Abatement of Forskolin-induced Intestinal Fluid Secretion by Modulation of Intracellular Camp With Penicillin

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Research Article

Keywords: diarrhea, intestinal secretion, penicillin, antibiotic, fluid loss

Posted Date: July 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-699067/v1>

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Abstract

Millions of people die every year due to diarrheal related diseases, with infants and the elderly making up the majority of these deaths. Deaths are caused by excessive intestinal fluid and electrolyte secretion and are especially common in impoverished developing countries. Antibiotics have been classically used as a method to treat diarrhea-related pathologies by modulating the gut microbiome. We recently reported that penicillin may protect against disease-induced excessive fluid and electrolyte secretion via a genetics-independent, microbiome-independent mechanism in individual colonic crypt cells. In this study we investigated whether microbial-independent protective effects of penicillin against fluid secretion can be observed in the rat small intestine at the whole-tissue level. Here we report that penicillin has a significant dose-dependent protective effect against fluid secretion in induced models of diarrhea in the microbiome deficient rat small intestine. Penicillin can rapidly bring fluid secretion down to levels comparable to healthy controls. Our results suggest, for the first time, an alternative function for penicillin G as a cost-effective and fast-acting treatment against diarrheal symptoms without dependence on modulating the behavior of the existing gut microbiome.

Introduction:

Even though there are currently several medications to prevent and treat diarrheal diseases, diarrhea is still a leading cause of death amongst the infant and elderly populations in many developing countries [1, 2]. These deaths could be easily avoided given necessary means; therefore, there is a demand for a direct and low-cost treatment.

Diarrhea is caused by severe intestinal fluid and electrolyte secretion and can lead to death when paired with the inability to replenish the lost fluids and nutrients. The most common cause of diarrhea comes from pathogenic microbes that release of toxins (for example, cholera toxin) leading to cyclic-adenosine monophosphate (cAMP) activation of Cl^- secretion, which in turn stimulates release of water [3, 4]. Activation of cAMP targets the protein kinase A (PKA) pathway and activates membrane transporters such as the cystic fibrosis transmembrane regulator chloride channel (CFTR) and calcium-activated chloride channels (CACC) leading to downstream secretion of intracellular Cl^- [5, 6]. The complication with diarrheal diseases is that ion transport influenced by stimulation of Cl^- secretion results in excessive secretion of water [4, 7].

Recent studies show that antibiotics are a promising avenue for such a remedy. Current research report that azithromycin, fidaxomicin, levofloxacin and ciprofloxacin are viable, antibiotic therapies for acute watery diarrhea and dysentery [8]. Treatments employing the use of antibiotics and loperamide, an adjunct therapeutic, have shown to shorten the duration of the symptoms associated with traveler's diarrhea when compared to any of the antibiotics on their own [8]. Because of previous antibiotic usage as treatment for diarrheal diseases, we investigated penicillin's ability to abate intestinal fluid secretion to prevent acute diarrhea.

As an antimicrobial, penicillin is a beta-lactam antibiotic containing a four-membered beta-lactam ring which blocks the activity of transpeptidases which are enzymes that form peptide cross-linkages in bacteria [9, 10]. Previous studies demonstrated that Penicillin can increase intracellular cyclic nucleotide levels in mammals [11]. We recently reported that members of the Penicillin family function in a microbiome-independent manner to activate H/K ATPase in isolated rat colonic crypt cells by possibly modulating the cAMP pathway [12, 13]. Due to the ability of H/K ATPase to uptake potassium, Penicillin may provide a potassium rescue mechanism that prevents life-threatening electrolyte and fluid losses. Although antibiotics are associated with diarrhea by altering host microbiome, Penicillin may trigger a compensatory response to reabsorb electrolytes in the colon [13]. However, whether Penicillin offers similar protective effects in the mammalian small intestine is still unknown. Previous reports elucidated that forskolin (FSK), the first main labdane diterpenoid isolated from the Indian plant *Plectranthus barbatus* Andrews, increases intracellular cAMP and can in turn affect transmembrane ion transport [3, 14]. Thus, FSK-induced cellular fluid secretion has been used as a model for studying secretory diarrhea [15]. In this study, we used FSK to mimic diarrheal conditions in the rat small intestine.

Here, we report penicillin's ability to abate FSK-induced intestinal fluid secretion in the rat small intestine. Thus, there is use for penicillin, given acutely, to treat diarrheal disorders as a fast-response short-term alternative at the beginning of the therapeutic window to quickly modulate excessive intestinal fluid secretion.

Results:

Modeling Diarrheal Conditions with Forskolin in the Small Intestine

To determine the intraluminal fluid secretion under control conditions in the intestinal segments we examined the change in FITC-Inulin concentration in the lumen in the absence of FSK or penicillin G (PenG). Under these conditions there was a 17.67% decrease in FITC-Inulin concentration over the period of 80 min (Table 1, Fig. 1). The fitted slope of this decrease was $-0.2032 \mu\text{M FITC-Inulin/min}$. This reflects the resting or basal level of fluid secretion (control). The fluorescence decreases as it is diluted with fluid secreted into the lumen. We next examined the effect of adding FSK to the intraluminal perfusate. The addition of FSK alone resulted in a $\sim 35\%$ decrease in FITC-Inulin concentration at 80 min with a slope of $-0.5495 \mu\text{M/min}$ (Fig. 2A, Table 1). This reflects the increased fluid secretion elicited by FSK. FSK stimulated fluid secretion was significantly greater with almost a 2-fold decrease in FITC-Inulin concentration compared to control conditions (34.92% vs 17.67%) (Table 1 and Fig. 1b).

Table 1

Table of average percent decrease, average slope of change in FITC-Inulin, and their standard errors from the mean over 80 min.

Treatment Group	Average Percent Decrease in FITC-Inulin Concentration (%)	SEM of Percent Decrease in FITC-Inulin Concentration	Average slope of Change in FITC-Inulin Concentration ($\mu\text{M}/\text{min}$)	SEM of Slope of Decrease in FITC-Inulin Concentration
FSK	34.92*	± 5.168	-0.5495*	± 0.09989
0.25 mM Pen G.	24.18	± 7.298	-0.3783	± 0.1338
0.50 mM Pen G.	19.30	± 6.110	-0.2267	± 0.1003
1.0 mM Pen G.	21.98	± 2.649	-0.2622 [§]	± 0.04459
2.5 mM Pen G.	19.36 [§]	± 2.101	-0.2723 [§]	± 0.02928
5.0 mM Pen G.	16.95 [§]	± 3.238	-0.2133 [§]	± 0.04327
Healthy	17.67	± 1.874	-0.2032	± 0.02987
*Values were significantly different compared to Healthy group. P-value = 0.03 for average percent decrease and p-value = 0.02 for average slope.				
[§] Values were significantly different compared to FSK. For average percent change in FITC-Inulin, p-value = 0.02 at 2.5 mM Pen G. and p-value = 0.03 at 5.0 mM Pen G. For average slope, p-value = 0.048 at 1.0 mM Pen G., p-value = 0.029 at 2.5 mM Pen G., and p-value = 0.026 at 5.0 mM Pen G.				

Dosage Dependent Protective Effects of Penicillin G. Against Forskolin

In a separate additional series, we studied the effects of PenG on the FSK stimulated fluid secretion. (Penicillin G: 0.25 mM, 0.50 mM, 1.0 mM, 2.5 mM, and 5.0 mM). HRBS containing a single dose of PenG were perfused intraluminally to each of the small intestinal segments treated with FSK (10 μM). There was a significant dose dependent decrease in fluid secretion as the concentration of Penicillin G was increased. The addition of 0.25 mM PenG resulted in a 24.18% decrease of FITC-Inulin concentration with a slope of -0.3783 $\mu\text{M}/\text{min}$ (Figs. 2 and 3, Table 1). In the presence of 5.0 mM PenG (our maximum dose) we observed a 16.95% decrease at -0.2133 $\mu\text{M}/\text{min}$ in FITC-Inulin concentration (Fig. 2a, Fig. 2b, Table 1). This dose (5.0 mM) elicited the maximal effect on fluid secretion we obtained. With the addition of intermediate PenG concentrations (0.50 mM, 1.0 mM, and 2.5 mM PenG), there was a 19.30, 21.98, 19.36 percent decrease in FITC-Inulin concentrations, respectively, with slopes of -0.2267 $\mu\text{M}/\text{min}$, -0.2622 $\mu\text{M}/\text{min}$, -0.2723 $\mu\text{M}/\text{min}$, respectively. Although there was no significant difference between all treatment groups compared to basal conditions, shown in Fig. 3 are the FSK induced rates of change in FITC-Inulin

detection in the presence of PenG concentrations of 0.25 mM, 1.0 mM. When compared to 5.0 mM PenG the concentration of FITC-Inulin approaches that of control or “healthy intestinal” secretion across segments. This can also be seen in Figs. 2A, 2B and Table 1. We also analyzed the decrease in fluorescence as a function of PenG concentration. This is shown in Fig. 4 where we plotted the data in a nonlinear fit (inhibitor vs. response curve) using GraphPad. The IC_{50} of PenG is calculated from the Prism analysis fit to be 0.18 mM. The linear line in Fig. 4 is drawn to demonstrate the control level in the decrease in FITC-inulin with application of FSK and no PenG. Thus, PenG is able to counteract the induced fluid secretion produced with FSK. This would indicate that PenG could be useful to reduce the excess fluid secretion observed in diarrheal diseases (Figs. 3 and 4).

Discussion:

Throughout the history of medicine, many medications have been found to have alternative uses outside their originally intended function. In the field of Gastroenterology, current research has shown that the antibiotics azithromycin, fidaxomicin, levofloxacin, and ciprofloxacin all have been deployed as alternative therapies for cases of acute watery diarrhea and dysentery. In this study, we show that penicillin G, another commonly prescribed antibiotic, also has the potential to be repurposed and used as a therapeutic measure against cases of diarrhea and dysentery. A major consideration is also the cost savings using penicillin provides. A quick check of pharmacy prices shows azithromycin cost about \$1.50 per tablet while penicillin is about \$0.23 in the United States.

The breakthrough discovery of penicillin occurred in 1928 when it first became widely recognized as the original modern antibiotic [16]. Penicillin originally found its use as a cure for various infectious diseases, such as bacterial endocarditis, meningitis, pneumococcal pneumonia, and streptococcal septicemia [9]. However, since then, penicillin is no longer being used to treat these infections due to the rapid spread of bacterial resistance to penicillin. Although penicillin isn't being widely utilized to treat bacterial infections, it has found new uses as a treatment for sexually transmitted diseases, and penicillin G currently remains the drug of choice for treating all stages of syphilis (Treatment of sexually transmitted diseases, 1986).

Our data suggests another off-label use for penicillin G for not only treating bacterial infections, but also as a treatment for diarrhea and dysentery. Our study using an ex-vivo perfusion device, demonstrates that the intraluminal application of penicillin G provides a protective effect on the distal small intestine against FSK induced fluid secretion (mimicking diarrhea or dysentery). The significant effects of penicillin G perfusion as a means to abate the secretory effects of FSK suggest that this therapeutic may be used at the first signs of secretory diarrhea. Penicillin G has the potential to be a low-cost and easily accessible short-term alternative for diarrheal diseases intended for use in developing countries.

At the moment, Ciprofloxacin remains the most commonly used pharmaceutical in the prevention of diarrhea [17]. However, Ciprofloxacin has been shown to possess arrhythmogenic properties and is linked to the development of cardiac arrhythmias and an increased risk of tears of cardiac vasculature [18]. This makes the potential of Penicillin G as an antidiarrheal a safer, low-risk alternative that can also be used

as a treatment option for patients with pre-existing heart conditions, as it has no reported cardiac side effects, unlike present conventional antibiotic treatments.

Our study demonstrated that intraluminal application of penicillin G. provides a dose dependent protective effect on the distal small intestine against FSK induced fluid secretion. Penicillin doses down to 0.5 mM in the presence of FSK prevented fluid secretion with no statistical difference in fluid secretion compared to a healthy intestinal segment perfused with control solutions. The counteracting effects of penicillin G on FSK exposed tissue indicate the antibiotic's ability to prevent excessive fluid loss that is associated with exposure to FSK and cellular fluid loss due to cAMP upregulation [14]. Our results suggest an alternative function for penicillin G as a cost-effective, rapid low-risk therapeutic measure against diseases with diarrhea related symptoms with the small and large intestines as potential therapeutic targets.

Materials And Methods:

Animal Model

Male Sprague-Dawley rats (Charles River, Wilmington, MA) with weights between 275g and 410g were housed in rooms with controlled climate, humidity, and 24-hour light cycles. Protocols for animal handling, euthanasia, and tissue harvesting were approved by Yale University's Animal Care and Use Committee (Protocol #2018–10253). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines (<https://arriveguidelines.org/>). A total of 35 rats were used with 5 rats per experimental group.

Tissue Harvesting

Rats were euthanized via the overdose of the anesthetic Isoflurane (IsoThesia TM, 99%/mL) (Dublin, OH) in an enclosed chamber. Midline laparotomy was performed on rats and the distal small intestine, defined as 10 cm segments of the ileum closest to the cecum, was isolated [19]. Cold (4°C) HEPES-Ringer buffer solution (HRBS) (115 mM of NaCl, 5 mM of KCl, 1.2 of MgSO₄, 1 mM of CaCl₂, 10 mM of glucose, 2 mM of NaH₂SO₄, and 32.2 mM of HEPES at pH 7.4 and an osmolarity of 300 mOsm) was used to wash the lumen of each segment removing any residual intraluminal debris and to prevent ischemic injury [19].

Intestinal Perfusion

An ex-vivo perfusion device originally described by Munoz-Abraham et al in 2015 was used to perfuse intraluminal and extraluminal fluids at a rate of 6 mL/min (mimicking biological fluid flow rates) at 37°C [20]. In all experiments, 37°C HRBS was perfused both intraluminally and extraluminally to prolong the viability of tissues by mimicking the biological extracellular conditions of the gut environment. Powdered fluorescein isothiocyanate coupled with inulin (FITC-inulin) (Sigma Aldrich, St. Louis, MO) added to the intraluminal HRBS solution obtaining a concentration of 100 µM and was used as a fluorescent indicator for the extent of fluid secretion. Penicillin G at various dosages were also added intraluminally to different

experimental groups to observe the protective effects against FSK induced fluid secretion. FSK was used to increase levels of cAMP within the cell and as seen in diarrheal diseases. Extraluminal perfusion contained warm HRBS alone. The pH and osmolality (mOsm) of all perfused solutions were adjusted to 7.4 and 300 ± 5 respectively at 37°C.

Experimental Conditions

In this study, we define an experiment as the harvest of the distal small intestine segment for use on our isolated perfusion apparatus [21]. Three separate series of experiments were performed: Control, FSK-induced, and FSK + penicillin G (PenG). Five separate experiments were performed under control conditions with standard perfusate in the absence of both FSK and penicillin G. as negative control. The standard perfusate contained HRBS and Inulin-FITC intraluminally, and HRBS only extraluminally. Five separate experiments were conducted under diarrheal conditions (enhanced fluid secretion compared to control) with 10 μ M FSK added to the intraluminal control perfusate. As mentioned previously, FSK induces fluid secretion via the upregulation of cAMP [14]. Twenty-five experiments were performed under diarrheal conditions (10 μ M FSK) with various concentrations of penicillin G (0.25 mM, 0.50 mM, 1.0 mM, 2.5 mM, and 5.0 mM) introduced into the lumen of the small intestine (five experiments per dose, 5 samples taken at each time point for each tissue). The data from the FSK-induced and FSK + penicillin G groups were then compared to the control data.

Data Collection

The nanofluorometer (Nanodrop 3300, Thermo Fisher Scientific Inc., Wilmington, DE) recorded instantaneous fluorescence intensity, measured in arbitrary fluorescence units, of intraluminal FITC-Inulin. An increase or decrease in fluorescence intensity indicates an absorption or secretion by the small intestine, respectively. Starting from time zero, replicates of five intraluminal fluid samples were collected every 20 minutes for a total duration of 80 minutes. A standard curve correlating known concentrations of FITC-Inulin in the standard perfusate (made that day for the experiment) with corresponding fluorescence intensities was used to determine intraluminal FITC-Inulin concentrations. Basolateral perfusate samples were also taken and measured periodically to ensure that there was no leakage or perforation of the intestinal segment. If there was a fluorescent signal detected in the basolateral perfusate, the experiment was discarded as this was indicative of a leak in the intestine [22].

Data and Statistical Analysis

We used the software package GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA) to calculate the linear regression of change in FITC-Inulin concentration over time to determine the slope of each experimental condition. The average of each slope and standard error was then recorded and graphed as previously described [23]. The percent change in FITC-Inulin concentration of each experiment was determined by dividing the change in FITC-Inulin concentration over the 80-minute period by the initial FITC-Inulin concentration. The average percent change with its standard error was graphed for each experimental condition. Student t-tests and ANOVA analyses were used to determine significance,

comparing negative control with treatment groups and positive control. P-values < 0.05 were considered statistically significant.

Declarations:

Funding

Charles Ohse Award from the Department of Surgery at the Yale University School of Medicine.

Conflict of Interest

None of the authors have any conflicts of interest.

Authorship Contribution

EPC: Wrote paper, analyzed data, performed research.

JLO: Wrote paper, analyzed data, performed research.

JO: Wrote paper, analyzed data, performed research.

ZT: Wrote paper, analyzed data, performed research.

BAD: Wrote paper, analyzed data

JPG: Wrote paper, conceived and designed study, analyzed data

Ethics approval

Animal protocols approved by Yale Animal Use and Care Committee

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

Figure 1

The decrease in FITC-Inulin concentration illustrated as the slope (A) and concentration vs time (B) over 80 min with and without FSK.

Figure 2

The average percent decrease in concentration (A) and average slope of FITC-inulin concentration change (B) with 0.25 mM, 0.50 mM, 1.0 mM, 2.5 mM, and 5.0 mM Pen G.

Selected Comparisons

Figure 3

The change in intraluminal FITC-inulin concentration in the presence or absence (control) of FSK and FSK with PenG (0.25 mM, 1.0 mM and 5.0 mM).

Figure 4

The data from Figure 2 and Table 1 (change in FITC-inulin concentration) was fit using a nonlinear least square fit (inhibitor vs response analysis, GraghPad). The linear line was drawn to indicate the basal (control) activity in the absence of FSK and PenG.