Original Paper

Acute Effects of Vitamin C Exposure On Colonic Crypts: Direct Modulation of pH Regulation

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Key Words

Intestines • Malignancies • PH • Fluid Movement • Rats • Nutraceutical • Tumors

Abstract

Background/Aim: Colorectal cancer is still considered a leading cause of death in the United States and worldwide. One potential way to improve survival besides detection is to look to new therapeutic agents that can be taken prophylactically to reduce the risk of tumor formation. For cancer cells to grow and invade, a higher (more alkaline) intracellular pH must occur. We chose to examine a specific nutraceutical agent, which is Vitamin C. The acute effect of Vitamin C exposure on normal colonic crypts has been studied, providing some insight into how Vitamin C achieve its effect. **Methods:** Distal colon was excised from rats. Following enzymatic digestion single colonic crypts were isolated. Colonic crypts were loaded with pH sensitive dye to measure the intracellular pH changes. Crypts were exposed to solutions +/- Vitamin C. **Results:** 10 mM Vitamin C decreased Na⁺-dependent intracellular pH recovery. Vitamin C modulates SVCT leading to changes in proton extrusion. Vitamin C entry occurs via either SVCT2 on the basolateral membrane or by transcellular passive diffusion through tight junctions to the apical membrane and then active transport via SVCT1. **Conclusion:** Acute addition of Vitamin C to the basolateral membrane maintains low intracellular pH for a longer period which could halt and/or prevent tumor formation.

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Introduction

Colorectal cancer is the third most common cancer worldwide and the third most common cause of death with just over one hundred thousand new cases diagnosed each year equally divided between men and women [1]. The United States has among one of the highest levels for all countries and has approximately 93,000 diagnosed cases per year, and an estimated death rate of 49,000 per year [2].

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One of the common pathophysiological pathways for cancer to grow, proliferate and invade neighboring cells, is that the cancer cell must maintain an elevated intracellular pH (pH_i) and concurrently a significantly lower pH of the extracellular environment (pH_e) [3, 4]. By understanding the intracellular ionic signaling pathways, it may be possible to manipulate the pH_i of a cancer cell thereby arresting its growth. Similarly, modulating the pH_e may prevent or retard the growth of the tumor. The modulation of these two environments (pH_i and pH_e) via targeting the specific ionic exchange proteins could thus be a promising novel treatment strategy. Vitamin C (Ascorbic Acid) is known to play a role in the differentiation process of cells [5], showing that Vitamin C has capabilities to modulate cells proliferation, which suggests a possible inhibitory action also on tumor cells [6]. As an antitumorigenic, Vitamin C has been proven to protect against several types of non-hormonal cancers in lung and colon [7-9]. Maulén et al. showed that the analysis of quantitative PCR of colonic carcinoma cell line, which revealed that the expression of the Na-dependent Vitamin C transporters SVCT 1 mRNA increased by 4 fold without changes in SVCT2 mRNA levels. Moreover, this study also showed that the V_{max} increased also by at least 2-fold [10].

Recent studies have shown that Vitamin C is absorbed throughout the intestine via an energy-dependent Na-dependent process that is saturable. In previous studies, this absorption of Vitamin C was associated with Glucose transport [11, 12]. If this relationship to Na-dependent Glucose transport can be exploited, it may be possible to enhance the uptake of Vitamin C and thus further provide an antitumorigenic effect [13]. Under these conditions, we would expect an enhanced modulation of the pH-dependent processes both intracellularly and extracellularly. Our studies presented below also focused on the uptake of Vitamin C via SVCT1 and SVCT2 [14-16], and glucose Transporters (GLUT) and their effects on acid extrusion or uptake. We further examined the concentration dependence of these effects by using 3 concentrations that were either 10-fold lower or higher than the suggested maximal daily dose [17]. The highest concentration used in this study was comparable to a dose used clinically in attempting to reduce tumor growth [18]. In the present study, we chose to investigate the physiological process(es) that occurs in an acute exposure of Vitamin C on normal colonic crypts and to determine if there are any modulations in sodium dependent hydrogen ion extrusion.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 300-650g were used in this study. All animals were kept in a climate controlled facility. The animals were fed standard chow and had ad lib access to water prior to experimentation. The animals were handled by animal care personnel according to the approved protocol by The Institutional Animal Care and Use Committee at Yale University (protocol number 2015-10253). The animals were fasted for 12 hours before tissue harvest with ad lib access to water during this period [19].

Colonic Crypt Isolation and Collection

All animals used in the study were exposed to an overdose of the inhaled anesthetic Isoflurane. We then performed a laparotomy to expose the colon and excise it. We identified the anal junction and made a transverse dissection. We then removed ~10 cm proximal to the anal colon junctional interface. The colon was then removed and flushed with 4°C cold HEPES Ringer solution (See Table 1) to remove the residual fecal material from the interior of the colon as previously described [20]. Briefly, we cut the clean distal colon into two longitudinal strips [21, 22]. The two strips were then immersed into a digestion solution that was composed of a low Calcium High EDTA/HEPES buffer. Following digestion, the colon was removed and discarded, and the remaining solution was centrifuged at 500 rpm for 45 seconds. Using our previously described technique, we then removed the digestion buffer and rehydrated the remaining pallet with a cold HEPES pH 7.4 at 4°C (see Table 1) [23].

Table 1. Composition of Solutions. All solutions were adjusted to a final osmolality of 300mOsm at pH 7.4 at 37 °C

Solution	Composition in mM	pH
Standard Na ⁺ Concentration HEPES (117 mM)	NaCl (117), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10).	7.4 at 37 °C
Low Na ⁺ Concentration HEPES (77 mM Na)	NaCl (77), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10), NMDG (40)	7.4 at 37 °C
Glucose-Free HEPES (117 mM)	NaCl (117), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2).	7.4 at 37 °C
Cold HEPES	NaCl (117), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10)	7.4 at 5 °C
Room temperature HEPES	NaCl (117), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10)	7.4 at 20 °C
NH ₄ Cl	NaCl (77), NH ₄ Cl (40), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10).	7.4 at 37 °C
Glucose-Free NH ₄ Cl	NaCl (77), NH ₄ Cl (40), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2).	7.4 at 37 °C
Ø Na ⁺	NMDG (N-Methylglucamine; 1-Deoxy-1-(methylamino)-D-glucitol) (132.8), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), Glucose (10)	7.4 at 37 °C
Glucose-Free Ø Na ⁺	NMDG (N-Methylglucamine; 1-Deoxy-1-(methylamino)-D-glucitol) (132.8), KCl (5), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2)	7.4 at 37 °C
High-K	KCl (105), CaCl ₂ *2H ₂ O (1), MgSO ₄ *7H ₂ O (1.2), HEPES (32.2), NMDG (32.8), Mannitol (5)	7.0 at 37 °C

Dye preparation and cell loading

A coverslip (Fisherbrand®, Fisher Scientific measuring 22 x 50 mm) pretreated with 0.1 µL of Corning® Cell-Tak™ (Cell and Tissue Adhesive, Polyphenolic proteins secreted by *Mytilus edulis*). We then transfer a 200 µL aliquot of the crypts HEPES solution to the chamber, then incubated the crypts with a 1 µL of BCECF/AM dye (Santa Cruz Biotechnology) a pH sensitive dye as previously described [22, 24].

Data collection

For each BCECF loaded crypt, 5 regions (1 control region and 4 experimental regions) were selected to monitor pH_i changes for a duration of a single experiment. The crypts were treated with standard HEPES buffer solution, alkalinized it with NH₄Cl, then acidified it by depleting the crypt from Na⁺ through ØNa perfusion, a reperfusion of the crypts with standard HEPES before lastly calibrated it with High-K/Nigericin. The pH_i monitored through measuring the average rate of Hydrogen ion extrusion through experiment cycle. The BCECF loaded crypts were excited at 490 and 440 nm±20nm respectively while collecting the emission at 530 nm±20nm every 15s. The pH was calculated using the High-K/Nigericin calibration technique [22] previously used in our laboratory.

Addition of Vitamin C and other drugs

L-Ascorbic acid (C₆H₈O₆) (CAS Number: 50-81-7) and Phlorizin Hydrate: CAS Number : 60-81-1 C₂₁H₂₄O₁₀·xH₂O were purchased from Sigma-Aldrich Co. (St Louis, MO USA).

Statistics

All data were analyzed with Prism 6 program from GraphPad using Students T- test, when comparing 1 condition to 1 other condition and ANOVA when all columns were compared together. Significance was recorded for each series under all conditions.

Fig. 1. Measurements of intracellular pH in isolated rat colonic crypts. Panel A and C, Isolated crypt loaded with the pH sensitive dye BCECF. Panel B Original tracing showing the rate of recovery from an acid load in control conditions. Panel D tracing from crypt following exposure to 10mM Vitamin C. Please note the suppressed Na-dependent pH recovery following Vitamin C exposure. Key: C= Control (Standard HEPES), NH= NH₄Cl, HK= High-K.

Results

Vitamin C Concentration Curve

To develop a concentration curve for Vitamin C effect (see Fig. 1 and 3), different concentrations have been chosen according to previous studies. The pharmacokinetics of Vitamin C show that the body has a tight control keeping the level of Vitamin C ~100 μ M [18]. The maximum tolerable concentration of Vitamin C has been reported to be around 1mM, while it is proven that a concentration of 10mM Vitamin C could kill and fight cancer *in vitro* and *in vivo* [18]. First, we compared the recovery rate of pH_i among control experiments (Δ pH_i /min 0.0160) and experiments containing 100 μ M of vitamin C (Δ pH_i /min 0.0284); the recovery rates were faster when we added 100 μ M of Vitamin C with a p value of <0.0001. On another set of experiments, we increased the concentration of Vitamin C to 1mM which is the same as the daily maximum dose [17]; we found that the recovery rate of pH_i (Δ pH_i /min 0.0203)

Fig. 2. Tracings showing the recovery rate of intracellular pH in isolated rat colonic crypts from an acid load in different conditions. Panel A tracing from crypt in control conditions. Panel B tracing from crypt following exposure to 100 μ M Vitamin C. Panel C tracing from crypt following exposure to 1mM Vitamin C. Panel D tracing from crypt following exposure to 10mM Vitamin C. Please note the suppressed Na-dependent pH recovery following Vitamin C exposure.

still faster in comparison to control experiments with p value of <0.0001 but slower than that of 100 μ M with a p value of <0.0001. However, when we further increase the concentration of Vitamin C to 10mM, a concentration proposed to kill cancer cells, we found that the pH_i recovery rate slowed down (ΔpH_i /min 0.0080) in comparison to control with a p value of <0.0001. If we compare the pH_i recovery rate between 100 μ M of Vitamin C and 1mM and 10mM, we observed that 100 μ M has the fastest recovery with p value of <0.0001. Conversely, the recovery rate of 10 mM of Vitamin C is slower than 100 μ M and 1mM with p value of <0.0001. Tracings of pH_i recovery rates are shown in Fig. 2 and the summary data shown in Fig. 3.

SVCT (Sodium Vitamin C Transporter) Role in Vitamin C-Dependent pH modification

To observe and establish a role of SVCT in Vitamin C-dependent pH modification and how it could modulate the efficacy of Vitamin C at different concentrations, we designed experiments with the same components of the solutions as found in Table1 except for reducing the Na^+ concentration to 77mM (see Fig. 5). Comparing control experiments of standard Na^+ concentration (117 mM) with control experiments of 77 mM Na^+ concentration, there was no significance difference in the recovery rate of pH_i , the ΔpH_i /min for 77 mM Na^+ was 0.0169 and 0.0160 for the 117 mM Na^+ respectively. The recovery rate of pH_i with 100 μ M of Vitamin C dissolved in 77 mM solution (ΔpH_i /min 0.0098) is slower than the control experiments of the same Na^+ concentration(77mM) with a p value of <0.0001. However, when we increased the concentration of Vitamin C to 1mM in the same 77 mM Na^+ concentration condition we observed a slower rate of pH_i recovery (ΔpH_i /min 0.0128) compared to 77mM Na^+ control rates with a p value of 0.0021. Interestingly, when we increased the

Fig. 3. Vitamin C Concentration Curve. Crypts perfused with 100 μ M, 1mM and 10mM. Please note the slow recovery rate of pH with 10mM in comparison with control experiments. The slower rate of pH recovery become faster with lower doses of Vitamin. n = 40.

Fig. 4. Tracings showing the recovery rate of intracellular pH in isolated rat colonic crypts from an acid load in different conditions. Panel A tracing from crypt in control conditions. Panel B tracing from crypt following exposure to 100 μ M Vitamin C. Panel C tracing from crypt following exposure to 1mM Vitamin C. Panel D tracing from crypt following exposure to 10mM Vitamin C. Please note the suppressed Na-dependent pH recovery following Vitamin C exposure.

Fig. 5. The effect of SVCT on Vitamin C-dependent pH modification: Please note the slow recovery rate of pH with 100 μ M in 177 mM Na⁺ concentration in comparison with control experiments of the same Na⁺ concentration. The slower rate of pH recovery become aberrant with lower doses of Vitamin C dissolved in low Na⁺ concentration solution. n = 40.

concentration of Vitamin C that is dissolved in 77mM Na⁺ to 10 mM; there was no significant difference in the recovery rate of pH_i ($\Delta\text{pH}_i / \text{min}$ 0.0190) from that of the control solution of 77 mM Na⁺. Tracings of pH_i recovery rates are shown in Fig. 4, and the summary data shown in Fig. 5.

GLUT Effect On Vitamin C-Dependent pH Modification

To study the effect of GLUT on Vitamin C-dependent pH_i modification, in the first set of this series of experiments we used the same solutions for the first set of experiments in Table 1, but excluding Glucose (117 NaCl) (see Fig. 7). The recovery rate of pH_i to resting physiological pH values with 10 mM Vitamin C in glucose-free solutions ($\Delta\text{pH}_i / \text{min}$ 0.0080) was slower with a p value of < 0.0001 in comparison to glucose-free control solutions ($\Delta\text{pH}_i / \text{min}$ 0.0133). However, there was no significant difference between 10 mM of Vitamin C dissolved in Glucose solutions and 10 mM Vitamin C dissolved in Glucose-free solutions (See Fig. 7).

In these second set of experiments we dissolved Phlorizin a known inhibitor of glucose transport (SGLT1 and SGLT2), and Vitamin C in Glucose-free solutions to blunt the activity of GLUT transport. The recovery rate of pH_i with 10 mM Vitamin C plus Phlorizin dissolved in Glucose-free solutions ($\Delta\text{pH}_i / \text{min}$ 0.0081) was slower

Fig. 6. Tracings showing the recovery rate of intracellular pH in isolated rat colonic crypts from an acid load in different conditions. Panel A tracing from crypt in control conditions. Panel B tracing from crypt in Glucose-free solutions. C. Panel C tracing from crypt following exposure to 10 mM Vitamin C in control conditions. Panel D tracing from crypt following exposure to 10mM Vitamin C in Glucose-free conditions. Panel E tracing from crypt following exposure to 10mM Vitamin C in Glucose-free condition with added Phlorizin. Please note the suppressed Na-dependent pH recovery following Vitamin C exposure.

Fig. 7. GLUT effect on Vitamin C-dependent pH modification: No significant difference in the recovery rate of crypts perfused with 10mM Vitamin C in presence or absence of Glucose. Additionally, there was no significance difference after blocking GLUT with Phlorizin in comparison to 10mM Vitamin C free of Glucose and Phlorizin. n = 40.

in comparison with control Glucose-free solutions ($\Delta\text{pH}_i/\text{min}$ 0.0133) with a p value of <0.0001. Moreover, there was no significance difference in pH_i recovery rate when we compared 10 mM Vitamin C +/- Glucose versus 10 mM Vitamin C plus Phlorizin dissolved in glucose-free solutions. Tracings of pH_i recovery rates are shown in Fig. 6 with summary data presented in Fig. 7.

The difference between GLUT and SVCT Effects On Vitamin C-Dependent Na-Dependent pH_i recovery

In this set of studies, we examined the difference in recovery rates between GLUT and SVCT on Vitamin C-dependent pH modification (See Fig. 9). We compared the rate of pH_i recovery rates between control experiments that either had no Glucose ($\Delta\text{pH}_i/\text{min}$ 0.0133) or had a Na^+ concentration of 77 mM plus 10 mM Glucose ($\Delta\text{pH}_i/\text{min}$ 0.0169). We observed that the control experiments that were Glucose free had a slower pH_i recovery rate than those experiments with 77 mM Na^+ concentration plus 10 mM Glucose concentration with a p value of 0.0002. When we added 10 mM Vitamin C to the various groups in a separate series

Fig. 8. Tracings showing the recovery rate of intracellular pH in isolated rat colonic crypts from an acid load in different conditions. Panel A tracing from crypt in control conditions of 77 mM Na. Panel B tracing from crypt in control Glucose Free conditions. Panel C tracing from crypt following exposure to 10 mM Vitamin C in 77 mM Na. Panel D tracing from crypt following exposure to 10mM Vitamin C in Glucose Free conditions. Please note the suppressed Na-dependent pH recovery following Vitamin C exposure.

Fig. 9. GLUT and SVCT effect on Vitamin C dependent pH modification: Please Note that there is a significant difference in pH recovery rate between the control of 77 mM Na⁺ with that of Glucose-free. When we add 10mM Vitamin C to the solutions, the recovery rate of pH become very slow except with 77 mM Na⁺ concentration that showed a stimulatory effect. n = 40.

of experiments, we found that the pH_i recovery rate in the absence of Glucose (ΔpH_i /min 0.0080) became dramatically slower than that of the 177 mM Na⁺ Glucose-free (ΔpH_i /min 0.0133) with a p value of <0.0001. Original tracings of pH_i recovery rates please refer to Fig. 8 with summary data presented in Fig. 9.

Discussion

Vitamin C is believed to be an anti-tumorigenic agent in chronic exposure to the colon, but the effect of Vitamin C in the acute condition is controversial and appears to only be antitumorigenic when infused directly into the bloodstream at high concentrations [18]. In retrospective studies published about the benefits of using Vitamin C in cancer treatment there appeared to be some effects [25, 26]. Later, double-blind trials showed no effect of Vitamin C as a tumoricidal agent [27]. However, in 2008, Chen et al. considered Vitamin C as a cancer therapy from a different perspective from the previous studies mentioned above, notably it was infused directly at high concentrations and was tumor cytotoxic at these high levels [28].

Pandey et al. [29]. established the risk of all cancers in the body being reduced significantly (39%) when >113 mg/d Vitamin C was consumed, compared to the control group who consumed <82 mg/d. However, as in all previous studies there were no published data about the effects of Vitamin C when taken acutely. We, therefore, decided in this paper to concentrate our focus on the acute effect(s) of Vitamin C on colonic crypts and observe any changes in cellular pH regulation. These potential acute changes in pH_i could have a positive effect in regards to carcinogenesis/chemoprevention in the colon according to Vaughan-Jones proposed theory of cancer development [3].

To do so, we noted the changes of intracellular pH before and after Vitamin C administration. Our results were obtained using the isolated colonic crypt model from Sprague-Dawley rats. Vitamin C shows a marked effect on the acidity of the crypt cells from intact isolated crypts. Maintaining the pH acidic intracellularly would inhibit the growth of tumor cells, as tumor cells require the intracellular milieu to be alkaline in order to proliferate.

Our first finding that Vitamin C appears to affect pH_i is supported by our experiments on the colonic crypts acidity when superfusing the crypts with solutions mixed with Vitamin C. Adding 10 mM Vitamin C induced a slower rates of pH_i recovery (ΔpH_i/min 0.0080) in comparison with the control experiment (ΔpH_i/min 0.0160) (Fig. 3). With intracellular pH becoming more acidic, an intracellular milieu has been created that is optimized to prevent tumor cell proliferation.

Furthermore, In the setting of acute Vitamin C administration, we observed in Fig. 3 that exposure to low concentrations (100 μM and 1 mM) of Vitamin C resulted in accelerated recovery rates of pH_i (ΔpH_i/min 0.0284 for 100 μM Vitamin C and ΔpH_i/min 0.0203 for

Vitamin C 1 mM) following an acid load which could mean a favorable environment for stimulation of tumor growth, this was reversed (tumor inhibitor effect, slow Na-dependent acid extrusion) with high acute Vitamin C exposure (10 mM). In other words, the effect of Vitamin C on the intracellular pH is concentration-dependent, as observed in Fig. 3 (Vitamin C Concentration Curve), when the colonic crypts were superfused with different concentrations resulting in a biphasic concentration dependent response, low concentrations of Vitamin C (100 μ M and 1 mM) maintains high (alkali) pH_i during the recovery period while high concentration of Vitamin C (10 mM) maintains low (acidic) pH_i during recovery period. This observation can be explained by dose dependent modulation of the Na⁺ dependent Vitamin C transporter (SVCT). This increased activity leads to both increases in H⁺ concentration within the cell as well as increases in intracellular Na⁺ concentration. The higher intracellular Na⁺ concentration prevents NHE from working efficiently due to the increased Na⁺ gradient within the cell. On the contrary, with lower concentrations of Vitamin C, the SVCT is less active which leads to a slower build up in the intracellular Na⁺ concentration. This results in a faster Na-dependent recovery rate.

As we have observed that Vitamin C transport is dependent on Na⁺ and since we use a 77 mM Na⁺ concentration for our NH₄Cl prepulse technique different from that of HEPES (177 mM) (Table1), we wanted to eliminate any influence on the recovery rate that may result from this Na⁺ concentration difference between solutions. We performed a new set of experiments with HEPES solution that made of 77mM Na⁺ concentration (Table1), contain or devoid of different Vitamin C concentrations to observe its ability to maintain an acidic intracellular environment of the crypts. There was no significant difference when we compare the recovery rate of pH_i between control experiments of 77 mM Na⁺ concentration ($\Delta pHi/min$ 0.0169) with control experiments of standard 117mM Na⁺ concentration ($\Delta pHi/min$ 0.0160). Comparing the results of the control experiments of 77mM Na⁺ concentration ($\Delta pHi/min$ 0.0169), to those of the 100 μ M Vitamin C dissolved in 77 mM Na⁺ solutions ($\Delta pHi/min$ 0.0098) we observed a slowing of the rate of pH_i recovery to the normal resting physiological rate (control conditions) with a p value of <0.0001. A similar slow recovery rate was observed when we increased the concentration of Vitamin C from 100 μ M to 1mM ($\Delta pHi/min$ 0.0128) with a p value of 0.0021. However, when we increased the concentration to 10mM ($\Delta pHi/min$ 0.0190) (a concentration that is used clinically to kill cancer cells), there was no significant difference in the recovery rate of pH_i from that of the control solution (0mM Vitamin C). We established that the difference in Na⁺ concentration effects the Vitamin C dependent intracellular pH changes, by the rate of Na dependent proton extrusion, at a (77 mM) Na⁺ concentration the opposite effects of Vitamin C dependent proton extrusion are observed, as seen in Fig. 5 (*SVCT Role in Vitamin C Dependent pH modification*). This reversal in the recovery rates (Fig. 5) in comparison to 117mM Na⁺ concentration (Fig. 3) can be explained by the difference in the Na⁺ concentration and the effects on the Vitamin C modulation of SVCT and subsequently on NHE activity, resulting in a modulation in the extrusion rate of H⁺.

In a recent study published by Maulén et. al. using CaCo-2 cells they determined that these cells express SVCT 1 selectively on the apical side. Moreover, in the same study they found that SCVT 1 and SVCT 2 have different Km values, SVCT 1 has a lower affinity for Vitamin C (Km 125 μ M) while SVCT 2 has a higher affinity (Km 8 μ M) [10]. Their study also finds that there was no transport of Vitamin C across the basolateral surface and that all of the transported Vitamin C is being transported on the apical membrane through SVCT1 [10]. However, in our study, we superfused the intact colonic crypts (isolated from rat colon) *in vitro* so that all effects were predominantly via changes in basolateral transport. With that being said, and since we did observe a basolateral Vitamin C effect as measured by changes in pH_i , these results show that there could be a direct difference in Vitamin C transport from cultured CaCo-2 cells and intact freshly isolated crypts. In our study Vitamin C could potentially be transported via SVCT2, or it could diffuse through the tight junctions to reach the apical side and interact with SVCT1, or alternatively there could be another protein transporter that has yet to be identified that allows Vitamin C to enter the cells.

The third outcome of our study is that GLUT transporters have a role to play in Vitamin C transport inside the cell and thereby could manipulate the effect(s) of Vitamin C. As shown in Fig. 7 10 mM Vitamin C still maintained an acidic pH ($\Delta\text{pHi}/\text{min}$ 0.0080) in the intracellular environment after removal of Glucose from the solutions.

When the drug Phlorizin was added to the glucose-free solutions to specifically bind and block SGLT transporters, Vitamin C still was able to change intracellular pH ($\Delta\text{pHi}/\text{min}$ 0.0081) in a similar pattern as previously seen. These results suggest that attenuating GLUT through glucose removal and blocking SGLT with Phlorizin did not modulate the Vitamin C effect on the recovery rate of pH to the normal physiological value. These results could mean that using Vitamin C as a therapeutic agent for colon cancer in diabetic patients would still lead to an elevation in intracellular acidity and potentially lead to reduced tumor burden.

Finally, we compared the influences of Glucose and Na^+ on Vitamin C-dependent proton extrusion rates. We observed that the pH_i recovery rate of 117mM Na is faster when we compared to control experiments of 77mM Na^+ concentration that contain 10 mM Glucose ($\Delta\text{pHi}/\text{min}$ 0.0169) with control experiments that have the standard concentration Na^+ and contain no Glucose ($\Delta\text{pHi}/\text{min}$ 0.0133) p value 0.0002. Remarkably, the rate of recovery become more aberrant when we add 10mM Vitamin C to the Glucose-free solution ($\Delta\text{pHi}/\text{min}$ 0.0080) with a p value of <0.0001 while there was no significant difference between 77 mM Na^+ control and 10mM Vitamin C with the same 77 mM Na^+ concentration in the extracellular environment. We believe that Na^+ concentrations have the major influences on Vitamin C-dependent proton extrusion (Fig. 9) through modulations in Na-Glucose exchange and Na-H Exchange.

In summary, our data for the first time demonstrate the acute effects of Vitamin C on modulating intracellular pH regulation in isolated colonic crypts. This data is highly suggestive that a positive effect of Vitamin C as an antitumorigenic agent during chronic exposure can also be achieved in the acute exposure setting.

Disclosure Statement

There are no competing interests or sponsors that affected this work. All studies are done in compliance with the Yale school of Medicine conflict of interest committee.

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