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REVIEW ARTICLE

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Preclinical screening techniques for anti-diarrheal drugs: a comprehensive review

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ABSTRACT

Diarrhea is a global epidemic mostly common in developing countries, especially among children below 5 years. The side effects posed by conventional anti-diarrheal agents necessitate the screening and development of alternative agents. This review assessed the current experimental techniques involved in screening agents with promising anti-diarrheal properties. The various models described include *in vivo* model (gastrointestinal transit time using charcoal meal, castor oil-induced diarrhea, castor oil-induced enteropooling, magnesium sulfate-induced diarrhea, prostaglandin (PGE_2)-induced enteropooling, and serotonin-induced diarrhea), intestinal enteroids model (enterotoxigenic *escherichia coli*-induced diarrhea, enteropathogenic *escherichia coli*-induced diarrhea, and cholera toxin-induced diarrhea) as well as *ex vivo model* (involving the use of isolated jejunum or ileum from guinea pigs, rabbits, and rats). Innovative screening areas covered include enkephalinase, intestinal ion channels, inflammatory bowel disease associated diarrhea and farnesoid X receptor target. Advantages and disadvantages of these techniques were also highlighted. Application of these models would aid researchers in the discovery of alternative anti-diarrheal agents.

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Introduction

Diarrhea, a word derived from Greek (*dia*, through) and Latin (*rheein*, to flow or run), involves the passage of loose or watery stools that occurs at least three times daily [1]. It is characterized by an increased frequency of bowel (more than two stool above 200 g per day), watery stools, presence of blood in stools, steatorrhea, nausea, loss of appetite and weight, vomiting, and abdominal pains [2].

According to the World Health Organization, diarrhea had been reported to be the major cause of morbidity and mortality in several developing nations where about three to five billion cases of diarrhea occur each year with children below 5 years of age accounting for about one billion of these cases [3,4].

Diarrhea may be due to exposure of an individual to contaminated or wrong diet, which could lead to infection as well as disruption in the intestinal absorptive and secretory functions [4]. Other causes include bacterial infections, salmonella, Vibrio cholera, Shigella, enterotoxigenic Escherichia coli, *Campylobacter* spp., enteric parasites (such as *Cryptosporidium* spp., *Giardia lamblia, Entamoeba histolytica*, and *Blastocystis hominis*), virus, psychological stress, anxiety, and side effects originating from some medications [5–7].

Diarrhea could be acute, caused by parasitic infections that may last for 1–2 days or chronic,

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associated with functional disorders including irritable bowel syndrome, Crohn's disease which may last up to 1 month [8].

Some complications associated with diarrhea include growth retardation, malnutrition, impaired cognitive development, and performance. These are common in developing countries having limited resources [9]. Dehydration, hyponatremia, and hypokalemia may also occur from excess loss of water and electrolytes [8].

The treatment approach to diarrhea include the use of oral rehydration therapy, supplements (zinc sulfate, vitamin A, folate, magnesium, and copper), adsorbents (pectin, kaolin, activated charcoal, and lactobacillus sporogens), and other medications such as diphenoxylate, loperamide, bismuth subsalicylate, codeine, lidamidine, and rececadotril [4,7]. Gastrogard also serves as a prophylactic agent against diarrhea that is caused by retroviral infections in children [10]. The use of antibiotics is also an important remedy against infectious diarrhea [2].

Statement of Problem

Although conventional therapies play significant roles in the treatment of diarrhea, such medications posed some limitations such as; constipation, abdominal discomfort, headache, dry mouth, nausea, and vomiting [8].

The use of alternative approaches remains the solution to the limitations posed by the conventional anti-diarrheal drugs.

Alternative and traditional treatment approaches to diarrhea include the use of various parts of herbs in the form of infusion, decoction, and enema [9]. Other methods include massage, acupressure, and acupuncture [10].

Some advantages derived from the use of herbal medicine in the treatment of diarrheal include tolerability, high safety profile, low cost, acceptability, accessibility, and wide spread availability [11]. Even though some alternative medicines posed limitations such as lack of dosage instruction, toxicity associated with some wild herbs, risk of interaction with other conventional drugs, and lack of regulation [10], the search for natural alternative anti-diarrheal molecules still remains the current scientific target.

The goal of discovering these alternative potential anti-diarrheal agents can be achieved through laid down experimental techniques.

To our knowledge, there is paucity of detailed and updated techniques in the screening of promising agents as alternatives in the treatment of diarrhea. This present review addressed this problem.

Materials

Materials such as apparatus, equipment, and chemical used in the screening of anti-diarrheal agents have their unique role to play based on the models and techniques that are involved.

Experimental animals

Experimental animals such as rats, mice, rabbits, and guinea pigs used for these screening should be handled in accordance with the ethical guidelines on the use of laboratory animals for research purpose.

Apparatus and equipment

These include organ or tissue baths, isometric transducer coupled with power lab data acquisition system attached to a computer or a digital recorder, water bath, top and analytical weighing balances, metabolic cages, fabricated cages, oral gavages (orogastric tubes), animal cages, hand gloves, syringes (1, 2, and 5 ml), dissecting kit, transparent meter rule, stop watch (timer), test tubes, graduated cylinder (5–10 ml), 37°C incubator with 5% CO_2 and 95% humidity, well plates, and among others.

Chemicals and reagents

These include atropine sulfate, acetylcholine (ACH), histamine (H), castor oil, Loperamide, Medicinal charcoal (activated charcoal), carboxymethylcellulose, tween-80, tragacanth powder, yohimbine, phentolamine, pilocarpine, propanolol, physiological salt solution (PSS, Tyrode's solution), anesthetic agents such as chloroform, diethyl ether, ketamine, and among others.

Other materials

These include cell lines, cholera toxins, distilled water, paper tape (for labeling), marker pen (for labeling of animals), beddings (sawdust or wood shavings for keeping animal cages clean), notebook or spreadsheet (for results tabulation) and statistical software(s) for data analyses, interpretation, and result presentations.

In Vivo Model of Screening Anti-diarrheal Drugs

This involves the use of whole animals (intact organisms such as rat, mice, and rabbit) to find out if a test substance has the potential to inhibit diarrhea. This

model has characteristics resemblance to human diarrhea because it provides the likely clinical effect when compared to isolated tissue or organ experiments, which eliminate pharmacokinetic and pharmacodynamics interactions. Various techniques in this model include gastrointestinal motility/transit time test using charcoal meal, castor oil-induced diarrhea, castor oil-induced enteropooling, magnesium sulfate-induced diarrhea, prostaglandin (PGE_2)-induced enteropooling, and serotonin-induced diarrhea. Adequate animal handling skills are required to achieve the objectives of these models. Some merits and demerits of these techniques are given in Table 1.

Gastrointestinal transit time using charcoal meal

Principle

During diarrhea, peristalsis is enhanced and absorption is diminished. Charcoal meal in this model serves as a marker or tracer to monitor the level of peristalsis that occurs in the intestine when a substance is administered. The lesser the distance travelled by charcoal meal, the lesser the peristalsis. Thus, agents that inhibit propulsive movement or gastrointestinal transit of charcoal meal could be spasmolytic and capable of reducing the frequency of stool associated with diarrhea [12]. The use of atropine sulfate (anti-cholinergic agent) as a reference anti-diarrheal agent is due to the fact that the hyper-motility is mediated by the cholinergic system [12].

Procedure

Mice and rats are the common specie used in this technique. Animals are deprived of feed between 12 and 18 hours (mice, 12 hours and rats, 18 hours), but with free access to water. They are weighed, labeled, and randomly allotted into groups of 5–10 animals each. The grouping should involve a vehicle group (negative control), standard anti-diarrheal group (positive control), and test groups (graded doses of the test substance). Animals in the vehicle group are administered with 5–10 ml/kg of the vehicle (distilled water or normal saline or 5% tween-80, etc.). Animals in standard group are administered with a reference anti-diarrheal agent such as loperamide 2–5 mg/kg (p.o.) or atropine sulfate, 3–5 mg/kg, IP, or other reference drug of choice. The test group(s) should be treated with at least two doses of the test drug(s) p.o. or i.p. [12].

One hour after treatment, 0.5 ml of 5% solution of charcoal suspension (prepared in a boiling solution of 2% carboxymethycellulose or in 10% tragacanth powder) is administered orally to each animal. Thirty minutes later, animals are sacrificed by cervical dislocation and their abdomen is cut open and the intestinal segment is carefully removed from the pyloric sphincter to the cecum [12]. Distance travelled by the charcoal meal from the pylorus to the caecum is measured with the aid of a graduated transparent meter rule and is expressed as a percentage of total distance from the pylorus to the cecum. Preferably, the intestine of each animal can be immersed in formalin to arrest peristalsis and then washed in clean tap water before measuring the distance travelled by the charcoal meal.

Charcoal movement is expressed as a peristaltic index (PI) as follows:

$$\text{PI: } \frac{A}{B} \times 100$$

where A = distance travelled by charcoal meal and B = length of full intestine.

Percentage inhibition is also calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{APIC} - \text{APIT}}{\text{APIC}} \times 100$$

where APIC = average PI of control and APIT = average PI of test group [4,13,14].

A modification of this method was recently made by Mulugeta et al. where animals (mice) were administered castor oil (0.5 ml, p.o.) 1 hour after treatment. Then 1 hour later, animals were administered 1 ml of 5% activated charcoal meal suspended in distilled water and animals were sacrificed after 30 minutes [7]. In this review, we recommend that treatments should be applied 30–60 minutes prior to castor oil or charcoal meal administration.

Merits: This technique is simple and less time consuming compared to other techniques.

Demerits: Charcoal meal dissolved in distilled water can obstruct or clog oral gavage, thereby making it difficult to administer the drug to animals. For easy administration, it is recommended to prepare the charcoal suspension using 2% carboxymethycellulose or 10% tragacanth powder.

Castor oil-induced diarrhea

Principle

Castor oil is a practical diarrhea inducer that had been used in experimental models of screening anti-diarrheal agents. Following the administration of castor oil, it is hydrolyzed by lipase in the upper part of the small intestine to ricinoliec acid (the active

form) which causes local irritation, inflammation of the gut, release of prostaglandin leading to intestinal hyperactivity, and fluid hyper-secretion. This will prevent water and electrolytes absorption, reduction in activity of sodium potassium–ATPase in the intestine, and finally, diarrhea manifestation [1,4,15,16]. Agents that can inhibit electrolytes permeability and secretion can prevent castor oil-induced diarrhea [5].

Procedure

Animals are allocated into groups (control, standard, and tests) and fasted for 12–18 hours. Loperamide (2–3 mg/kg, p.o.) could be used as a reference drug. Aleem and Janbaz [12] used 10 mg/kg of Loperamide in their study. One hour after treatment, castor oil (5 ml/kg, 10 ml/kg, or 0.5 ml per animal) is administered via the oral route and animals are individually placed in metabolic cages whose floors are lined with clean white filter paper [3]. Onset of diarrhea should be noted [7]. Observation should be done for 4–6 hours. Animals are removed from their cages and weight of feces is obtained by subtracting the weight of filter paper from the weight of feces and filter paper.

In a study carried out by Mahmood et al. [11], Verapamil (50 mg/kg) and Loperamide (10 mg/kg) were used as standard drugs. In a study by Adeniyi et al. [17], four receptor blockers, which inhibit motility and secretions from the gut, Prazosin, 1 mg/kg (an alpha adrenergic receptor blocker), Propanolol, 10 mg/kg (a beta adrenergic receptor blocker), nifedipine, 2.5 mg/kg (a calcium channel blocker), and atropine (a muscarinic receptor blocker), were used as reference drugs.

Diarrhea can be graded as follows:

Normal pelleted feces (0), discrete soft-formed feces (1), soft-formed feces (2), soft watery stool (3), and watery stool with little solid matter (4).

Percentage diarrhea inhibition for wet and dry feces can be determined using the following formula:

$$\%\text{age Inhibition} = \frac{\text{AWFC} - \text{AWFT}}{\text{AWFC}} \times 100$$

where AWFC = average weight of feces in the control group and AWFT = average weight of feces in the test group [15].

Merits: This technique is simple to carry out.

Demerits: This technique takes longer observation duration (4–6 hours) compared to charcoal meal test. It may be difficult to procure sufficient numbers of metabolic cages for this study. Alternatively, fabricated cages whose bottoms are lined with white paper can be used. Also, animals (mouse or rat) can be placed on an already weighed filter paper and a 250, 500, or 1,000 ml beaker (depending on the size of the animal) is placed over the filter paper and the animal. A weight can be placed on the top of each beaker to prevent the movement of animals from their positions. The open end of the beaker is meant to provide aeration for the animal. The filter paper is usually replaced every 60 minutes for a period of 4–6 hours.

Castor oil-induced enteropooling

Principle

This technique is similar to that of castor oil-induced diarrhea. The difference is that animals used in the model are sacrificed and the fluid and electrolyte content of the small intestine are measured. It is not based on intestinal hyper motility but enteropooling (accumulation of fluid in the small intestine). The lesser the volume of intestinal content, the more the anti-diarrheal activity produced by the test agent. Rats or mice can be used in this model.

Procedure

Animals are allocated into groups (control, reference, and tests) of 5–10 animals each and are usually fasted 12 hours prior to the experiment. Animals in the control group receive the vehicle, animals in the reference group receive a standard anti-diarrhea agent (Loperamide, 2–5 mg/kg) while animals in the test groups receive graded doses of the test drug whose anti-diarrheal properties is to be evaluated. After 1 hour of treatment, castor oil (2 ml/kg or 0.5 ml per rat/mouse) is administered by the oral route (p.o.). One hour later, animals are sacrificed by cervical dislocation and both ends of the small intestine of each animal are tied with thread to ensure that the content does not spill out. The tied intestine is weighed, its content is emptied into a graduated cylinder and its volume is measured. The emptied intestine is weighed and the difference between the empty and intact intestine is used to calculate percentage inhibition (reduction in fluid accumulation) of intestinal secretion relative to control group using the formula as follows:

$$\%\text{age Inhibition} = \frac{(\text{A} - \text{B})}{\text{A}} \times 100$$

where A = average volume or weight of intestine in control group and B = average volume or weight of intestine in test groups [4,14,15].

Merits: This technique is simple to carry out and does not require the use of metabolic cages when compared to castor oil-induced diarrhea.

Demerits: Little or none.

Magnesium sulfate-induced diarrhea

Principle

This technique involves the use of magnesium sulphate to induce diarrhea. Diarrhea induced by magnesium sulfate is due to osmotic imbalance, increase in electrolyte secretion, and prevention of water and electrolyte absorption [13].

Magnesium sulfate also enhances the release of cholecystokinin from duodenal mucosal thereby promoting secretion and motility of the small intestine [13]. Thus, agents that could prevent increase electrolyte secretion caused by the magnesium sulfate could be useful anti-diarrhea agents [5].

Procedure

In this technique, animals are fasted for a period of 12–18 hours and are grouped into control, reference, and test groups. After 1 hour of treatment, animals are dosed with magnesium sulfate (2 g/kg, p.o.) and are housed in their separate cages for 4 hours [2,5,8].

In a study by Ramdas et al. using this technique, magnesium sulfate (2 g/kg, p.o.) was administered 30 minutes before treatment [13]. This is a curative approach unlike the preventive approach where the test substance is administered before induction of diarrhea.

Merits and demerits: These are similar to those in castor oil-induced diarrheal.

Prostaglandin (PGE_2)-induced enteropooling

Principle

The presence of digested food in the intestine stimulates the absorption of water and electrolytes. PGE_2 induces diarrhea by inhibiting absorption of glucose, thereby resulting in accumulation of fluid in the intestinal lumen. PGE_2 agonists act on prostaglandin receptors coupled to G-protein that makes use of inositol triphosphate (IP_3), diacylglycerol (DAG), or cyclic adenosine monophosphate (cAMP) transducer mechanism [1]. Activation of E-type prostanoid receptor-1 (EP1) causes contraction of smooth muscles via IP_3/DAG or cAMP, which results in secretion of water and electrolytes [18]. Agents that have the potential to inhibit the activity of prostaglandins could be useful in preventing the enteropooling effect of PGE_2 [5].

Procedure

In this technique, prostaglandin serves as a diarrhea producing agent. Animals are fasted for a period of 12–18 hours and are grouped into control, reference, and test groups. Prostaglandin may be administered immediately or 1 hour after treatment. One hour later, animals are sacrificed and the small intestine (from the pylorus to the caecum) is isolated and its content is measured [2,18]. In a study conducted by Bimlesh et al. [1], 100 µg/kg of PGE_2 was used to induce diarrhea in rats 1 hour after treatment. In a study carried out by Lakshminarayana et al. [5], PGE_2 (100 µg/kg) was administered to animals immediately after treatment with test and standard drugs.

Merits: This technique is simple, time saving and does not involve the use of metabolic cages.

Demerits: Little or none limitations.

Serotonin-induced diarrhea

Principle

Serotonin, 5-hydroxytryptamine (5-HT) plays a significant role in the gastrointestinal tract due to its high level of distribution in the enterochromaffin cells of the gastrointestinal mucosal epithelia and enteric neurons. It is involved in smooth muscle contraction, regulation of gastrointestinal motility, gastric acid secretion, and pancreatic fluid secretion as well as intestinal chloride secretion [19]. Agents that could prevent this form of diarrhea may be useful in the treatment of serotonin-mediated diarrhea.

Procedure

Animals are fasted for 12–18 hours and are randomized into groups (control, reference, and test). After 30 minutes of treatment, animals are administered with serotonin (600 µg/kg, i.p). Animals are kept in separate cages and observed for diarrhea for a period of 4–6 hours [8].

Merits and demerits: These are similar to those in castor oil-induced diarrhea.

Intestinal Enteroids Model of Screening Anti-diarrheal Drugs

This model evaluates the structure and function of the normal intestinal epithelium. This technique is

useful in investigating agents with anti-diarrheal activities against *E. coli* using high-throughput process [20].

Here, stem cells isolated from the intestinal crypt of mouse or human are propagated using a three-dimensional culture platform that gives resemblance of the normal intestinal mucosa with interest, paint, goblet, and entero-endocrine cells that are self-organized into the crypt- and villus-like structures [21,22]. Enteroids prepared from rodents or human intestine would help to prevent or minimize interspecies limitations involved in translating animal results to human [23].

Cells derived from human and mouse can efficiently replicate the pathophysiological signaling mechanisms associated with secretion caused by the cholera toxin, heat-labile enterotoxins, and rotavirus [20,21]. This had provided an exclusive avenue in the search for agents against enterotoxigenic diarrhea [24–26]. They also operate based on receptor signaling in various channels [23,26].

Enterotoxigenic E. coli-induced Diarrhea technique

Among the six categories of *E. coli* (*Enterotoxigenic E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and enterodispersion *adhesive E. coli*), ETEC is the leading cause of bacterial diarrhea in humans and farm animals. This occurs via colonization of the small intestine as well as production of enterotoxins which alters the small intestine's function [2].

A preliminary study is usually carried out by challenging animals with various concentrations of bacterial cell suspension adjusted to different McFarland standards to obtain a final density of approximately 5, 3, 2, and 1×10^8 CFU/ml, respectively. This would help to determine the effective concentration of bacteria that can cause diarrhea. Clinical signs of diarrhea are identified within 72 hours after infection [27].

In the main study, animals are randomized into groups, control (normal and negative controls), reference, and test groups. Animals (usually mice) are intraperitoneally injected with bacteria, 0.25 mL/10 g of 10^8 CFU/ml of *E. coli*. After 3 hours, animals are treated with the vehicle, reference, and test drugs. Treatment can be done twice daily for 5 consecutive days. Body weights are also monitored daily. After the fifth day, the treatment is repeated once and animals are placed in their individual cages for fecal examination (frequency of diarrhea). The animals are euthanized using anesthesia before necropsy is carried out. Blood samples can also be collected into ethylenediaminetetraacetic acid (EDTA) tubes for WBC count. Sera obtained from whole blood can be used for tumor necrosis factor alpha (TNF-α) and interleukin assays. Cecum microflora content is also collected under a sterile condition for the determination of the number of bacteria present. Anaerobic atmosphere (80% N_2, 15% CO_2, and 5% H_2) without agitation is required for this procedure. Viable counts of organism per gram of feces are calculated and expressed as log CFU/ml [28].

Pathogenic (E. coli)-induced diarrhea technique

EPEC is a major causative organism of diarrhea in children. Its resistance to available anti-biotics had revitalized the need to search for alternative agents.

In this technique, bacterial strain (*E. coli*) can be procured from patients suffering from gastrointestinal disorders. Procured organisms are grown to lag phase before being inoculated in the liquid broth medium for activation. Cultures (0.1 ml) are inoculated in 10 ml broth media. Then, 0.5 ml of standard inoculum is spread on an agar with the aid of sterile spreader and allowed to dry. The test sample is introduced at various concentrations into the bored agar in a plate. The mixture is allowed at room temperatures for proper diffusion to take place. Incubation is carried out at 37°C for 24 hours and thereafter the inhibition zone diameter is measured [29].

Alternatively, the total plate count method can be used to determine the number of viable bacteria. In this method, 0.1 ml of a suspension mixture of the test sample and bacteria inoculums is used for re-plating on Muller–Hinton agar plates. Various dilutions (concentrations) of sample (0.1 ml) are spread on Muller–Hinton agar and then incubated at 37°C for 24 hours [30]. Minimum inhibitory concentration can be determined using agar well diffusion assay method [14,31].

Merits: This technique is simple to carry out and does not involve the use of experimental animals. It promotes easy translation of *in vitro* results to humans because such pathogenic organisms are from human source.

Demerits: This technique is only useful for pathogenic diarrheal and does not evaluate anti-motility or anti-secretory potentials of a test substance. Thus, it may not be sufficient to validate the anti-diarrheal properties of an agent.

Cholera toxin-induced diarrhea

This model is useful for the evaluation of cholera associated diarrhea. Lyophilized powder of cholera toxin (1 mg) is suspended in 5 ml of sterile water and serial dilutions are made to a concentration of 10 µg/ml. Animals (usually mice) are randomized into groups (control, standard, and test groups) and 0.5 ml of 10 µg/ml of cholera toxin is administered to mice (p.o.). Racecadotril (100 mg/kg) is administered to mice. Animals are placed individually in cages lined with absorbent paper and are observed for a period of 4 hours for fecal droppings. Fecal outputs are weighed and percentage inhibition of diarrhea is calculated [6].

Merits and demerits: This technique is simple to carry out. It involves little or no limitations.

Ex Vivo Model of Screening Anti-diarrheal Drugs

This involves the use of isolated tissue segments (ileum or jejunum) of rabbits, guinea pigs, or rats to evaluate the anti-diarrheal potential of test substances. This technique is useful in evaluating anti-motility mechanism(s) of an anti-diarrheal agent. Merits and demerits of these techniques are highlighted in Table 1.

Principle

The gastrointestinal motility, which is regulated by the enteric nervous system, is mediated by several transmitters such as acetylcholine (ACH), histamine, 5-hydroxytryptamine (5-HT), bradykinins, prostaglandins, substance P, and cholecystokinins. The activation of these transmitters causes elevation in cytosolic calcium level [32,33].

It had been reported that calcium plays a significant role in producing smooth muscle contraction. Acetylcholine-induced smooth muscle contraction occurs via the inositol triphosphate (IP_3) pathway which causes the release of calcium from the sarcoplasmic reticulum [4]. During membrane depolarization due to stimulation by agonists or mechanical activation, there is a release of cytoplasmic calcium, which binds to calmodulin and then activates myosin light chain kinase, which initiates contraction. Calcium from intracellular storage also has a role to play in smooth muscles contraction [4].

Histamine released from the enterochromaffin-like cells present in the fundic mucosa also plays a significant role in contracting smooth muscles, including those in the gut [34].

Table 1. Advantages and disadvantages of anti-diarrheal screening techniques.

Model	Advantages	Disadvantages
Gastrointestinal transit time test	It is simple, less technical and less time consuming. It does not require the collection of fecal matter.	It is useful only for studying agents with anti-motility activity. It requires additional studies to elucidate mechanism of action.
Castor oil-induced diarrheal	It is simple to carry out.	Several cages are required to collect and measure fecal output. It is not useful for studying agents with anti-motility activity.
Castor oil-induced enteropooling	It is simple to carry out. Animals do not need to be housed separately to measure fecal output.	None or little limitations.
Magnesium sulfate-induced diarrhea	It is simple to carry out.	Several metabolic cages may be required for all animals.
PGE_2-induced enteropooling	It is simple to carry out.	None or little limitations.
Serotonin-induced diarrhea	It is simple to carry out.	None or little limitations.
Intestinal enteroid techniques	It requires no or fewer number of animals.	Results translation to intact (*in vivo*) condition may be difficult.
Ex vivo model (isolated tissue)	It is anti-motility mechanism based. Isolated tissue segments from one animal can be used for several experiments when properly isolated, aerated and preserved in PSS.	Tissue handling requires expertise. Some isolated tissues may fail to respond. Isolated organ bath coupled to a computer acquisition system may not be available in some laboratories.
EPEC-induced diarrhea technique	It is cheaper and does not require the use of animals.	None or little limitations.
Cholera toxin-induced diarrhea	It is simple, less costly and less technical.	None or little limitations.
Other innovative models highlighted	They are useful for better understanding of the mechanism (s) of anti-diarrheal activity of test agents.	Some equipment, chemicals and reagents are difficult and expensive to access in some countries and laboratories.

Preparation of isolated tissues

Animals are deprived of food, but with free access to water for 24 hours before the study. They are sacrificed by cervical dislocation and exsanguinated to ensure that blood does not flow into the visceral organs and tissues to be isolated. The abdominal cavity is opened up and segments of the small intestine (ileum and jejunum) are dissected from adhering mesentery. While some tissue segments are aerated and preserved with PSS (Tyrode's solution) for later use, a 2–3 cm portion is fixed in the organ or tissue bath immediately. Fixation is done in such a way that the upper portion is tied to the isometric force transducer lever with the aid of a needle and cotton thread while the lower portion is attached to the hook of the organ bath in the opposite direction to the upper portion. The 20–25 ml tissue bath housing the tissue should contain Tyrode's solution that is aerated with air or carbogen gas, 95% O2, and 5% CO_2. The system is allowed to stabilize for a minimum of 30 minutes to ensure proper acclimatization of the tissue to the new environment. During this process, the tissue undergoes rhythmic contraction [4]. Composition of Tyrode's solution per liter include sodium chloride, NaCl (8.0 g), potassium chloride, KCl (0.2 g), calcium chloride, $CaCl_2$ (0.2 g), sodium carbonate, $NaHCO_3$ (1.0 g), magnesium chloride, $MgCl_2$ (0.1 g), sodium hydrogen phosphate, Na_2PO_4 (1.0 g), and glucose (2.0 g). Components of the Tyrode's solution have their various functions on the tissue. Calcium chloride aids in tissue contraction, glucose provides energy to the tissue, NaCl, $MgCl_2$, and KCl aid in depolarization and repolarization of the tissue while $NaHCO_3$ and Na_2PO_4 serve as a buffer to the tissue [34].

Effect of the test samples on isolated tissues

Before introducing the test sample into the tissue, it is important to do some preliminary studies by adding graded concentrations of acetylcholine (ACH) or histamine (H) consecutively. This would help to ensure that the viability of the tissue is established. This is necessary because some isolated tissues may not respond due to errors in their preparation. This preliminary study would also aid in establishing a dose response curve for ACH and H [12]. In the case of rat or rabbit ileum or jejunum, ACH can the used as an agonist, while histamine can be used as an agonist for guinea pig ileum [33].

Having established a dose response curve for ACH or H, the tissue is washed off of the initial drugs and allowed to rest for 15–30 minutes. Thereafter, various concentrations (1, 2, 4, 8, 16, 32, 64, 128, and 256 µg/ml) of the test drug are interacted with the tissue to establish its spasmodic or anti-spasmodic effect. The tissue is washed off and allowed to rest for 15–30 minutes. This is followed by the interaction of the test drug with the tissues in the presence of sub-maximal concentration of ACH or H that elicited contraction in the preliminary studies. The contact time of each concentration of the test drug on the tissue should be between 15 and 60 seconds depending on the speed of the setup [34].

Changes in tension produced by ACH or histamine and the test substance are recorded isometrically with the aid of a transducer coupled to a data acquisition system connected to an output display [4,12].

Pheniramine maleate, ketanserin, and atropine could be used as histamine, 5-HT_3, and Acetylcholine antagonists, respectively. Application of atropine to isolated ileum is meant to abolish the contractile effect of acetylcholine [33]. 5-HT_3-induced contraction of smooth muscle in animals can be inhibited by ketanserin. Histamine (H1)-induced smooth muscle contraction in guinea pig ileum can be inhibited by pheniramine maleate. Most H1 antagonist had been reported to also inhibit ACH receptors in the intestinal tissues [33,35].

Effect on *K*-induced contraction

In order to evaluate whether the spasmolytic activity of the test drug is due to calcium channel blockade, a high concentration (>30 mM) of potassium (80 mM) in the form of KCL is added to the tissue. In the presence of high concentration of potassium, a sustained contraction is expected to take place due to opening of voltage gated calcium channel [11].

Thereafter, various concentrations of the test drug are added in a cumulative manner to find out whether a concentration dependent inhibition will be produced by the test drug. Test substance that would inhibit potassium-induced contraction may have calcium channel blocking activity [11].

This can be further confirmed by allowing the tissue to get acclimatized to a calcium-free Tyrode solution containing 0.1 mM of EDTA. Here, potassium is used to replace calcium in the fresh Tyrode solution. After 30 minutes of incubation, a control concentration response curve (CCRC) of calcium chloride ($CaCl_2$) is established. Thereafter, various concentrations of the test drug are interacted with the CCRC [12,36].

Merits: It is easy to achieve an extreme condition that does not require hormonal and biochemical interactions. Segments of isolated tissue from the same animal can be used for several experiments provided that they are well aerated in a PSS. The use of metabolic cages is not required in this experiment, thereby making it less expensive and tasking. Very few numbers of animals (1 or 2) are required in this model.

Demerits: High technicality is required in the preparation of isolated tissues. Some isolated tissues may undergo fatigue or death due to insufficient supply of oxygen. Medical oxygen (carbongen gas, 95% oxygen, and 5% carbon dioxide) may not be readily available in some laboratories. There may be difficulty in translating results of these experiments to *in vivo* condition where whole animal tissues, organs, and hormonal systems function homeostatically. Also, pro-drugs may be discarded in *ex vivo* conditions because pharmacokinetic and pharmacodynamics features are not involved. Also, any interruption in power supply (electricity) may hamper the success of the experiment. Modern isolated organ baths may not be available in some laboratories that still rely on obsolete equipment (smoke drums/kymographs) due to poor funding.

Innovative Techniques of Screening Anti-diarrhea Agents

Recent advanced techniques had paved way in the discovery of anti-diarrheal agents and their possible mechanism(s) of action(s).

Enkephalinase inhibition as a potential drug target for secretory diarrhea

Natural and systemic opiates such as enkephaline, morphine, codeine, and loperamide have long been known to inhibit intestinal fluid and electrolyte secretion thereby possessing anti-diarrheal activities [39]. Enkephalin apart from being an endogenous opioid is also a neurotransmitter in the enteric nervous system and exerts its anti-secretory effects through δ-receptors, which when activated inhibits the action of the adenylate cyclase.

The actions of enkephalins are rapidly inactivated through degradation by neuropeptidase–enkephalinase. It is therefore logical that increasing the half-life of the enkephalins could be of therapeutic advantage in potentiating the anti-secretory effect of enkephalin. The drawbacks posed by older anti-diarrheal agents such as constipation effects can be overcome through enkephalinase inhibition.

Enkephalinase inhibition assay

Principle

Fluorometric quantification of enkephalinase activity is based on the principle that the enzyme cleaves Gly-Phe(pNO2) peptide bond of the substrate dansyl-D-Ala-Gly-Phe(pNO2)-Gly (DAGNPG) leading to a fluorescence increase related to the disappearance of intramolecular quenching of the dansyl-fluorescence by the nitrophenyl residue [37]. Decrease in fluorescence relative to blank is used for monitoring enzyme inhibition.

Enzyme source

Fresh rat kidney can serve as the source of enkephalinase enzyme. The kidney is homogenized in 10 ml of cold 0.05 M Tris-HCL buffer, pH 7.4. The homogenate is centrifuged for 5 minutes at 1,000 g. The pellet is discarded and the supernatant centrifuged at 60,000 g for 60 minutes. The resulting pellet is re-suspended in 50 mM Tris-HCl buffer, pH 7.4.

Experimental procedure

The assay is carried out using DAGNPG as substrate at 37°C. A 0.1 ml of 50 nm Tris-HCL buffer, pH 7.4 containing 50 μm DAGNPG is pre-incubated for 15 minutes at 37°C. Graded concentrations of the test substance (inhibitor) are prepared by dissolving them in the same buffer as the substrate. Racecadotril can be used as reference inhibitor (positive control). Both samples and standards are to be prepared in triplicate of 0.1 ml. The reaction is initiated by the addition of 50 μM Captopril. The reaction tubes are incubated for 30 minutes in a water bath with constant shaking. The enzymatic reaction is stopped by boiling at 100°C for 5 minutes. The reaction mixture in each tube is then diluted with 1.35 ml of Tris-HCL buffer and centrifuged at 500 g for 30 minutes. An aliquot of 1 ml of the supernatant is transferred to the spectrofluorometric cells and read at 562 nm with excitation wavelength of 342 nm.

Percentage inhibition of enzyme activity is calculated.

The IC_{50} can be determined from regression analysis of the plot of percentage inhibition against concentration. Drug candidates with good potency comparable to standard can further be evaluated *in vivo* using any model of secretory diarrhea such as castor oil-induced diarrhea.

Merits and demerits: This technique can screen several samples. Thereafter, samples with enkephalinase inhibitory activities are selected for *in vivo* studies using rodents. Non-availability of assay kits and modern facilities in some developing countries are limitations to this technique.

Intestinal ion channels as anti-diarrheal screening target

Secretory diarrhea can be triggered by increase in permeability of luminal chloride (Cl^-) channel which result to a parallel decrease in intestinal sodium absorption [38].

Improper homeostasis of the intestine could lead to too much secretion of sodium and chloride ions thereby resulting in the accumulation of water in the lumen which manifests as diarrhea.

At the basolateral membrane, chloride ion is transported into the cell through the aid of $Na^+/K^+/2Cl^-$ co-transporter, which is mediated by Na^+ and Cl^- concentration gradients developed by Na^+/K^+/ ATPase at the basalateral potassium channels. Establishment of this gradient leads to secretion of chloride across the luminal membrane of Cl^- channels which mainly involves the cAMP-activated channel cystic fibrosis transmembrane conductance regulator (CFTR) and CA-activated Cl channels (CaCC) [39].

Thus, inhibition of the enterocyte chloride channels would serve as a target of screening agents against secretory diarrheal and agents with chloride inhibitory potential could serve as adjunct in diarrhea therapy [38]. This technique is useful in studying the malabsorptive or anti-secretory mechanism of an agent [40].

Agents that could modulate intestinal chloride transport facilitated by the CFTR and CaCC chloride channels can be subjected to high-throughput screening against chloride channels before testing the promising agents in animal models when the need arises.

Procedure

This involves the use of cell lines, such as Fischer rat thyroid epithelium cells stably co-transfected with the YFP-H148Q, fluorescence protein and human wild-type CFTR cDNA or Anoctamin 1, ANO1, complementary DNA, cDNA, and HT-29 cells, which should be cultured using appropriate media and incubation in 5% CO_2 at 37°C and 95% humidity before they are used for iodide influx fluorescence study and short-circuit current measurement as described by Jiang et al. [41].

Some herbal products had been identified to prevent CFTR and CaCC chloride conductance during *in vitro* studies as well as mouse models of cholera retroviral diarrhea [38].

In vitro studies revealed that crofelemer, a heterogenous proanthrocyanidin from the bark latex of *Croton lechleri* elicited high inhibition on CaCC and less inhibition of CFTR [42].

Alternatively, the use of HT-29/B6 cells and human colonic biopsies can be applied in screening for reduction in chloride secretion *in vitro*. The test substance can be screened on basal and on forskolin- or cholera toxin-induced Cl^- secretion. This can be achieved by measuring short-circuit current (I_{SC}) and tracer fluxes of Na^+ and Cl^-. Also, para- and transcellular resistances are determined by two-path impedance spectroscopy. Measurement of enzymatic activity of the Na^+/K^+-ATPase as well as intracellular cAMP levels (ELISA) are also carried out. Study by Schulzke et al revealed that the root extract of a South African plant, *Xysmalobium undulatum elicited anti-diarrheal effect by inhibition of* active chloride secretion via Na^+/K^+-ATPase inhibition and reduction in intracellular cAMP responses and paracellular resistance [40].

Merits: This technique can screen several samples. Afterward, promising samples are subjected to *in vivo* studies in rodents when the need arises.

Demerits: High technicality is involved. It is also expensive to procure the assay kits and other materials required for this assay.

Screening of drugs against inflammatory bowel disease (IBD) associated diarrhea

Inflammatory bowel disease (IBD) is characterized by chronic inflammatory dysfunction of the intestine resulting from unknown causes. Patients with IBD develop diarrhea as a symptom apart from rectal bleeding, abdominal pain, weight loss, and vomiting. Studies had shown that inflammation and epithelial tight junction play significant roles in IBD [43].

Also, disruption in epithelial barrier of the intestine during inflammation can result to passive diffusion of water and ions from the circulation to the intestinal lumen which results to leak flux diarrhea. Moreover, passage of some pathogens across the epithelial barrier to the basal side can also cause pathogen invasion via secretion of pro-inflammatory cytokines such as interferon gamma, TNF-α, and interleukin-1 beta (IL-1β). Thus, the levels of these cytokines can be measured [43,44].

The agent 2,4,6-trinitrobenzenesulfonic acid (TNBS) can model IBD conditions in humans [45].

Procedure

Animals, usually mice are randomized into groups as follows: vehicle control, TNBS control, and treatment groups. They are meant to receive a single rectal dose of 0.5 mg of TNBS to induce colitis. The treatment groups are meant to receive various doses of the test drug by oral route daily for 6 days. Duration of treatment may be extended and doses of TNBS can be varied.

Diarrhea frequency and body weights are measured on a daily bases for 6 days. At the end of day 6, animals are sacrificed. Length of the colon is measured and the colon is fixed in formalin and processed for histology.

Mice colonic tissues are homogenized using phosphate buffer saline and the supernatant, after centrifuging is used for the determination of the following parameters: TNF-α and interleukin, interferon gamma (IF-γ) using Elisa kits procedure.

Demerits: It is expensive to procure the assay kits and relevant facilities for this assay. High technicalities are also involved. It is time-consuming, as drugs have to be administered daily when compared to other techniques that require only single dose administration.

Farnesoid X receptor (FXR) as a potential target for anti-diarrheal screening

A research undertaken by the Royal College of Surgeons in Ireland alongside with Trinity College Dublin and Johns Hopkins University in Baltimore unveiled a novel approach for the discovery of anti-diarrheal agents.

Farnesoid X receptor (FXR), a nuclear hormone receptor which binds bile acid is a vital controller of intestinal function. However, impairment in ileal bile acid transport system can occur due to improper absorption of bile acids. Agents which act on FXR in the tissue of the intestine can prevent the movement of water into the gut thereby preventing diarrhea. Drugs that could target these receptors may have higher efficacy with fewer side effects than the already existing anti-diarrheal drugs [46].

The study involves a change in short-circuit current across voltage that measured alteration in electronic ion transport clamped T84 cell monolayers or mouse tissues. Chambers are used for quantitative electrogenic ion transport. In Caco-2 cells, sodium–hydrogen exchanger 3 activity is measured as bis (carboxyethyl)-5(6)-carboxyfluorescein fluorescence. Immunoblotting and cell surface biotinylation is used to measure protein expression [46].

Merits: This technique is inventive and useful in identifying possible targets of anti-diarrheal agents. Also, it requires less number of animals.

Demerits: High cost of facilities and difficulty in translating *in vitro* results to human condition is a draw back to this model.

Precautions and Other Essential Information

Precautionary measures for in vivo technique

It is necessary to acclimatize animals coming from other animal facilities to the laboratory condition before they are used in these experiments. Animals of the same strain and age range should be procured for these studies. This would help to eliminate variability, which may result in outlier in the data acquired. Fasting helps to ensure uniform drug absorption. During fasting of animals, it is important to remove feed and beddings, saw dust and other materials that animals may feed upon due to lack of food. Only water should be left in the cage during fasting. To avoid coprophagia (consumption of feces), which usually occur during the night, it is necessary to restrict animals in a gridded floor during fasting to prevent accumulation of feces in the cage. Volume of administration (e.g., 10 ml/kg) should be similar in all the groups. It is also important to change the filter paper of animals every hour during fecal collection period to ensure neatness of the cage. Pilot studies may be required to guild on the choice of dosage selection for diarrhea producing agent or reference anti-diarrheal drugs. Cervical dislocation should be used by trained personnel.

Precautionary measures for ex vivo technique

Some portions of the isolated intestine should be aerated in a PSS to serve as an alternative source of tissue whenever the tissues in the organ baths go fatigued and require replacement. Great care should be exercised to avoid stretching, squeezing, and crushing of isolated tissues. Handling isolated tissue with dirty hands should be avoided. Tissues submerged in the organ/tissue baths should be incubated at 37°C in a temperature regulated water bath. The setup should also be aerated with oxygen or carbogen throughout the experiment. Inverter should be made available to supplement the

available power supply to the isolated tissue set up. Direct administration of cold drug to the isolated tissues should be avoided, as this may result in a dramatic fall in tissue temperature and less tissue response.

Data collation, analyses, and results presentations

Results gathered from these experiments can be tabulated on a record sheet and later imputed in a spread sheet such as Microsoft Excel for easy storage and analysis. Statistical packages such as Graph pad prism, Statistical Package for Social Sciences among others can be used for data analyses. Results obtained from these studies are presented as mean ± Standard error of mean (or standard deviation) of replicate experiments. Differences in mean values among groups are determined using one way analyses of variance followed by *post hoc* test(s). Independent Student's *t*-test should be used when comparison is done between only two groups. The level of statistical significance can be established at $p < 0.05$ or 0.01. EC_{50} (median effective concentrations) values of test results in *ex vivo* or *in vitro* studies can be obtained from the CRCs using regression equations.

Conclusion

In this review, various techniques in screening agents with anti-diarrheal properties were compiled. Most studies employed the *in vivo* technique. While the enteriod technique is useful in screening *E. coli* associated diarrhea, the *ex vivo* technique is useful in evaluating possible anti-motility mechanism(s) of anti-diarrheal agents. It was also observed that most *in vivo* techniques employed cervical dislocation compared to the use of anesthesia as a method of animal euthanasia. Very few studies employed the use of light anesthesia prior to cervical dislocation, as recommended by the animal welfare bodies. It is not advisable to employ only one technique in the screening of agents with promising anti-diarrheal properties. Hence, the involvement of *in vivo, in vitro*, and *ex vivo* approaches would be more efficient.

From this review, researchers should ensure that all necessary precautions are taken in order to achieve good research outcome.

Conflict of Interest

The authors declare that they have no conflict of interest.

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