Assessing Alaskan Blueberry Bioavailability *in vitro*

Savanah Owen, Aline Collin, and Thomas Kuhn

Abstract

There is a strong correlation between type II diabetes (T2D) and obesity determined by body mass index (1). T2D is characterized by a lack of insulin-sensitive regulation of blood glucose despite all components of the regulatory system being properly expressed. Notably, rural Alaskan communities exhibit a strange phenomenon where the prevalence of T2D is considerably lower than that of obesity, which may be attributed to the berry rich diets as a part of their subsistence lifestyle. Rescue or restoration of insulin sensitivity has been shown in cell cultures assays but it remains to be shown whether this potency is actually transported from the digestive system (intestine) to the blood. Hence we assessed in vitro the bioavailability of blueberry botanicals relying on the widely used Caco-2 cell model. These cells form a diffusion barrier in vitro modeling the quantitative transport of pharmacological and/or botanical compounds across the intestine. Our study showed that blueberry botanicals presented unique problems for the caco-2 system, including cytotoxicity and interference with insert filters. Resolution of the issues presented in this paper will allow us to use the Caco-2 bioavailability assay to collect Alaskan blueberry metabolites in vitro for further analysis using LC-MS.

Photo of Alaskan Blueberries picked at Skiland in 2019 by Savanah Owen

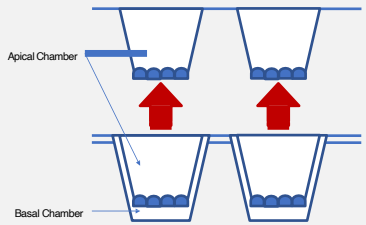
Background

Berries contain high levels of vitamins, minerals, fibers and polyphenols with the latter likely responsible for most of the health benefits of berries (2). There is mounting evidence, obtained in in vitro studies, that polyphenols from Alaskan blueberries potently enhance sensitivity to insulin (1,3) . The principal obstacle remains in the establishment of causation between in vitro studies and in vivo outcomes, which are largely determined by bioavailability and biotransformation.

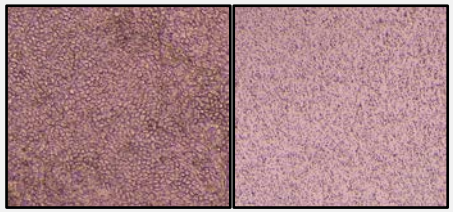
Bioavailability addresses the crucial step of absorption of botanicals across the intestinal barrier while, chronic exposure to botanicals has been shown to influence their absorption (4).

Methods

Caco-2 cell monolayers, that can mimic transport across the intestinal lining were grown in DMEM+20% FBS until adequate concentrations (~2.4 x 10^5) were obtained. Cells were then transferred onto transwell membrane inserts with 0.4µm pore filters. Once cao-2 cells became confluent on inserts, they were chronically exposed to different concentrations of botanical extract: 0 µg/ml, 5 µg/ml, 10 µg/ml. After complete maturation, 21-29 days post-seeding, monolayers were put through control transport testing that evaluated monolayer intactness.



(Above) removable transwell membrane inserts that mature Caco-2 cells were transferred to. These wells can be placed in a standard 12-well plate and when cells are confluent and intact they are able to form a selectively permeable layer that mimics the intestinal wall.



(Left) Confocal microscopy photo (100x) of confluent, and mature caco-2 monolayer grown on a transwell insert at D21. Tight junctions can be seen between cells.

(Right) Confocal microscopy photo of a blank transwell insert. 0.4µm pores can be seen, and appear as small, dark specs on the insert.

Fluorescently labeled 3kDa dextran was used as the reporter molecule for control testing prior to botanical transport assays. A 0.4ml dose of 100µg/ml dextran in culture medium was applied to each mature monolayer. At various time points: 0min, 30 min, 60 min, 90 min, 120 min, and 240 min, samples were collected from the basal chamber and fluorescence was measured. Monolayers with 1% dextran transport were considered to be fully intact.

Intact monolayers were then used in botanical transport studies. In trial 1,100 µg/ml solution of crude berry extract in PBS, phosphate buffered saline was applied to the apical chamber (trial 2 was conducted with 50 mg/ml and without chronic treatment) and at various time points: 0 min, 30 min, 60 min, 90 min, 120 min, and 240 min, basal chamber contents were collected. These samples were then kept frozen until being sent for LC-MS analysis.. Due to current budget restrains LC-MS analysis has been postponed.

Once botanical transport studies were completed, monolayers were again tested for intactness. For post-botanical control testing, lucifer yellow (LY) was used as the reporter molecule. 0.4 ml of 1.0 mg/ml lucifer yellow in culture medium solution was applied to each of the monolayers. At various time points: 0min, 30, min, 60min, and 120min, samples from the basal chamber were collected and their fluorescence was measured. Monolayers that showed <3% LY transport were considered intact.

The effect of botanical exposure on cell viability was measured via an MTT assay. Viable cells are capable of reducing MTT into formazan.Caco-2 cells were grown on 96 well plates and then exposed to the various concentrations of botanical extracts: 5 µg/ml, 10 µg/ml, and 100µg/ml. After 4 hrs. (100 µg/ml) and 7 days (5 µg/ml, 10 µg/ml) MTT was applied. At t= 2 hrs post-MTT, DMSO solvent was applied, and at t=4 hrs post-MTT, absorbance at 595 was measured in order to quantify Formazan concentration.

Results

Trial 1 showed monolayers were intact prior to berry transport (Fig 1. & Fig 2.), but were compromised after berry transport (Fig 3. & Fig 4.), leading us to suspect berry cytotoxicity.

MTT tests showed that acute concentrations from trial 1 i.e. 100 µg/ml killed cells (64.2-69.2% viability) and compromised monolayers, while chronic concentrations i.e. 5, and 10 µg/ml actually increased cell proliferation (105.5-108.6% & 107-114.6% viability respectively).

Trial 2 testing, using a reduced acute concentration, showed controls inconsistent with trial 1. Controls appeared to have some form of transport occlusion making transport rates nearly identical to that of experimental groups (Fig 5. & Fig 6.) invalidating results that would otherwise read as intact.

Figures

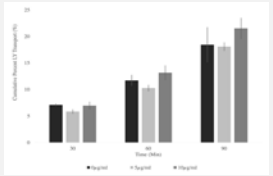


Fig 1.Trial 1, cumulative percent Dextran transported for experimental groups: 0, 5,and 10 µg/ml (n=9, for each). Standard error is expressed by gray bars.

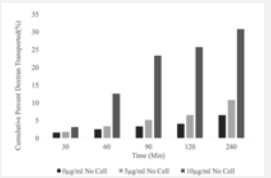


Fig 2. Trial 1, cumulative percent Dextran transported for no-cell control groups: 0, 5, and 10 µg/ml (n=1, for each).

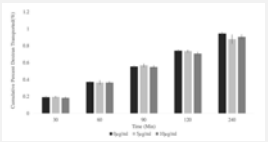


Fig 3. Trial 1, cumulative percent LY transported for experimental groups: 0, 5 ,and 10 µg/ml (n=9, for each). Standard error is expressed by gray bars.

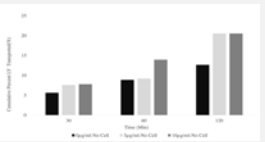


Fig 4.Trial 1, cumulative percent LY transported for no-cell control groups: 0, 5 ,and 10 µg/ml (n=1, for each).

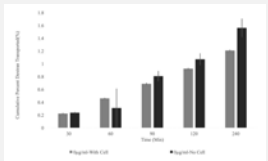


Fig 5. Trial 2, cumulative percent transport dextran in both experimental-0 µg/ml (n=8)and no-cell control-0 µg/ml (n=2) groups. Standard error is expressed by gray bars.

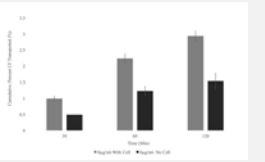


Fig 6. Trial 2, cumulative percent transport of LY for both experimental-0µg/ml (n=8)and no-cell-0µg/ml (n=2) groups. Standard error is expressed by gray bars.

Conclusions

Within the two trials performed monolayers were not sufficiently intact. To improve upon the caco-2 bioavailability assay monolayer integrity must be improved. Further investigation into berry caco-2 cytotoxicity could indicate the maximum non-toxic berry concentration, thus eliminating the destructive effect of berry solutions on monolayers. Investigation into the impact of berry solutions on insert filters is needed to ensure that berry particles do not impact transport into the basal chamber. Growth of caco-2 cells appears to be successful, however being able to grow sufficient amounts of cells in a timely manner would make this assay more useful. During this study, we found that use of ECM MAXgel™ along with high seeding count improved cell proliferation speeds.

A functional caco-2 bioavailability assay accompanied by LC-MS analysis could be an in vitro method for evaluating bioavailability of Alaskan blueberry botanicals. By using this system the active, anti-dietetic compounds in Alaskan blueberries could be identified and further studied bringing us one step closer to a novel dietary therapy for T2D.

References

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Research reported in this publication was supported by the National Institute Of General Medical Sciences of the National Institutes of Health under Award Numbers UL1GM118991, TL4GM118992, or RL5GM118990. The content is solely the responsibility of the authors and not necessarily represent the official views of the National Institutes of Health.