressed in genetically modified crops that are difficult or impossible to isolate in the quantities necessary to conduct *in vivo* toxicology studies (Bushey et al., 2014; Delaney, 2017; Hurley et al., 2016b).

Alternative methodologies to animal acute toxicology studies for protein hazard characterization have recently been explored. These include human IEC polarized monolayers and primary human small intestinal barrier models cultured on permeable Transwell® filters (Hurley et al., 2016a, 2016b; Eaton et al., 2017; Markell et al., 2017; Zimmermann et al., 2018). Such *in vitro* approaches have demonstrated a capability of distinguishing between known hazardous and non-hazardous proteins after intestinal monolayers were exposed to a single dose of various test proteins for a duration of up to 24 h (Hurley et al., 2016a, 2016b; Eaton et al., 2017). More recent investigations implemented a protocol that extended the duration of test protein exposure beyond 24 h. These studies revealed that single dose exposures for 48 or 72 h further increase the effectiveness of the model to distinguish between hazardous and non-hazardous proteins (Markell et al., 2017; Zimmermann et al., 2018). While an experimental design with single dose exposures of increasing duration yielded data that more clearly resolved hazardous and non-hazardous proteins, it remained unclear whether a repeated exposure protocol using this *in vitro* experimental platform would represent an effective and feasible approach. The study herein compares the impact of a single overnight exposure to various test proteins versus repeated IEC monolayer overnight exposures to the same concentrations and proteins. Both approaches were evaluated for their effectiveness at discerning hazardous from non-hazardous proteins.

2. Materials and methods

2.1. Test substances

Proteins and other test substances used in this study are listed in Table 1. Fibronectin (Fbn), Porcine Serum Albumin (PSA), Streptolysin O (SLO), and Triton X-100 (TX-100) were purchased from Sigma Chemical Company (St. Louis, MO). Wheat Germ Agglutinin (WGA) and Unconjugated *Phaseolus vulgaris* hemagglutinin (PHA-E) were purchased from Vector Laboratories (Burlingame, CA). *Clostridium difficile* Toxin A (ToxA) was purchased from List Laboratories, Inc. (Campbell, CA). Bovine serum albumin (BSA) was purchased from Fisher Scientific (Waltham, MA). All proteins were diluted in assay media (phenol red free, serum free, antibiotic free DMEM/F12) purchased from Invitrogen Corporation (Carlsbad, CA) and examined at the concentrations indicated in Table 1.

2.2. Cell culture

Intestinal epithelial carcinoma adherent cell lines derived from colon (T84 and Caco-2) were purchased from American Type Culture Collection (ATCC; Manassas, VA). IECs were grown on the inner well membrane of 0.4 μm Transwell™ inserts purchased from Corning Incorporated/Life Sciences (Tewksbury, MA). Cultures were provided with culture media (DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum and 1X penicillin-streptomycin) purchased from Invitrogen Corporation (Carlsbad, CA) as previously described (Hurley et al., 2016a).

2.3. Evans Blue-BSA flux assay

A 10X Evans Blue-BSA stock solution was prepared by adding 85 mg Evans Blue dye purchased from Sigma Chemical Company (St. Louis, MO) and 5 g BSA purchased from Fisher Scientific (Waltham, MA) to 50 ml assay medium. Assay media containing Evans Blue and BSA was filter sterilized through the Rapid-Flow Filter Unit with 0.2 μm CN membrane purchased from Thermo Fisher Scientific (Waltham, MA). Epithelial inserts were washed 3X in assay media and placed in wells containing 1.0 ml of assay medium in the bottom compartment. Evans Blue-BSA stock solution was diluted 10-fold in assay medium, with or without specific test proteins at concentrations listed in Table 1 and 100 μl was added to the apical surface of the epithelial monolayers (inner well of the Transwell). Epithelial monolayers with Evans Blue-BSA solution ± test protein were incubated overnight (37°C, 5% CO_2). A volume of 100 μl of solution from the bottom compartment of the Evans Blue-BSA assay plate was transferred to a 96 well plate in duplicate, and the optical absorbance was measured at 590 nm to determine the quantity of Evans Blue-BSA flux across the epithelial monolayer over 24 h. After collection and transfer of samples to a 96-well plate, epithelial monolayers were washed 3X, moved into a separate plate containing culture media, and placed at 37°C, 5% CO_2 until needed for additional testing.

Table 1
Protein and controls evaluated.

Protein/toxin	Abbreviation	Category	Vendor[c]	Dose Tested
Bovine Serum Albumin	BSA	Non-hazardous protein	Fisher Scientific NC0582624	1 mg/ml
Streptolysin O	SLO	hemolysin	Sigma-Aldrich S0149	2500 units/ml
Clostridium difficile Toxin A	ToxA	enterotoxin	List Laboratories #152B	2 μg/ml
Wheat Germ Agglutinin	WGA	food toxin	Vector Laboratories L1020	1 mg/ml
Porcine Serum Albumin	PSA	Non-hazardous Protein	Sigma-Aldrich A4414	1 mg/ml
Phaseolus Vulgaris Erythroagglutinin	PHA-E	food toxin	Vector Laboratories L1120	1 mg/ml
Fibronectin bovine plasma	Fbn	Non-hazardous Protein	Sigma-Aldrich F4759	100 μg/ml
Control	**Abbreviation**	**Category**	**Vendor[c]**	**Dose Tested**
Assay media	(−)	(−) control	Invitrogen	(−)
TritonX-100	TX-100	(+) control[a]	Sigma-Aldrich	0.1%
Ethylenediamine tetraacetic acid	EDTA	(+) control[b]	Invitrogen	5 mM

[a] Treatment of the apical surface of IECs with 0.1% TX-100 serves as a (+) control for induced cytotoxicity of IECs and for disruption of IEC monolayer barrier integrity.

[b] Treatment of the apical surface of IECs with 5 mM EDTA serves as a (+) control for reversible disruption of IEC monolayer barrier integrity.

[c] Fisher Scientific (Waltham, MA), Sigma-Aldrich (St. Louis, MO), List Biological Laboratories, Inc. (Campbell, CA), Vector Laboratories, Inc. (Burlingame, CA), and Invitrogen Corporation (Carlsbad, CA).

Table 2
Experimental design for single dose exposures.

1 [a]	2	3	4	5	6	7	8
Assay media [b]	measure [c]	Assay media	measure				Assay media
9	10	11	12	13	14	15	16
measure	Assay media	measure				Assay media	measure
17	18	19	20	21	22	23	24
Assay media	measure				Assay media	measure	Assay media
25	26	27	28	29	30		
measure				Test protein [d]	Final measure [e]		

[a] Experiment involving treatment of intestinal epithelial cell (IEC) monolayers with a single dose of a test protein are exposed to assay media on days 1,3,8,10,15,17,22, & 24. One day 29, IEC monolayers are exposed to the test protein for the first time. Each treatment exposure is 24 hours in duration. The experiment begins on day 1 and ends on day 30.
[b] As a control to pair with a parallel repeated exposure of a test protein, epithelial monolayers are evaluated for TEER and treated with assay media containing sterile Evans blue-BSA on days 1,3,8,10,15,17,22, & 24 prior to being subjected to a single treatment with the test substance/protein on Day 29.
[c] On the days indicated (2,4,9,11,16,18,23, & 25), epithelial monolayers were evaluated for TEER as well as the magnitude of Evans Blue-BSA flux that was added the previous day.
[d] For single exposure treatment experiments, the test protein/substance is only added once, on day 29, along with HRP and FITC-inulin to measure the magnitude of flux the following day.
[e] On the day following the administration of the test substance/protein, a final measurement of TEER, HRP flux, inulin flux, as well as the magnitude of MTT conversion is assessed

2.4. Exposure to proteins

IECs seeded on transwell inserts were grown for one week in culture medium to achieve confluence, monolayer integrity, and polarity. On the first day of exposure, IEC monolayers were washed, equilibrated in serum free assay media, and Trans-Epithelial Electrical Resistance (TEER) was evaluated using a voltohmmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc., Sarasota, Florida, USA). A total of 48 individual IEC monolayers grown on transwells were separated into two plates containing 24 transwells/plate; one plate for single exposure (Table 2) and one plate for repeated exposure (Table 3). The experimental design depicted in Table 3 represents the repeated dose exposure protocol. The design detailed in Table 2 represents the single dose exposure protocol conducted in parallel to control for repeated feedings, periodic exposure to assay media with Evans blue (EB) dye + BSA, and handling of IEC monolayers over the course of the 30 day-long experiment. Within the repeated exposure plate, IEC monolayers were exposed to test proteins or control substances (4 monolayers/condition) resuspended in assay medium containing EB-BSA multiple times and incubated at 37 °C, 5% CO_2 for a duration of 24 h each time (Table 3). The IEC monolayers within the single exposure plate were exposed multiple times in parallel to assay medium containing EB-BSA alone for a duration of 24 h (Table 2). Following each 24-h incubation period, aside from the final treatment, IECs were washed and evaluated for barrier integrity (TEER, EB-BSA flux) and returned to culture medium and incubated at 37^{O}C, 5% CO_2

Table 3
Experimental design for repeated dose exposures.

1 [a]	2	3	4	5	6	7	8
Test protein [b]	measure [c]	Test protein	measure				Test protein
9	10	11	12	13	14	15	16
measure	Test protein	measure				Test protein	measure
17	18	19	20	21	22	23	24
Test protein	measure				Test protein	measure	Test protein
25	26	27	28	29	30		
measure				Final Test protein [d]	Final measure [e]		

[a] Experiment involving treatment of intestinal epithelial cell (IEC) monolayers with repeated doses of a substance/protein are exposed on days 1,3,8,10,15,17,22,24, & 29. Each treatment exposure is 24 hours in duration. The experiment begins on day 1 and ends on day 30.
[b] Epithelial monolayers are evaluated for TEER and then treated with test substance/protein suspended in assay media containing sterile Evans Blue-BSA on days 1,3,8,10,15,17,22, & 24 prior to the final treatment with the test substance/protein on Day 29.
[c] On the days indicated (2,4,9,11,16,18,23, & 25), epithelial monolayers were evaluated for TEER as well as the magnitude of Evans Blue-BSA flux that was added along with test substance/protein the previous day.
[d] For repeated exposure treatment experiments, the test substance/protein is added multiple times with the ninth exposure on day 29, along with HRP and FITC-inulin to measure the magnitude of flux the following day.
[e] On the day following the final administration of the test substance/protein, a final measurement of TEER, HRP flux, inulin flux, as well as the magnitude of MTT conversion is assessed.

for 24 h. Exposure was conducted in this manner twice a week for a total of 8 exposures. For the 9th and final exposure, IEC monolayers in both the repeat and single exposure plates were exposed to either a protein of interest or control substance along with the barrier integrity probes FITC-inulin (500 μg/ml) and horseradish peroxidase (HRP) (450 ng/ml) and incubated for 24 h at 37 °C, 5% CO_2. Following the designated incubation period, IECs were evaluated for barrier integrity (TEER, FITC-inulin flux, and HRP flux) and cytotoxicity (MTT conversion to formazan) as described previously (Hurley et al., 2016a). FITC-inulin and HRP were purchased from Sigma-Aldrich (St. Louis, MO). The vybrant® MTT Cell Proliferation Assay Kit was purchased from Fisher Scientific (Waltham, MA).

2.5. Data analysis/statistics

All experiments were performed on at least 3 separate occasions (≥3 biological replicates). Each data point within an internally controlled experiment represents the mean and standard deviation of quadruplicate IEC monolayers (4 technical replicates/internally controlled experiment). Differences from the negative control (no protein) were considered statistically significant when $p < 0.05$ using an unpaired two-tailed student's T test within an internally controlled experiment. Statistical significance was calculated only within internally controlled experiments and not across separate experiments. A statistically significant decrease in barrier integrity or increase in cytotoxicity caused by a test protein was interpreted as an indication of hazard only if the observation was reproducible (observed in multiple independent experiments). The magnitude of effect elicited by a test protein was not factored into the assignment of hazard or non-hazard.

3. Results

3.1. Evaluating intestinal barrier integrity at intervals following exposure to test proteins

Figs. 1–4 depict results of TEER and EB-BSA flux for single (no protein added yet) and repeated dose exposures of the IECs to the test proteins or controls for day 1-day 28. Figs. 5 and 6 show the results of TEER, FITC-inulin flux, HRP flux and MTT reduction for the single and repeated dose exposure of the IECs to the test proteins or controls for day 29-day 30. Data generated from the single dose exposure protocol (no protein day 1-day 28) is depicted in green and data generated from the repeated dose exposure protocol is depicted in orange.

As a control for the experimental design, repeated exposure to assay media was compared to a parallel but separate group of identically treated IEC monolayers also exposed repeatedly to assay media. These were arbitrarily designated as either single (green) or repeat (orange) and no significant differences in TEER between these two experimental groups of T84 monolayers identically treated with assay media were noted at any point in time (Fig. 1, Media). The impact of repeated exposure to TX-100, a known disrupter of barrier integrity (repeated, orange), revealed a rapid decrease in TEER to essentially background levels following the initial dose of TX-100 (Fig. 1, TX-100). TEER never recovered for T84 monolayers throughout the remainder of the experiment following the initial TX-100 exposure. Exposure to assay media alone (single, green), in parallel, resulted in maintenance of TEER well above background throughout (Fig. 1, TX-100). Fluctuations in TEER were observed with assay media controls that ranged from approximately 2500 Ω on the low end and 7500 Ω on the high end. Interestingly, the increase in TEER appeared to coincide with media feedings built into the 30 day-long experiment. This pattern was consistently observed for all assay media parallel controls depicted in green (Fig. 1).

EDTA, a reversible disrupter of barrier integrity, also caused an immediate and sustained decrease in TEER following the initial exposure. In contrast to TX-100, however, evidence of partial TEER recovery was detectible during the gap periods, where cells were not

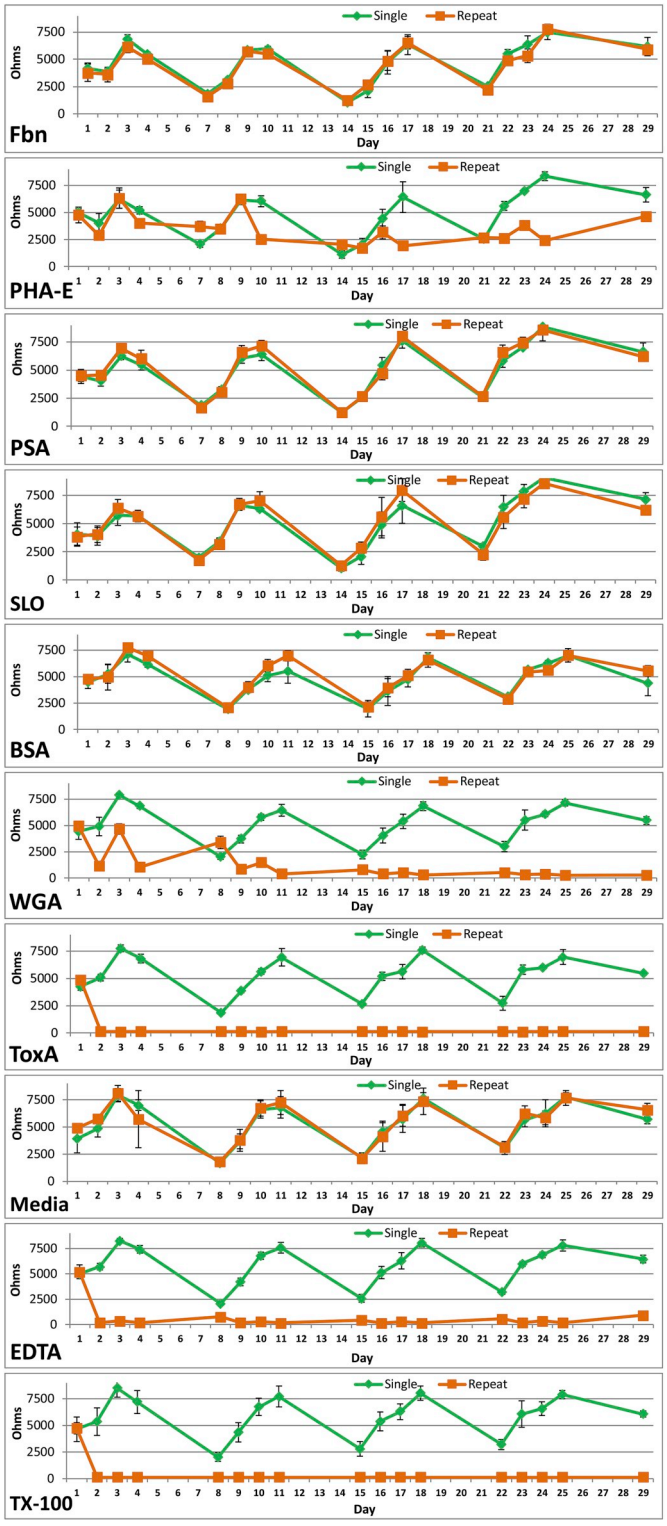

Fig. 1. Fig. 1: Measurement of TEER in Ohms across T84 IEC monolayers before and after eight separate exposures to a test protein, (−) control, or (+) controls (repeated, orange line) or exposed to assay media alone in parallel (single, green line). Each exposure to test substance or assay media was 24 h in duration. The single exposure monolayers (green line) receive the sole exposure of test substance at the end of the experiment on day 29 following measurement of TEER, while the monolayers exposed repeatedly to test substances (orange line) received the ninth exposure on day 29, following measurement of TEER. The impact of the single and nine repeated exposures on IEC monolayers are depicted in Fig. 5. The experimental design to examine the impact of single and repeated dose exposures on IEC monolayers are outlined in Tables 2 and 3 respectively.

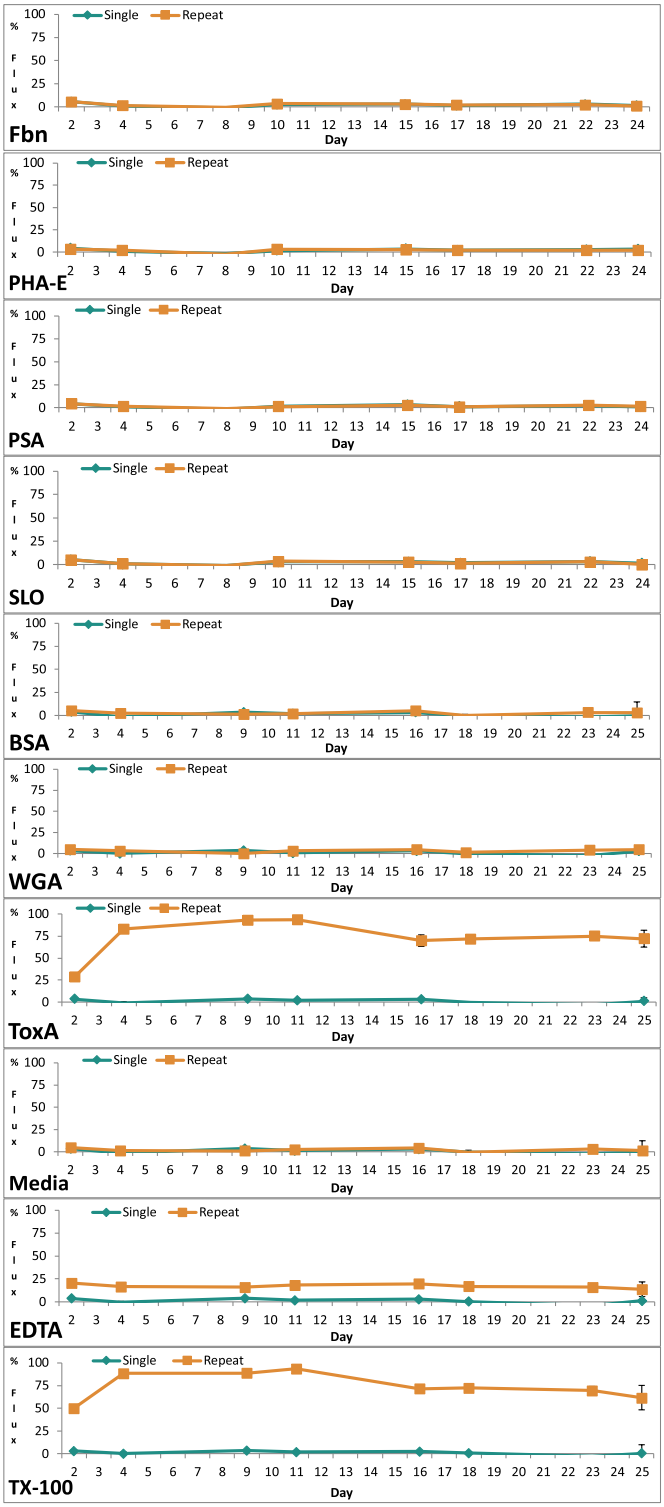

Fig. 2. Fig. 2: Measurement of the % EB-BSA flux across T84 IEC monolayers following eight separate exposures to a test protein, (−) control, or (+) controls (repeated, orange line) or exposed to assay media alone in parallel (single, green line). Each exposure to test substance or assay media was 24 h in duration and the % EB-BSA flux was assessed during that time. The single exposure monolayers (green line) received the sole exposure of test substance at the end of the experiment after day 25 while the monolayers exposed repeatedly to test substances (orange line) received the ninth exposure after day 25. The impact of the single and nine repeated exposures on IEC monolayers are depicted in Fig. 5. The experimental design to examine the impact of single and repeated dose exposures on IEC monolayers are outlined in Tables 2 and 3 respectively.

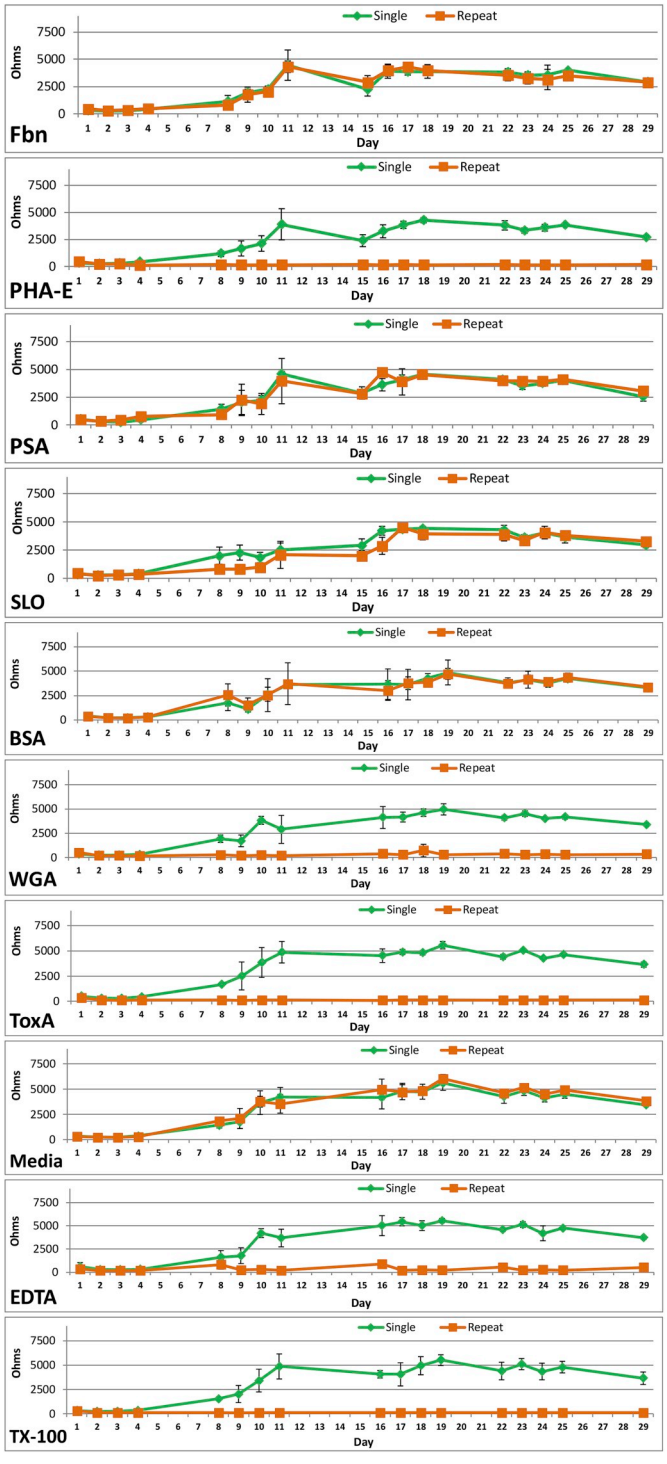

Fig. 3. Fig. 3: Measurement of TEER in Ohms across Caco-2 IEC monolayers before and after eight separate exposures to a test protein, (−) control, or (+) controls (repeated, orange line) or exposed to assay media alone in parallel (single, green line). Each exposure to test substance or assay media was 24 h in duration. The single exposure monolayers (green line) receive the sole exposure of test substance at the end of the experiment on day 29 following measurement of TEER, while the monolayers exposed repeatedly to test substances (orange line) received the ninth exposure on day 29, following measurement of TEER. The impact of the single and nine repeated exposures on IEC monolayers are depicted in Fig. 6. The experimental design to examine the impact of single and repeated dose exposures on IEC monolayers are outlined in Tables 2 and 3 respectively.

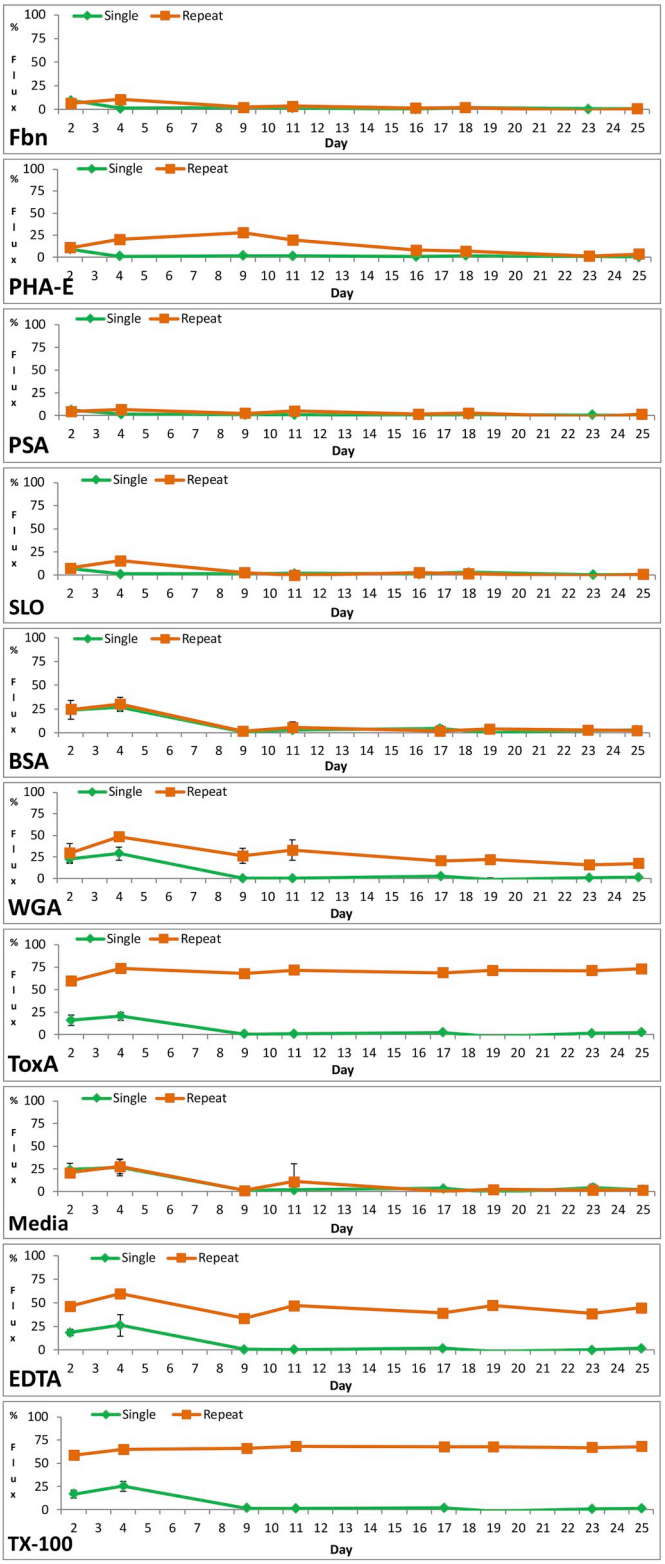

Fig. 4. Fig. 4: Measurement of the % EB-BSA flux across Caco-2 IEC monolayers following eight separate exposures to a test protein, (−) control, or (+) controls (repeated, orange line) or exposed to assay media alone in parallel (single, green line). Each exposure to test substance or assay media was 24 h in duration and the % EB-BSA flux was assessed during that time. The single exposure monolayers (green line) receive the sole exposure of test substance at the end of the experiment after day 25 while the monolayers exposed repeatedly to test substances (orange line) received the ninth exposure after day 25. The impact of the single and nine repeated exposures on IEC monolayers are depicted in Fig. 6. The experimental design to examine the impact of single and repeated dose exposures on IEC monolayers are outlined in Tables 2 and 3 respectively.

subjected to test substance, such as between days 4 and 8 or 11 and 15 (Fig. 1, EDTA). When repeatedly exposing T84 monolayers to non-hazardous proteins Fbn, PSA, and BSA, (repeat, orange) no significant difference in TEER was observed at any point during the 30 day-long experiment when compared with assay media alone (single, green) (Fig. 1).

A feature of the assay design to allow measurement of EB-BSA flux involves addition of 10 mg/ml BSA complexed with Evans Blue (EB)

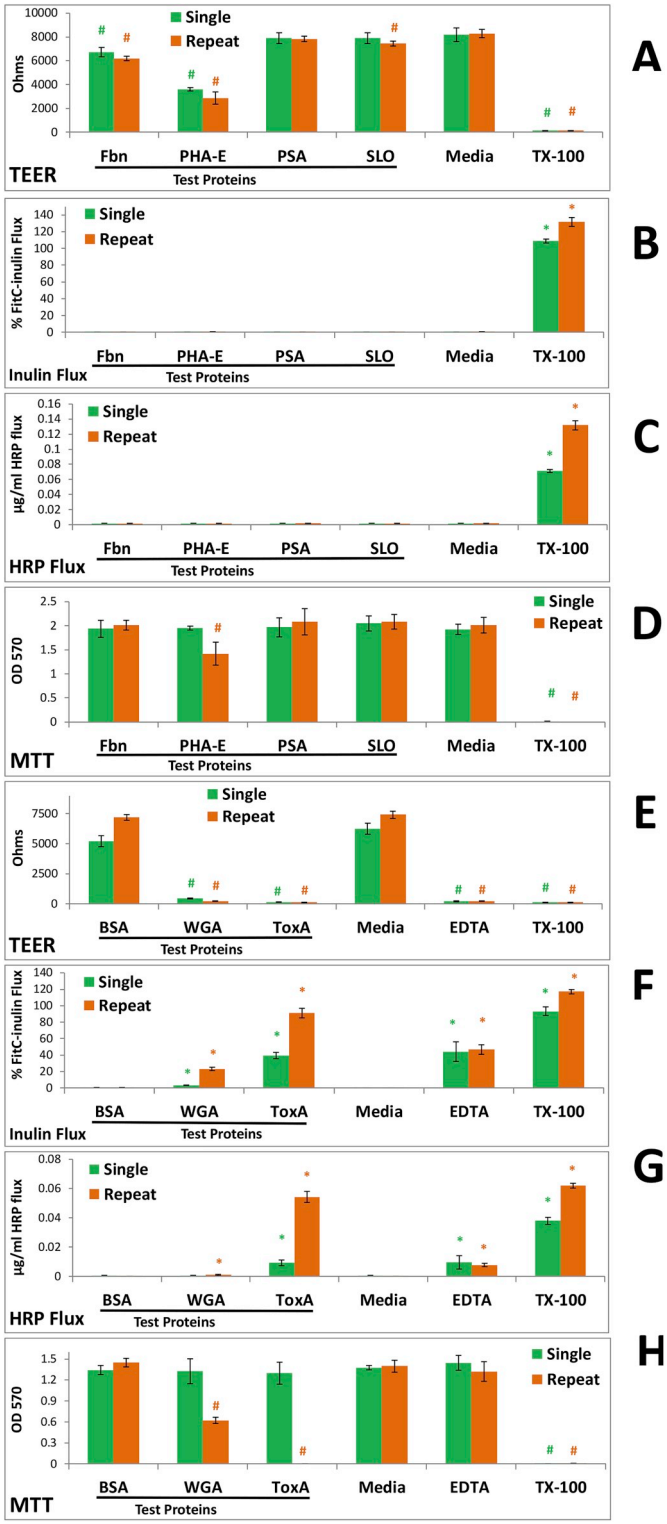

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Fig. 5. Fig. 5: Exposure of T84 Polarized Intestinal Monolayers to test proteins as well as (−) control (Media) and (+) controls (TX-100 & EDTA) for a single exposure of 24-h in duration (green) or 9 repeated exposures, each 24-h in duration (orange). Data represents the final measurements on day 30, with final treatment on day 29 of monolayers monitored in Figs. 1 and 2 depicting TEER and EB-BSA flux over the course of 30 days. (A) TEER measurement following single and repeated exposures to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (B) FITC-inulin flux following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (C) HRP flux following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (D) MTT conversion following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (E) TEER measurement following single and repeated exposures to controls and test proteins; BSA, WGA, & ToxA. (F) FITC-inulin flux following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. (G) HRP flux following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. (H) MTT conversion following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. Data depicted represent two separate internally controlled experiment (A-D & E-H) that were repeated on at least three separate occasions in which all four assays were performed each time. The symbol (*) indicates that a statistically significant increase ($p < 0.05$) over (−) control (Media) was observed in this internally controlled experiment and this significant increase was reproducible in separate independent experiments. The symbol (#) indicates that a statistically significant decrease ($p < 0.05$) from (−) control (Media) was observed in this internally controlled experiment this significant decrease was reproducible in separate independent experiments.

dye to all wells. One of the test proteins examined in this study was BSA (1 mg/ml). It was therefore determined whether this repeated addition of EB-BSA, in and of itself, was capable of impacting TEER at any point during the experiment. No significant differences in TEER were detected between T84 monolayers exposed to assay media alone versus assay media supplemented with EB-BSA (Supplemental Fig. 1).

Repeated treatment with food toxins PHA-E and WGA both caused reduced TEER compared to their respective assay media controls (Fig. 1). Repeated treatment with PHA-E reduced, but did not eliminate, TEER. WGA, on the other hand, led to a progressive decline in TEER upon repeated exposures, eventually resulting in background levels of TEER. Treatment with repeated exposures of the enterotoxin ToxA caused rapid and sustained loss of TEER, similar to TX-100 treatment (Fig. 1). SLO, a toxin that causes hemolysis and is not known to specifically target IECs had only minor effects. However, on day 29 a slight but significant drop in TEER was detected in the context of repeated challenge with SLO (Fig. 1).

TEER is an exquisitely sensitive metric of IEC barrier integrity and may not necessarily indicate overt leakiness of the epithelial barrier to large molecules such as proteins. To determine whether repeated exposure by any of the agents resulted in an increase in translocation of EB-BSA across IEC monolayers, the amount of EB-BSA in the basolateral well was measured following addition of EB-BSA ± test substance to the apical surface on the previous day. The experimental design control comparing two identical groups, both repeatedly treated with assay media (single green vs. repeat orange), exhibited only very low levels of %EB-BSA flux. Further, there was no significant change in the magnitude of flux at any time throughout the experiment (Fig. 2, Media). Identical results were observed with all three non-hazardous proteins (Fbn, PSA, and BSA). In fact, only repeated treatment with ToxA elicited overt increases in the magnitude of EB-BSA flux, as PHA-E, SLO, and WGA failed to cause increased permeability to EB-BSA (Fig. 2). As expected, TX-100 resulted in rapid and substantial EB-BSA flux. EDTA caused an increase in EB-BSA permeability, but this increase was significantly lower in magnitude when compared to ToxA or TX-100 at each day post-treatment tested (Fig. 2).

The studies described above were also conducted using a second polarized intestinal epithelial cell line (Caco-2). Caco-2 IECs were repeatedly exposed to various test substances and compared with a set of Caco-2 IECs identically treated but without protein exposure as described above. The pattern of TEER measured throughout the multi-week experiment was distinct for Caco-2 IECs when compared to T84 IECs (Figs. 1 and 3). For the experimental design control, TEER values were initially low, albeit significantly higher than background, and steadily increased in magnitude over time leveling off by day 19 (Fig. 3, Media). There was no significant difference between the two groups, which were treated identically (single green vs. repeat orange).

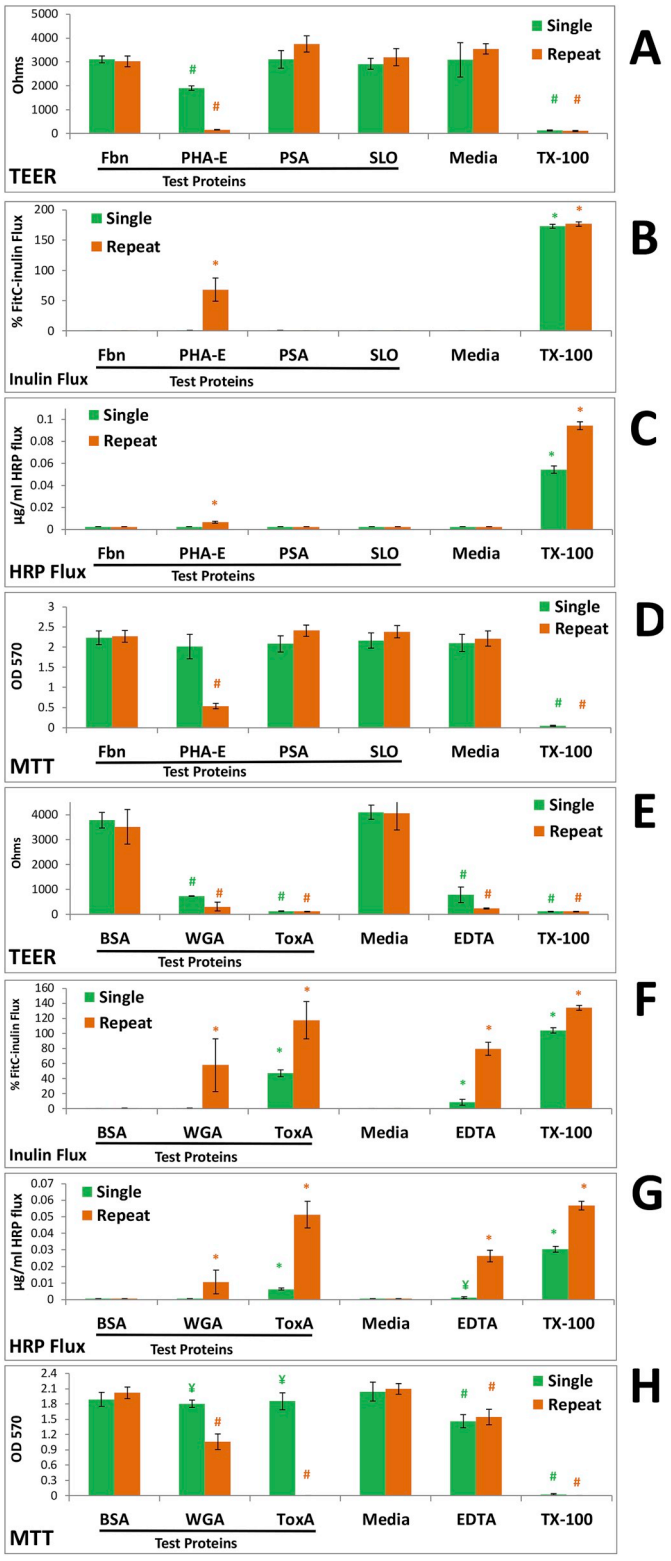

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Fig. 6. Fig. 6: Exposure of Caco-2 Polarized Intestinal Monolayers to test proteins as well as (−) control (Media) and (+) controls (TX-100 & EDTA) for a single exposure of 24-h in duration (green) or 9 repeated exposures, each 24-h in duration (orange). Data represents the final measurements on day 30, with final treatment on day 29 of monolayers monitored in Figs. 3 and 4 depicting TEER and EB-BSA flux over the course of 30 days. (A) TEER measurement following single and repeated exposures to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (B) FITC-inulin flux following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (C) HRP flux following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (D) MTT conversion following single and repeated exposure to controls and test proteins; Fbn, PHA-E, PSA, & SLO. (E) TEER measurement following single and repeated exposures to controls and test proteins; BSA, WGA, & ToxA. (F) FITC-inulin flux following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. (G) HRP flux following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. (H) MTT conversion following single and repeated exposure to controls and test proteins; BSA, WGA, & ToxA. Data depicted represent two separate internally controlled experiments (A-D & E-H) that were repeated on at least three separate occasions in which all four assays were performed each time. The symbol (*) indicates that a statistically significant increase ($p < 0.05$) over (−) control (Media) was observed in this internally controlled experiment and this significant increase was reproducible in separate independent experiments. The symbol (#) indicates that a statistically significant decrease ($p < 0.05$) from (−) control (Media) was observed in this internally controlled experiment and this significant decrease was reproducible in separate independent experiments. The symbol (¥) indicates a significant difference was observed when compared to the (−) control in two separate identical experiments, however, the difference in the depicted representative experiment trended the same way but failed to reach statistical significance.

Measurable TEER above background never developed with repeated exposure to TX-100 (Fig. 3). Exposure to the reversible disrupter of barrier integrity, EDTA, produced slight increases in TEER during gaps of EDTA exposure; however, TEER remained far lower than assay media control (single, green) at each day tested (Fig. 3). The toxins PHA-E, WGA, and ToxA all dramatically impacted TEER levels across Caco-2 IECs following repeated exposures. TEER remained quite low throughout the multi-week experiment when IECs were exposed repeatedly to each of these toxins, whereas the corresponding assay media controls displayed a steady increase in TEER followed by a plateau of maximal TEER (Fig. 3). Repeated SLO treatment did cause a slight delay in achievement of maximal TEER on certain days, but overall did not impede Caco-2 IEC monolayers from achieving a level of TEER comparable to the respective assay media control (Fig. 3). Repeated exposure to all three non-hazardous proteins (Fbn, PSA, & BSA) resulted in TEER values that were no different from their respective assay media controls (Fig. 3). Furthermore, when comparing Caco-2 IECs treated repeatedly with assay media either in the presence or absence of EB-BSA, a similar level of TEER was reached by day 22, however, monolayers without EB-BSA exposure lagged behind in the development of TEER (Supplemental Fig. 2).

Flux of EB-BSA across Caco-2 monolayers was increased as expected by repeat exposure to reversible and irreversible barrier integrity disrupters EDTA and TX-100 respectively (Fig. 4). Toxins PHA-E, WGA, and ToxA all elicited increases in EB-BSA flux of varying magnitudes, with ToxA being the most potent and PHA-E exhibiting smaller effects after the first few exposures, which are no longer observed at later exposures. SLO exerted minimal impact on EB-BSA flux as did all three non-hazardous proteins tested (Fbn, PSA, and BSA) (Fig. 4).

3.2. Evaluating endpoint intestinal monolayer barrier integrity and IEC viability

A variety of endpoints were examined for polarized IEC monolayers which were either repeatedly exposed (nine times) to test substance suspended in assay media + EB-BSA or assay media + EB-BSA alone over the course of 30 days. The cumulative impact on barrier integrity and IEC viability of multiple exposures to test substance versus a single exposure to test substance, each relative to a parallel assay media control, were determined and assessed for statistical significance (Figs. 5 and 6). Treatment of T84 monolayers with Fbn caused a slight but significant decrease in TEER following a single treatment exposure, which was not enhanced any further in the content of repeated treatment with Fbn (Fig. 5A). PHA-E caused a more substantial decrease in the TEER of T84 monolayers that occurred similarly with repeated exposures. The non-hazardous protein PSA did not elicit any decreases in TEER when applied to T84 monolayers as a single exposure or repeatedly. The hemolysin SLO also did not elicit a decrease in TEER following exposure to a single dose; however, repeated exposure to SLO caused slight but significant decrease in TEER (Fig. 5A). As expected, both single and repeated exposure to the irreversible disruptor of barrier integrity TX-100 reduced TEER to background levels indicating a complete loss of a functional integrity (Fig. 5A). When examining the occurrence of potentially greater damage to barrier integrity impacting permeability to macromolecules, flux of ~ 3 kDa FITC-inulin (Fig. 5B) and ~45 kDa HRP were assessed (Fig. 5C). Neither Fbn, PHA-E, PSA, nor SLO caused any notable leakage of either of the two probes when applied as a single exposure or exposed repeatedly to T84 monolayers. TX-100 caused substantial flux of FITC-inulin (Fig. 5B) and HRP (Fig. 5C) whether exposed for a single treatment or repeatedly.

To determine whether any of the test proteins exposed either once or repeatedly caused cytotoxic effects on T84 monolayers, the magnitude of MTT conversion was measured. The test proteins Fbn, PSA, and SLO did not cause any significant cytotoxicity when applied to T84 monolayers either once or repeatedly (Fig. 5D). PHA-E elicited cytotoxic effects that were small in magnitude, but significant when repeatedly applied to T84 monolayers, however, no impact was observed following a single exposure to PHA-E. TX-100 resulted in complete loss of T84 monolayer viability following either a single or repeated exposure (Fig. 5D).

Treatment of T84 monolayers with the hazardous proteins WGA and ToxA both caused substantial and significant loss of TEER following single as well as repeated exposures. Exposure to the non-hazardous protein BSA resulted in no change in TEER whether T84 monolayers experienced a single exposure or repeated exposures (Fig. 5E). Reversible and irreversible barrier disruptors EDTA and TX-100 respectively also caused substantial and significant loss of TEER regardless of exposure pattern (Fig. 5E). WGA and ToxA exposure caused an increase in flux of FITC-inulin that was substantially greater in magnitude when these toxins were applied repeatedly rather than only once (Fig. 5F). The magnitude of FITC-inulin flux was similar whether T84 monolayers experienced a single exposure or were repeatedly exposed to EDTA. The magnitude of FITC-inulin flux was substantial in response to TX-100 treatment, with slightly greater flux occurring with the repeated treatments (Fig. 5F). The non-hazardous protein BSA, whether applied once or repeatedly, did not cause any increase in the flux of FITC-inulin (Fig. 5F), nor did it cause increased flux to the larger paracellular probe HRP (Fig. 5G). Repeated exposure to ToxA resulted in much more HRP flux than a single exposure, although a single exposure did significantly increase HRP flux above media control (Fig. 5G). HRP flux increases across T84 monolayers in response to WGA compared to media control were only observed with repeated exposures and not with a single exposure. HRP flux in response to EDTA increased but was no different whether treated once or repeatedly, whereas TX-100 caused substantially more HRP flux upon single or repeated exposure, with the magnitude higher when repeatedly experiencing TX-100 Fig. 5G).

Cytotoxic responses were also examined for BSA, ToxA, and WGA along with positive and negative controls. As expected, TX-100 caused complete loss of T84 monolayer viability even upon a single exposure. EDTA and BSA did not elicit any cytotoxicity, nor did a single exposure to WGA or ToxA (Fig. 5H). Repeated exposures to WGA caused a significant degree of loss in MTT conversion indicating loss of cell

viability. Repeated exposures to ToxA caused complete loss of T84 monolayer viability, a strikingly different response than observed with a single exposure to ToxA, which elicited no change in T84 monolayer viability (Fig. 5H).

Endpoint assay analysis for Caco-2 IEC monolayers exposed to various test protein were also examined in 30 day-long experiments to determine whether single or repeated exposures elicited any significant decreases in barrier integrity or increases in cytotoxicity compared with assay media treated controls (Fig. 6). Hazardous proteins PHA-E, WGA, and ToxA all caused significant reduction in TEER whether exposed to Caco-2 monolayers once or repeatedly (Fig. 6A and E). In the case of PHA-E and WGA, the magnitude of TEER decrease was greater when repeatedly challenged with protein. The hemolysin SLO displayed no impact on TEER when applied once or repeatedly (Fig. 6A). The non-hazardous proteins (Fbn, PSA, and BSA) did not cause any significant changes in TEER upon exposure of Caco-2 monolayers to a single dose or repeatedly exposed to the same dose (Fig. 6A and E). The leakiness of Caco-2 monolayers to small (FITC-inulin) and large (HRP) paracellular probes was observed with repeated exposure to PHA-E, WGA, and ToxA (Fig. 6B, 6C, 6F, & 6G). The only observation of increased permeability in response to a single exposure, aside from positive controls TX-100 and EDTA, was observed with exposure to ToxA (Fig. 6F and G).

Similar to T84 monolayers, very little test protein-elicited cytotoxicity was observed in response to a single exposure to protein (Fig. 6D and H). There was a very small, but reproducible impact on Caco-2 monolayers following a single exposure to ToxA and WGA. Repeated exposures to hazardous proteins PHA-E, WGA, and ToxA, however, caused a substantial and significant reduction in Caco-2 monolayer MTT conversion, indicating increased cellular toxicity. No cytotoxicity was observed following single or repeated exposures to non-hazardous proteins Fbn, PSA, and BSA as well as hemolysin SLO (Fig. 6D and H). As expected, single and repeated exposures to TX-100 caused complete loss of Caco-2 monolayer viability. Single and repeated exposures to EDTA did cause some minor cytotoxicity in Caco-2 monolayers, however the monolayers remained capable of converting MTT, unlike TX-100 treated cells (Fig. 6D and H).

3.3. Overall analysis

To effectively discern hazard from non-hazard, we devised a two-color heat map that reflects aggregate data derived from T84 and Caco-2 monolayers (Fig. 7). Each protein tested on IEC monolayers for each assay performed was designated either as a hazard (positive symbol, red square) or as not revealing any evidence of hazard (negative symbol, blue square). The hazard designation depended on whether the observation for a protein within a given assay of an IEC monolayer met the pre-determined criteria for a hazard described in the methods section. If statistically significant differences in a barrier integrity or cytotoxicity assay were observed in T84 or Caco-2 monolayers in response to single or repeated protein exposures and the observation was reproducible in multiple independent experiments, then it was designated a hazard and assigned a positive symbol within a red box. Otherwise the observation was designated as a non-hazard and assigned a negative symbol within a blue box. Hazards were more commonly revealed among proteins known to be hazardous and repeated IEC exposures appeared to increase the number of hazards detected compared to single exposures (Fig. 7). Although not reflected in Fig. 7 because the magnitude of a hazard is not considered in the red/blue designation analysis, repeated exposures to hazardous proteins tended to cause increased magnitude of impact on barrier integrity and/or cytotoxicity as well.

4. Discussion

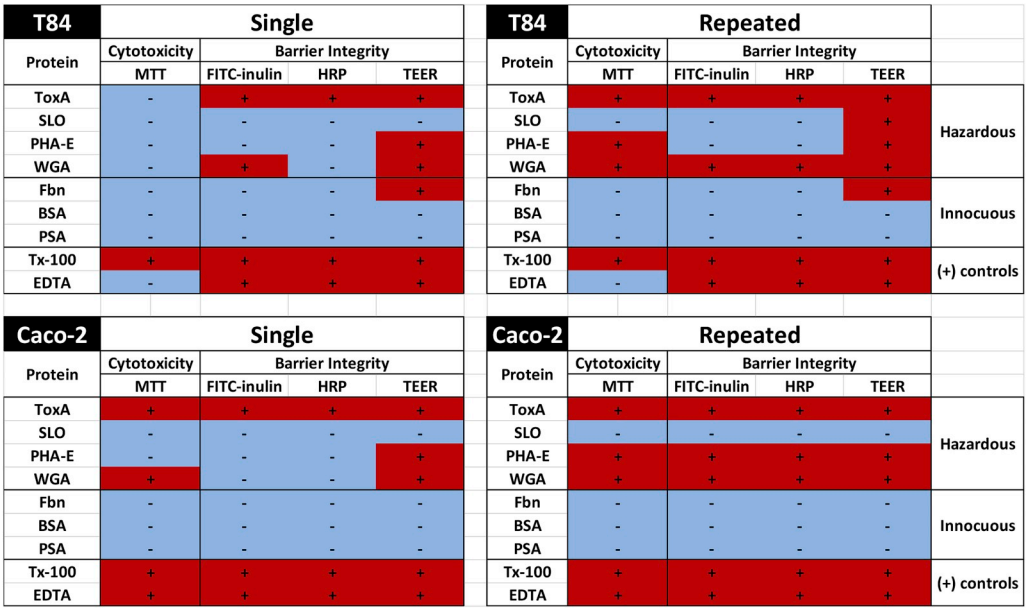
Proteins genetically engineered to be expressed by plants and confer specific advantageous attributes to the crop are subjected to a multi-component hazard *identification* process, which is used to assess potential hazard of a newly introduced protein. This hazard identification process is one component of the food and feed safety assessment of GM crops. Only if a newly expressed protein has an identified hazard should additional studies be necessary to characterize the hazard. Nevertheless, many of these proteins undergo Tier II hazard characterization studies (i.e. toxicology studies requiring the use of a murine *in vivo* model). The development of a human-based *in vitro* system could prove to be a useful alternative approach to identify potentially hazardous GM crop proteins and may ultimately result in the reduction or elimination of requirements for animal testing.

We have developed a repeated exposure protocol to investigate a polarized human intestinal epithelial barrier *in vitro* method for protein hazard characterization using proteins known to produce adverse effects following oral exposure often resulting in damage to the intestinal epithelium (Dalla Pellegrina et al., 2009; Lafont et al., 1988; Lorenzsonn and Olsen, 1982; Nusrat et al., 2001; Pusztai et al., 1993; Ramadass et al., 2010; Rossi et al., 1984; Weinman et al., 1989). Studies to date have demonstrated that the addition of hazardous proteins to human IEC monolayers for 24, 48 and 72 h results in compromised monolayer integrity and in some cases overt cytotoxicity, while the addition of non-hazardous proteins has little effect on the IEC monolayers. The current studies were designed and executed to investigate if nine repeated (24-h in duration) exposures followed by periods of recovery conducted over the course of 30 days would represent an effective approach that would allow IEC monolayers to distinguish hazardous from non-hazardous proteins.

Non-hazardous proteins BSA and PSA did not elicit any evidence of hazard whether T84 or Caco-2 monolayers received single exposures or repeated exposures. This is consistent with previous studies involving single exposures of various durations (Hurley et al., 2016a; Zimmermann et al., 2018). Fbn, a protein selected to represent non-hazardous proteins, was largely incapable of impacting IECs, although a slight but significant impact on TEER of T84 monolayers was noted following a single exposure, which was not further exacerbated by repeated exposures. It is difficult to determine if this is a biologically meaningful indication of hazard, particularly in the context of the aggregate negative data from all other assays involving Fbn exposures. However, since Fbn met our pre-determined criteria of assigning a hazard if any reproducible adverse finding from any assay was observed on either cell line, irrespective of magnitude, it was indexed as a red positive. Further, previous studies using higher concentrations of Fbn do in fact reveal more convincing evidence that this protein may cause adverse effects in IECs (Markell et al., 2017).

The enterotoxin produced by the bacterial pathogen *Clostridium difficile* known as toxin A (ToxA) was the test protein that indicated the greatest degree of hazard in this study. A single exposure of T84 monolayers caused evidence of barrier integrity disruption by all three assays (TEER, FITC-inulin flux, & HRP flux), however, no evidence of cytotoxicity was detected by MTT conversion. When T84 monolayers were exposed repeatedly to ToxA, complete loss of MTT conversion was observed, suggesting that repeated T84 monolayer exposure to ToxA reveals a greater hazardous impact than a single exposure. Furthermore, the degree of barrier integrity disruption is notably enhanced by repeated exposures when assessing FITC-inulin and HRP flux. Caco-2 monolayers exposed to ToxA exhibit hazardous indices among all assays performed, including both barrier integrity and cytotoxicity. This was the case following either a single exposure or repeated exposures, however, the magnitude of the effect following a single exposure of Caco-2 monolayers to ToxA was substantially smaller for all assays aside from TEER, where a single exposure was sufficient to effectively eliminate TEER. Despite the clear increase in the magnitude of impact that repeated ToxA exposure exerts upon T84 and Caco-2 monolayers, the single exposure protocol for ToxA was sufficient to confidently identify ToxA as a hazardous protein.

Repeated exposure of both IEC monolayers to PHA-E and WGA revealed increases in both the number of hazards identified as well as the



T84	Single			
Protein	Cytotoxicity	Barrier Integrity		
	MTT	FITC-inulin	HRP	TEER
ToxA	-	+	+	+
SLO	-	-	-	-
PHA-E	-	-	-	+
WGA	-	+	-	+
Fbn	-	-	-	+
BSA	-	-	-	-
PSA	-	-	-	-
Tx-100	+	+	+	+
EDTA	-	+	+	+

T84	Repeated				
Protein	Cytotoxicity	Barrier Integrity			
	MTT	FITC-inulin	HRP	TEER	
ToxA	+	+	+	+	Hazardous
SLO	-	-	-	+	
PHA-E	+	-	-	+	
WGA	+	+	+	+	
Fbn	-	-	-	+	Innocuous
BSA	-	-	-	-	
PSA	-	-	-	-	
Tx-100	+	+	+	+	(+) controls
EDTA	-	+	+	+	

Caco-2	Single			
Protein	Cytotoxicity	Barrier Integrity		
	MTT	FITC-inulin	HRP	TEER
ToxA	+	+	+	+
SLO	-	-	-	-
PHA-E	-	-	-	+
WGA	+	-	-	+
Fbn	-	-	-	-
BSA	-	-	-	-
PSA	-	-	-	-
Tx-100	+	+	+	+
EDTA	+	+	+	+

Caco-2	Repeated				
Protein	Cytotoxicity	Barrier Integrity			
	MTT	FITC-inulin	HRP	TEER	
ToxA	+	+	+	+	Hazardous
SLO	-	-	-	-	
PHA-E	+	+	+	+	
WGA	+	+	+	+	
Fbn	-	-	-	-	Innocuous
BSA	-	-	-	-	
PSA	-	-	-	-	
Tx-100	+	+	+	+	(+) controls
EDTA	+	+	+	+	

Fig. 7. Fig. 7: Overall Hazard Analysis: Individual proteins were considered (+) with a red box if they produced a significant ($p < 0.05$) and reproducible effect (≥two independent experiments out of three performed), irrespective of magnitude in each specific assay performed on T84 or Caco-2 IEC monolayers on the final day of the 30 day-long experiment comparing single versus repeated exposures. If no reproducible effect was observed for a specific assay, the response was considered (−) and assigned a blue box. For identification of cytotoxicity MTT conversion was performed. For identification of disruption of barrier integrity, three separate assays were performed (TEER, FITC-inulin flux, and HRP flux). Each assay was performed on at least three separate occasions for every protein analyzed. Relative magnitude of response is not reflected in this analysis.

magnitude of the effect measured when compared to IECs exposed to these food toxins only once. Over the course of the 30 day-long experiment, T84 monolayers receiving multiple exposures to PHA-E exhibited diminished TEER and repeated WGA exposures caused a clear progressive decline in TEER that eventually resulted in baseline TEER levels. In Caco-2 monolayers, both PHA-E and WGA prevented the development of higher levels of TEER and caused increases in EB-BSA flux. A single dose of PHA-E applied to T84 and Caco-2 monolayers caused a reduction in TEER, whereas repeated dosing caused a more substantial decrease in TEER along with increased evidence of cytotoxicity in both IECs monolayers. Moreover, Caco-2 monolayers also displayed leakage of FITC-inulin and HRP following repeated exposure to PHA-E. Repeated exposure of T84 and Caco-2 monolayers to WGA resulted in loss of TEER, increased flux of FITC-inulin and HRP, as well as decreased MTT conversion, indicating cytotoxicity. A single exposure of T84 monolayers to WGA exhibited fewer indices of hazard as only a reduction in TEER and a slight, but significant increase in FITC-inulin flux were observed. Moreover, a reduction in TEER and a very small decrease in MTT conversion were noted following a single exposure of Caco-2 monolayers to WGA. Although repeated exposures to both food toxins clearly amplifies the magnitude and indices of hazard measured in IEC monolayers, single exposures to PHA-E and WGA remain adequate to clearly identify these proteins as hazardous in this human IEC monolayer *in vitro* model system.

SLO is a hemolytic exotoxin produced by *Streptococcus pyogenes*. Unlike ToxA, SLO does not specifically target IECs. Whether the IEC model presented herein is capable of detecting hazardous effects following exposure to this toxin is unclear. A decrease in TEER in response to repeated exposure of T84 monolayers to SLO was observed and this did not occur following a single exposure. This effect was minor but significant, and therefore indexed as a hazard in the overall analysis. Caco-2 cells were largely unimpacted by SLO, however, we did note a slight delay in the development of higher levels of TEER and increased EB-BSA flux early on during multiple exposures compared with assay media control. Since these observations were not included as part of the overall hazard analysis, Caco-2 monolayers were essentially deemed unable to reveal SLO to be a hazardous protein. Purified bacterial toxins such as ToxA and SLO are only available in small quantities compared to the other proteins employed in the study. It is possible that a higher dose, comparable to the other proteins employed in this study, may resolve clearer indices of hazard for SLO. Given the case of Fbn and SLO, it may be advantageous to establish guidelines with appropriate concentration ranges for testing proteins using this experimental platform in order to optimally resolve hazardous from non-hazardous proteins.

While toxins were successfully identified as hazardous in this study, there were notable differences in sensitivity to different proteins between the cell lines. This is consistent with previous work (Hurley et al., 2016b; Zimmermann et al., 2018) and exemplifies the additional benefits of using more than one cell line for a collective analysis when evaluating the safety of a protein under this *in vitro* experimental platform.

The magnitude of response was not taken into consideration when assigning hazard using the previously developed overall hazard analysis (Hurley et al., 2016a; Zimmermann et al., 2018). For future implementation of this experimental platform towards evaluating proteins of unknown toxicity, devising an analytical approach for considering the magnitude of response as part of the overall hazard analysis may be useful in minimizing potential of false positives. Minor reductions in TEER or MTT with no accompanying differences in other assays may have marginal biological significance and therefore may not be indicative of a hazard per se. Additional studies will be necessary to effectively address these issues.

Taken together these results demonstrate that repeated exposure to known hazardous proteins increases the magnitude of adverse reactions revealed by IECs and increases the indices of hazard as part of an overall hazard analysis. However, since hazardous analysis protocols are mainly designed to simply alert that a hazard exists, the single exposure protocol appears to be sufficient for this purpose as the repeated exposure protocol does not necessarily offer a substantial improvement in this regard. Investigation of additional test proteins is warranted to continue to evaluate whether repeated exposure of a hazardous protein is capable of unveiling a hazard that is not recognized by a single exposure protocol. Regardless of the outcome of this assessment, the repeated exposure protocol using human IEC monolayers does offer a robust system to analyze cellular and molecular mechanisms of toxic proteins and substances that has multiple advantages over single exposure approaches. These advantages include providing an opportunity to investigate progressive versus rapid effects or reversible versus irreversible impacts by a test protein or substance.

5. Conclusions

The repeated exposure protocol (multiple 24-h in duration exposures) resulted in more substantial loss of barrier function and loss of

IEC viability upon encounter with hazardous proteins when compared to a single 24-h in duration exposure. However, the repeated exposure protocol introduces multiple technical challenges that likely diminishes its utility in hazard assessment analysis when compared to single exposure protocols. Aside from the increased time of conducting single experiments (a couple days versus 30 days), there is increased risk of contamination due to the requirement of handling the same set of IEC monolayers over the course of 30 days. Previous studies suggest that extending the duration of exposure from 24 to 48 h provides an increased ability to discern hazardous from non-hazardous proteins without the substantial increase of time and resources associated with the repeated dose protocol. Therefore, an extended duration single exposure protocol may be optimal for protein hazard characterization using the human polarized IEC experimental platform (Zimmermann et al., 2018).

The development of an *in vitro* assay to test GM crop proteins for hazardous effects on human IECs has numerous advantages over existing *in vivo* assays. In addition to reducing the need for animal testing, the quantity of the protein to be isolated for hazard assessment would be dramatically reduced. Additionally, this experimental system is human cell based as opposed to murine. To date, studies indicate that the results obtained from *in vitro* protein hazard characterization assays with human IEC monolayers are effective at discriminating between hazardous and non-hazardous proteins (Hurley et al., 2016a; Eaton et al., 2017). This *in vitro* protein hazard assessment experimental platform using human IEC monolayers may serve as a useful component of Tier II (hazard characterization) testing for proteins that have been examined initially through Tier I evaluation, thereby potentially reducing the necessity of *in vivo* toxicological experiments.

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Appendix A. Supplementary data

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Transparency document

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