Effects of Extracellular Vesicles on Caco-2 Cell Differentiation Using Transepithelial Electrical Resistance as Endpoint

Abstract:

In this study, a Caco-2 cell model was used to replicate a baby's small intestines to study the effects of exosomes on the model. Necrotizing Enterocolitis (NEC) is a severe disease that affects the intestines of preterm babies. Up to seven percent of preterm infants have been affected by NEC (Lim, 2015). This disease attacks the baby's small intestines, resulting in increased inflammation of the small intestines wall. Exosomes are known to contain extracellular vesicles, which include anti-inflammatory factors and help protects the baby's immunity. Since Caco-2 cells are differentiative cells, they transform into epithelial cells. The epithelial cells contain similar functions to enterocytes by forming tight junctions between one another to access cellular communication. In the model used, the enterocytes were being measured by a Transepithelial Electrical Resistance device, TEER, to measure the integrity of the cells and to make sure they are closely packed with one another. In addition, the tight junctions are formed to prevent any seeping bacteria into the small intestines, furthermore, causing more swelling.

For the experimentation, different exosomes have been collected from a variety of donor mothers. Also, formula fed milk exosomes have been collected because research has shown that formula fed milk is more likely to cause NEC in comparison to human feeding milk.

Using the Caco-2 cell model, the data compared was between the transwell inserts of the different derived exosomes. The exosomes that were collected were preterm mothers breast milk, term mothers breast milk, and formula feeding milk. These different types of exosomes were being compared to one another to see if there was statistical significance between those wells. The results showed that there was a statistical significance in TEER readings between when extracellular vesicles were present. However, there was very little statistical significance between the different extracellular vesicle groups: Preterm EVS, Term EVS, and Donor EVS. Thus, this information could be informative to mothers who have just delivered regarding benefits of extracellular vesicles and how they could potentially decrease their child's risk of NEC.

Introduction:

Necrotizing Enterocolitis (NEC) is a destructive disease that affects the intestines of preterm babies. Up to seven percent of preterm infants have been affected by NEC (Lim, 2015). This disease affects the wall of the small intestines which becomes inflamed due to the invasion of bacteria; furthermore, resulting in the destruction of the intestines in the baby's adult life (CHLA, 2018). Some may say that broad spectrum antibiotics could be a treatment for NEC, but this treatment may or may not be effective (Silverman, 2017). If NEC is not treated, the bacteria outside of the intestine can seep into the abdomen and cause a severe infection. This could potentially lead to death.

Enterocytes are intestinal absorptive cells that line the inner surface of the small intestines: they are very similar to epithelial cells. Epithelial cells are generally found on your skin, blood vessels, organs, etc. Enterocytes are essential for the ability of the baby to absorb nutrients. Caco-2 cells are currently used as a model to study enterocyte functions. Caco-2 cells are extended heterogeneous human epithelial colorectal adenocarcinoma cells (Lea, 1970). After two to three weeks, Caco-2 cells develop into a monolayer of cells formed by coupled tight junctions and express several functional features of enterocytes. Coupled tight junctions are formed between the enterocytes helping to form a protective barrier around the small intestines to prevent the seeping bacteria. The state of tight junctions between the enterocytes is directly related to permeability. Therefore, measuring of tight junctions could be used as an important indicator to test the overall health of the small intestines. In order to measure these coupled tight junctions, a transepithelial electrical resistance device is used. It measures an electrical resistance across a cellular monolayer. Also, this method is very reliable to confirm the permeability of the monolayer as well.

Figure #1: Displayed structures making up Enterocytes and its functions for the absorptive cell. (Adapted from Siumed, 2016)

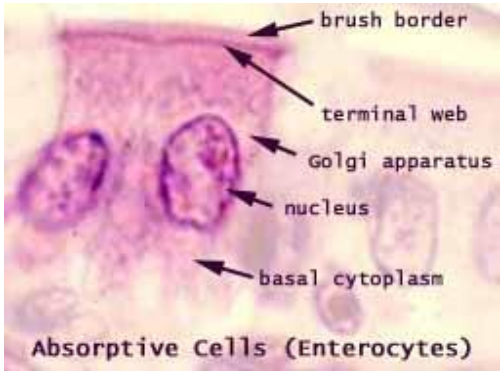
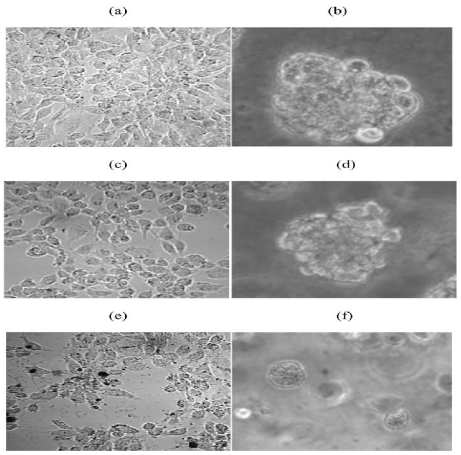


Figure #2: Different magnifications were used to view Caco-2 cells under the microscope. (Adapted from Zhipan, 2017)



One of the leading causes of NEC is formula fed milk, such as Enfamil (Admyre, 2007). Research has showed that the only accepted approach for the prevention of NEC is human feeding milk containing exosomes (Admyre, 2007). Exosomes are known to contain extracellular vesicles, which include antibodies to protect newborns against diseases and carry nutrients for the baby's intestines (Admyre, 2007). Extracellular vesicles, also known as exosomes, are one of the most important features inside our body. They consist of lipids, nucleic acids, and proteins from donor cells (Raposo, 2013). In addition, they are known to facilitate intercellular communication processes between cells. When released into the extracellular space they can enter body fluids and potentially reach distant tissue. Most importantly, extracellular vesicles stimulate antitumoral immune responses to lower inflammation (Snoeck, 2005).

Caco-2 cells were used as a model of a baby's small intestines. Thus, the purpose of my research was to combine Caco-2 cells and exosomes obtained from human feeding milk to study the effects on the Caco-2 cells' tight junctions. This was assayed using transepithelial electrical resistance (TEER).

In this way, TEER indicates the integrity of the tight junctions produced by the Caco-2 cells as a measure of their permeability. The impact of exosomes from human feeding milk was compared to exosomes from formula feeding milk, such as Enfamil, was compared in the Caco-2 cell model.

It was hypothesized that presence of exosomes, delivering anti-inflammatory signals and additional nutrients, could potentially be a treatment for Necrotizing Enterocolitis and improve overall baby wellness.

Methods:

Culturing and Maintaining Caco-2 cells:

Caco-2 cells are a widely used model and known to be a continuous line of human epithelial colorectal adenocarcinoma cells. The advantage of using Caco-2 cells is that they form tight junctions forming a monolayer. The purpose of the Caco-2 cells is to develop a model like a small intestine. Caco-2 cells consists of properties such as cell differentiation. They transform into epithelial cells which is very similar to enterocytes, cells that line the small intestine. These Caco-2 cells were stored in a humidified incubator at 24 degrees Celsius. The cells were maintained in DMEM High Glucose with minus exosome fetal bovine serum (FBS) and DMEM High Glucose serum free media. Since the cells were placed in a transwell insert, the Caco-2 cells were exposed to the serum free media with DMEM High Glucose on the apical side. On the basal, or bottom of the well, the minus exosome with DMEM High serum was placed to prevent drying of the cells. The purpose of growing these Caco-2 cells were for the TEER readings later on in the experimentation.

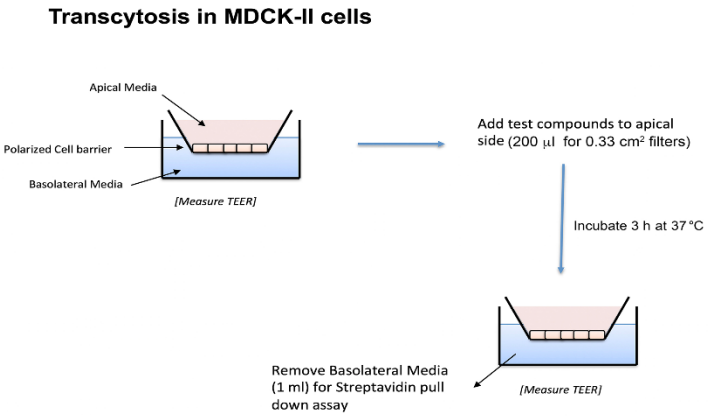


Figure #3: Transwell insert setup portraying Caco-2 cell model. (Adapted from Castillo, 2018)

Transepithelial Electrical Resistant Readings (TEER):

The Transepithelial Electrical Resistance device is a widely used technique for measuring tight junctions of cultured cells. This device contains electrodes, which measure electrical resistance across the cells. The measurements were executed by very carefully placing the electrodes on both sides of the cell layer. This measures both the voltage and current across the cell monolayer without disrupting the cells. I recorded this measurement for each reading. Since a transwell insert contains three sections in one well, I calculated the TEER readings by measuring each of the three sections and compared those readings to other wells to see how compact the other cell junctions were.

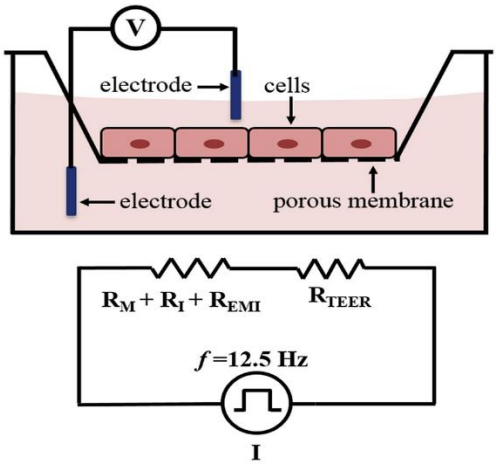


Figure #4: Diagram showing how a Transepithelial Electrical Resistant device is being used in the transwell insert. (Adapted from Srinivasan, 2018)

Extraction of Exosomes:

Many studies have convincingly shown a benefit of human feeding milk by reducing the risk of NEC. Human feeding milk contain vast amount of supplements such as protein, calcium, sodium, exosomes, etc. In my experimentation, the exosomes were being isolated by serial ultracentrifugation. After thawing donated colostrum samples from healthy mothers, they were subjected to 17,000 x g for 60 minutes at 4 degrees Celsius in Thermo Sci centrifuge (A27 -8x50 rotor). Next, the filtration process included filtering through 0.8 um, 0.45 um, and 0.22 um filters to remove any cell waste. The supernatants were centrifuged at 120.000 x g for 3 hours at 4 degrees Celsius in Beckman Coulter Optima XPN-90 Ultracentrifuge, with a SW50.2 rotor (6mm tube). Thus, ultracentrifugation forms a firm pellet of vesicles at the bottom of the tube. Next, I had to aspirate the supernatant with a pipette tip, avoiding contact with the pellet. Cold PBS

was then added on the opposite side of the pellet. After, ultracentrifugation took place for 45 minutes at 120.000 x g at 4 degrees Celsius in Beckman Coulter Optima XPN-90 Ultracentrifuge. Lastly, the tubes were covered with parafilm and refrigerated overnight at 4 degrees Celsius.

Addition of Exosomes to Caco-2 Cell Model:

After filtering thawed donated colostrum samples from healthy mothers, a BCA was performed. A BCA is a protein assay used for the quantification of the total amount of protein in a sample. Using the BCA Protein Assay Kit, I was testing to see the number of exosomes to obtain and use for the Caco-2 cell model. After establishing the quantity of exosomes to use in the cell model, the desired exosome amount was placed in different eppendorf tubes labeled K34W1 (control), K36W1 (term), K38W1(preterm), and TK13W1 (donor). Working under a fume hood, I pipetted 10 ul of the different eppendorf tube samples into the correct wells labeled on the Caco-2 cell model. I carefully placed the 10 ul of exosomes in the center of the transwell, where Caco-2 cells are present, along with the addition of new DMEM High Glucose serum free media. Next, the Caco-2 cell model was placed in a humidified incubator for 24 hrs.

Results:

Table #1:

Net TEER Readings, Experiment 11, Day 3, 5, 12, 19

Day	No Evs	Evs-Term	Evs-Preterm	Evs-Donor
3	286.2	279.8	298.0	297.7
5	224.7	353.9	405.0	425.9
12	348.0	429.8	473.2	371.0
19	617.0	755.3	770.8	722.7

Key: No Evs- well containing no extracellular vesicles.

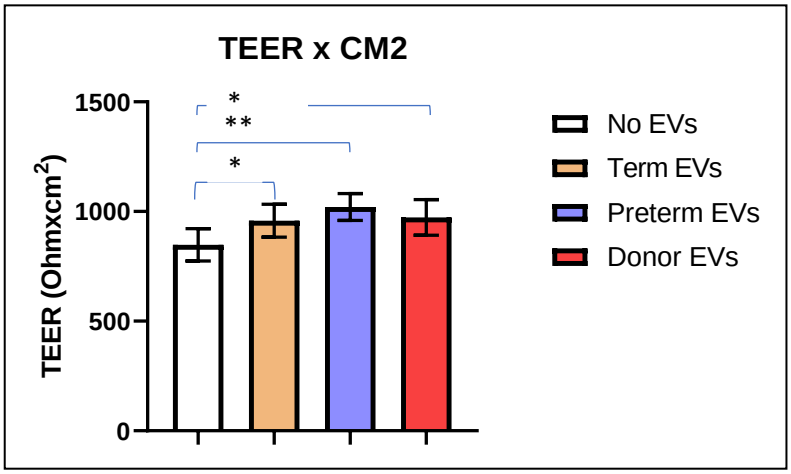
Evs-Term- well containing extracellular vesicles derived from mothers who delivered term babies.

Evs-Preterm- well containing extracellular vesicles derived from mother's wo delivered preterm babies.

Evs-Donor- well containing extracellular vesicles derived from formula fed milk

Graph #1:

TEER Measurements of Tight Junctions of Caco-2 Cells Including Extracellular Vesicles



Graph #1: Bar graph created for the measurement of tight junctions including Extracellular Vesicles by using TEER readings.

*Statistical Significance at p < .05 (significant)

**Statistical Significance at p < .01 (very significant)

*Statistical Significance at p < .001 (very significant)

Table #2:

Statistical Significance Summary

Holm-Sidak's multiple comparisons test	Mean Difference	Significant?	Summary	Adjusted P Value
No EVs vs. Term EVs	-110.5	Yes	*	0.0384
No EVs vs. Preterm EVs	-173.3	Yes	**	0.0019
No EVs vs. Donor EVs	-125.1	Yes	*	0.0217
Term EVs vs. Preterm EVs	-62.78	No	ns	0.3027
Term EVs vs. Donor EVs	-14.6	No	ns	0.7012
Preterm EVs vs. Donor EVs	48.18	No	ns	0.3857

*p < .05, **p < .01, *p < .001

Discussion:

A Caco-2 cell model was used to replicate a baby's small intestines to study the effects of exosomes and its reduced inflammation factors. In this study, as a control in the Caco-2 cell model, the first column of the transwell insert was only Caco-2 cells that are cultured and maintained without any exosomes present. In the next two columns, exosomes were present, but from different sources: Preterm-EVS and term-EVS. Lastly, the final column contains formula fed milk, which was not derived from human feeding milk: Donor-EVS.

In one of the eleven experiments performed, different numbers of extracellular vesicles were used for each column. Before the extracellular vesicles were used, a BCA, also known as a protein assay, was used in order to measure and calculate the right amount of exosomes used for the experimentation. The filtered breast milk and formula fed milk were used for the BCA. After 24 hours, the extracellular vesicles were then placed into the Caco-2 cell model. Next, after every other day, the cell culture had to be maintained by "feeding" the model more exosomes and media to maintain the Caco-2 cells liveliness. To measure the integrity of the Caco-2 cell model, the TEER was used to measure the tight junctions around the enterocytes, to prevent any leakage of media, containing exosomes, into the basal compartment of the transwell model.

There were eleven experimentations performed with these repeated steps. Data has been averaged to find the net TEER readings of the Caco-2 cells from all eleven experimentations to test the validity of the Cco-2 cells and the exosomes being used. Also, the Holm-Sidak's Multiple Comparisons Test was used in order to analyze the statistical significance between the different derived exosomes.

On day 3 through 19, experimentation 11, the exosomes ranged from 286.2 to 617.0 in the column containing no extracellular vesicles. Exosomes ranging from 279.8 to 755.3 were in the extracellular vesicles-term column. Exosomes ranging from 298.0 to 770.8 were in the extracellular vesicles-preterm column. Lastly, exosomes ranging from 297.7 to 722.7 were in the extracellular vesicles-donor column.

The Holm-Sidak's Multiple Comparisons Test, available online software, was used to measure the statistical difference between these columns and their exosome values. The system concludes that the mean difference between the following: No EVs vs. Term EVs was -110.5 with a p-value of 0.0384, No EVs vs. Preterm EVs was -173.3 with a p-value of 0.0019, No EVs vs. Donor EVs was -125.1 with a p-value of 0.0217, Term EVs vs. Preterm EVs was -62.78 with a p-value of 0.3027, Term EVs vs. Donor EVs was -14.6 with a p-value of 0.7012, and Preterm EVs vs. Donor EVs was 48.18 with a p-value of 0.3857. Since statistical significance is stated with any p-value less than or equal to 0.05, my results conclude that No EVs vs. Term EVs, No EVs vs. Preterm EVs, and No EVs vs. Donor EVs have a statistically significant difference as shown in Table #2.

In Graph #1, TEER measurements of tight junctions of Caco-2 cells including extracellular vesicles were being analyzed. This device was used for measuring the tight junctions between the extracellular vesicles. The different extracellular groups were being compared to one another to see if there was a significant difference between the different exosomes. There was statistical significance between No EVs vs. Term EVs. Also, there was a very significant difference between No EVs vs. Preterm EVs and between No EVs vs. Donor EVs.

The overall results of the experiment show that there was statistical significantly greater TEER readings between No EVS and EVS rather than EVS to EV wells. However, there is very little significance between wells containing extracellular vesicles, not including the wells containing no exosomes. There is only statistical significance between no extracellular vesicles and extracellular vesicles.

Conclusion:

The main goal of my research was to find a way to create a model, similar to a baby's small intestines, to see the impact of an exosomes' anti-inflammatory properties. Previous research states that up to seven percent of preterm infants have been affected by NEC (Lim, 2015). Necrotizing Enterocolitis is a serious

medical condition of bacteria attacking the walls of a baby's small intestines. However, people may say that general antibiotics could solve the problem. Therefore, in this case the treatment may not be as effective in resolving NEC. Since Caco-2 cells are differentiative cells they transform into epithelial cells, very similar to enterocytes. These packed cells contain antitumoral and anti-inflammatory factors. Potentially, a baby dealing with NEC could use these anti-inflammatory exosomes to decrease the swelling of the baby's intestines, which later affects his/her health.

When comparing the difference between which derived exosomes were more significant, there was no difference, but there was significance less than a p value of .05 between no exosomes compared to a large change. This data was interpreted by the TEER readings, measured in $Ohmxcm^2$, which shows a drastic change in $ohmxcm^2$ readings between the wells.

With this new knowledge, a potential treatment for babies impacted with Necrotizing Enterocolitis could be achieved. With the many benefits of breast milk, such as reducing risks of viruses, prevention of respiratory infections, and anti-inflammatory factors, this research could inform mothers who have just delivered to breastfeed their child to potentially decrease their risk of NEC and protect their baby's immunity.

Bibliography:

Frost, B. L., Modi, B. P., Jaksic, T., & Caplan, M. S. (2017, January 1). New Medical and Surgical Insights Into Neonatal Necrotizing Enterocolitis: A Review. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/27893069.

Lim, J. C., Golden, J. M., & Ford, H. R. (2015, June). Pathogenesis of neonatal necrotizing enterocolitis. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25854935.

Manca, S., Upadhyaya, B., Mutai, E., Desaulniers, A. T., Cederberg, R. A., White, B. R., & Zempleni, J. (2018, December 1). Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. Retrieved from https://nebraska.pure.elsevier.com/en/publications/milk-exosomes-are-bioavailable-and-distinct-microrna-cargos-have-.

de la Torre Gomez, C., Goreham, R. V., Bech Serra, J. J., Nann, T., & Kussmann, M. (2018, March 27). "Exosomics"-A Review of Biophysics, Biology and Biochemistry of Exosomes With a Focus on Human Breast Milk. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29636770.

Pegtel1, D. M., & UMC, S. J. G. A. (n.d.). Exosomes. Retrieved from https://www.annualreviews.org/doi/abs/10.1146/annurev-biochem-013118-111902.

Admyre, C., Johansson, S. M., Qazi, K. R., Filén, J.-J., Lahesmaa, R., Norman, M., … Gabrielsson, S. (2007, August 1). Exosomes with immune modulatory features are present in human breast milk. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17641064.

Epithelial Cells in Urine: MedlinePlus Lab Test Information. (2019, September 30). Retrieved from https://medlineplus.gov/lab-tests/epithelial-cells-in-urine/.