L-arginine in low concentration improves rat intestinal water and sodium absorption from oral rehydration solutions

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Abstract
Background—The nitric oxide (NO) precursor L-arginine has been shown to produce variable effects on intestinal absorptive function, including ion transport.

Aims—To determine whether there is an optimal concentration of L-arginine, promoting proabsorptive effects from oral rehydration solutions (ORS) with 90 or 60 mM sodium.

Subjects and methods—In vivo perfusion of rat jejunum with determination of net water absorption, unidirectional fluid exchanges, sodium and calcium transport, and glucose absorption.

Results—L-Arginine (1 mM) added to the 90 mM sodium ORS increased intestinal absorption of both sodium and water. Higher concentrations of L-arginine (2 to 10 mM) lacked this stimulatory effect. At 20 mM, L-arginine decreased sodium absorption below baseline. With a 60 mM sodium ORS, 2 mM L-arginine had a maximal fluid and electrolyte proabsorptive effect. At 20 mM L-arginine, net water absorption was indistinguishable from that obtained in the absence of L-arginine, and lower than with 2 mM L-arginine. Sodium absorption remained raised above baseline in perfusions with 10 and 20 mM L-arginine. Morphologically, villi from perfusions with increased absorption showed a large expansion of intercellular and lamina propria intercellular spaces.

Conclusions—Low concentrations of L-arginine seem to stimulate water and electrolyte absorption by the small intestine. This effect is consistent with NO induced vasodilation, whereas higher L-arginine concentrations may be vasoconstrictive and thereby reverse fluid and electrolyte transport.

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Keywords: L-arginine, nitric oxide, oral rehydration solutions, sodium absorption.

Nitric oxide (NO) is known to have multiple physiological roles. In the gastrointestinal system NO can act as an inflammatory mediator, a regulator of intestinal ion transport, and as an inducer of soluble guanylate cyclase, which effects the relaxation of gastrointestinal smooth muscle. Production of NO contributes to the preservation of vascular integrity in endotoxin generated gut damage. It has been postulated that endogenous NO regulates the integrity of the intestinal mucosa and decreases mucosal permeability due to reperfusion injury.

Several studies suggest that NO may have favourable effects on transport in the small intestine during normal and pathophysiological states. NO donor compounds stimulate electrolyte transport in the guinea pig intestine in vitro. Both L-arginine and the NO donor sodium nitroprusside reduce water and electrolyte secretion in isolated rabbit ileum. NO also reduces cholera toxin induced fluid secretion in the rat, as deduced from the systemic administration of L-arginine. Similarly, inhibition of NO production seems to be beneficial in experimental colitis. However, under certain circumstances, NO production leads to colonic electrolyte secretion. The principal physiological precursor of NO is L-arginine, a non-essential amino acid which is metabolically processed by the urea cycle. The effects of L-arginine are stereospecific, the natural L-isomer being the active form. NO is synthesised via NO synthase (NOS). Three isoforms have been identified: two constitutive (NOS-I and NOS-III; cNOS) and an inducible form (NOS-II; iNOS). Known inhibitors of iNOS, such as N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), are stereospecific. L-NAME can also modulate water transport in rat jejunum. In the intestine it seems as if the dominant effects of NO vary according to the localisation of NO generators or NO inhibitors.

Oral rehydration solutions (ORS) have undergone numerous modifications in search of a formulation that would be effective in the replacement of water and electrolyte losses in diarrhoea as well as reducing the purging rate, providing a direct inducement for use at home by medically untrained care givers. Amino acids have been the most often tested low molecular weight additives to experimental ORS. L-alanine, glycine, and L-glutamine have reached the stage of clinical trials. However, results with these amino acids have generally fallen below expectations. Other additives – namely, starch hydrolysates, especially from rice or glucose polymers – have gained greater acceptance and are probably more effective. Because NO may stimulate intestinal water and electrolyte absorption, the addition of the NO precursor L-arginine to ORS is of potential value. The goal of the
present study was to determine the concentration of L-arginine in ORS that would produce a maximal enhancement of absorption, using a standard in vivo perfusion model in rats.

Methods

EXPERIMENTAL PROTOCOL

Sprague-Dawley male rats (Zivic-Miller Labs, Pittsburgh, PA, USA) weighing 70 to 90 g were allowed to acclimatise for at least 48 hours and then fasted overnight before laparotomy. Briefly, under urethane anaesthesia (intraperitoneal, 1·3 g/kg), a 15–30 cm segment of the jejunum immediately distal to the ligament of Treitz, was identified and cannulated with flexible, plastic tubing at the proximal and distal ports. The experimental ORS described below, prewarmed to 37°C, were delivered with a peristaltic pump (Harvard Instruments, Boston, MA, USA, model 1203) at a rate of 10–12 ml/h. The precise pumping rate was determined by measuring the rate of flow before and after the perfusion. Approximately 2 µCi/1·74 MBq/l, NEN-Dupont, Boston, MA, USA) of tritiated water (3H2O) was added to all solutions to determine water influx ([J]w) from lumen to serosa. Based on previous determinations, the recirculation of the marker was estimated to be negligible during the course of the experiment. Water secretion (serosa to mucosa efflux) ([J]s) was estimated by calculating the difference between influx and net water absorption. The algorithms applied to compute rates of absorption have been published previously.12 Groups of 12 rats were perfused simultaneously, and several solutions were tested in the same experiment to minimise interindividual variation. The total number of rats used for each treatment appears in the legend of the figures and in the tables. After a one hour equilibration, perfusates were collected for eight 15 minute periods (Ilman and coworkers, 1965). The rate of net water absorption was computed by the formula:

\[
\text{[flow rate (min)]/ [outflow rate (min)] = 1000 / mL/mm cm}
\]

The rate of water influx ([J]w), also expressed in µl/min/cm, was calculated as follows:

\[
\frac{\text{[H₂O dpm] / flow rate (min)]}}{\text{[H₂O dpm] / outflow rate (min)]} = 1000 / IL (cm)
\]

Electrolyte and glucose absorption were computed and expressed in nmol/min/cm:

\[
\text{[h] / flow rate (min)] / [s] / outflow rate (min)] = 1000 / IL (cm)
\]

where IL=intestinal length (cm); f=dilution factor×1000.

The assay results of each collection fraction were averaged and only one value was taken for each rat.

Once the perfusion ended, the rats were killed by exsanguination from the abdominal aorta. The intestinal segment between the cannulae was extended with a 4 g weight and measured. Tritiated water was counted in a Beckman LS-3800 β-scintillation counter; sodium and calcium were determined by atomic absorption spectrophotometry (SpectraAA 10, Varian Instruments Inc, Sunnyvale, CA, USA) against external standards, and osmolality was measured by vapour pressure changes (Model 5500, Wescor Inc, Logan, UT, USA). Free glucose was determined enzymatically (Sigma 510). Nitrite accumulation in the perfusates was quantified by a method based on the Griess reaction.12 The protocol for this study was approved by the Institutional Animal Care and Utilization Committee.

The basal WHO-ORS contained sodium chloride (30 or 60 mM) and trisodium citrate (10 mM), making a total sodium concentration of either 60 or 90 mM, potassium chloride (20 mM) and glucose (111 mM=20 g/l). L-arginine (Sigma, St Louis, MO, USA) was added to the solutions at a 0·5, 1·0, 2·0, 10·0, or 20·0 mM concentration. Table 1 presents the osmolality of the basal and modified ORS. Results are expressed as means (SEM).

LIGHT AND ELECTRON MICROSCOPY

For study by light and electron microscopy the jejunum was fixed by perfusion through the lumen with 2% glutaraldehyde in 0·05 M cacodylate buffer (pH 7·3) at room temperature, followed by immersion in the same buffer at 4°C for two to 18 hours. The tissue was then rinsed in cold buffer, dissected into well oriented fragments to include regions spanning from the serosal surface to villus tip. Tissue fragments were postfixed in 2% buffered osmium tetroxide at 4°C for two hours, treated with 1% sodium cacodylate, rinsed in buffer, dehydrated in a series of ethanol dilutions, and embedded in epoxy resin (E Fullam, Schenectady, NY, USA). One micrometre thick plastic sections, stained with toluidine blue, were examined by light microscopy. Thin sections were studied and stained with uranyl acetate and lead citrate, on a JEOL-JEM 100CXII electron microscope.

Samples from both the 60 mM and 90 mM sodium ORS perfusions were studied by light and electron microscopy in the absence of L-arginine, at the concentration of maximal sodium and water absorption for each ORS and at the highest L-arginine concentration used (20 mM). Light microscopical studies were done “blind” to assess histological differences and to compare the relative amount

<table>
<thead>
<tr>
<th>L-Arginine (mM)</th>
<th>0</th>
<th>0-5</th>
<th>1-0</th>
<th>2-0</th>
<th>10-0</th>
<th>20-0</th>
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<tbody>
<tr>
<td>90 mM sodium</td>
<td>307</td>
<td>311</td>
<td>319</td>
<td>324</td>
<td>365</td>
<td>377</td>
</tr>
<tr>
<td>60 mM sodium</td>
<td>271</td>
<td>276</td>
<td>279</td>
<td>271</td>
<td>296</td>
<td>308</td>
</tr>
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The values correspond to two to four measurements per group.
of tannic acid stained material adherent to the microvillous brush border under the various experimental conditions.

**Statistical Analysis**
For transport studies, the significance of the differences among means was assessed by the Kruskal-Wallis test with non-parametric multiple contrasts. A test for trend was applied to determine L-arginine concentration versus dependent variable outcomes. The minimum level of significance was set at \( p < 0.05 \).

**Results**

**Water and Electrolyte Absorption**
The addition of a low concentration of L-arginine enhanced water and electrolyte absorption from both the 90 mM and 60 mM sodium ORS (Figs 1 and 2). When L-arginine was added to the 90 mM sodium ORS at a 1 mM concentration, there was a significant increase in the absorption of both sodium and water (Fig 1). A lower concentration of L-arginine (0.5 mM) produced no alterations over baseline. An increase in L-arginine concentration to 2 or 10 mM dissipated the stimulatory effect. The addition of 20 mM L-arginine resulted in sodium absorption rates lower than the baseline. For both net water and sodium absorption the uptake at the three highest concentrations of L-arginine was significantly lower than the peak values obtained with the addition of 1 mM L-arginine.

In ORS perfusions containing 60 mM sodium, L-arginine exerted its maximum proabsorptive effect on water and sodium absorption at 2 mM (Fig 2). At this concentration of L-arginine net transport of the electrolyte was absorptive. At lower L-arginine concentrations, the values obtained for sodium transport were indicative of a balance in sodium movement to and from the lumen, with modest mean secretory values at 1 mM L-arginine. Net water absorption at 2 mM and at 10 mM L-arginine remained higher than baseline. However, when L-arginine was increased to 20 mM, net water absorption became indistinguishable from that obtained in the absence of L-arginine, and lower than at the maximum effective concentration of 2 mM L-arginine. Sodium absorption remained higher than baseline in perfusions with 10 mM and 20 mM L-arginine.

**Water Fluxes**
With the 90 mM sodium ORS, \([J]\), as determined by the disappearance of tritiated water, was increased with 1 mM L-arginine and decreased with 20 mM L-arginine, paralleling net water absorption data (Table II). Water efflux \([J]\) also declined with 20 mM L-arginine as well as at 2 mM L-arginine. The ratio \([J]_2/[J]_1\) was unaffected by the L-arginine concentration.

In 60 mM sodium ORS perfusions (Table II) \([J]\) increased at 2 mM L-arginine, paralleling net water absorption, and a decline in \([J]\) was evident at 20 mM. The \([J]_2/[J]_1\) ratio peaked at 2 mM and remained raised at 10 mM and 20 mM L-arginine. With 2 mM L-arginine, this ratio was greater than at 0.5 mM and 1.0 mM L-arginine. The trend toward a higher ratio as the concentration of L-arginine increased was consistent with the decline in water \([J]\). Despite the lower osmolality of the 60 mM sodium ORS, baseline net water absorption, unidirectional fluxes, and flux ratios were indistinguishable from those of the 90 mM sodium ORS.

**Glucose, Calcium, and Nitrite Studies**
L-Arginine, in concentrations ranging from 1 to 20 mM, generally stimulated glucose absorption from both the 90 and 60 mM sodium ORS (Table III).

No relation was evident between calcium secretion and water and sodium data. Calcium was secreted under all perfusion conditions, but was greater with 60 mM than 90 mM sodium (Table III). With the 90 mM sodium ORS, calcium secretion was maximal at 20 mM L-arginine. With the 60 mM sodium ORS, calcium secretion was maximal at 10 mM L-arginine. Calcium release into the lumen was about three orders of magnitude smaller than sodium absorption rates.
Nitrite measured in the outflow of the perfusates containing 90 mM sodium showed no direct relation with the concentration of L-arginine present. The baseline value of 13.3 (1.6) pmol/min/cm² only increased to 30.3 (7.7) pmol/min/cm² at 10 mM L-arginine. Nitrite production and release was not determined in perfusions with 60 mM sodium.

**MORPHOLOGICAL FINDINGS**

Light microscopical studies of jejunal villi focused on the extracellular space of villus mucosa and lamina propria histological compartments. Generally, morphological changes of jejunal villi reflected L-arginine induced water and sodium transport alterations from perfusion experiments. Jejunal villi perfused with an L-arginine free 90 mM sodium ORS showed some dilation of intercellular spaces between enterocytes and lamina propria (Fig 3A). The addition of 1 mM L-arginine to the 90 mM sodium ORS perfusion led to a greatly increased expansion of the mucosal and lamina propria intercellular spaces, a finding consistent with the stimulation of water and sodium translocation from the lumen across the mucosa (Fig 3B). By contrast, in ORS perfusions with 20 mM L-arginine, mucosal and lamina propria intercellular spaces were reduced and were not readily distinguishable from villi in L-arginine free perfusions (Fig 3C). The pattern of morphological findings with the 60 mM sodium ORS paralleled those seen with 90 mM sodium (Figs 4A, B, and C). With the 60 mM sodium ORS, intercellular space expansion was most evident at 2 mM L-arginine (Fig 4B), but was reduced in jejunal perfused with 20 mM L-arginine (Fig 4C).

There was no evidence of structural damage to jejunal villi, or of changes in the number of goblet cells under any of the perfusion conditions. The amount of tannic acid positive material adherent to the brush border varied between animals, but no effect produced by the differing L-arginine perfusion conditions was distinguishable. When examined by electron microscopy villus absorptive cells from 90 mM and 60 mM sodium ORS perfusions were normal in appearance and unaltered by the addition of L-arginine at the concentration producing maximal proabsorptive effect (Figs 5A and B).
Figure 4: Light photomicrographs of typical jejunal villi perfused with a 60 mM sodium ORS, with no added L-arginine (A), with 2 mM L-arginine (B), and with 20 mM L-arginine (C). The pattern of changes in the amount of mucosal and lamina propria intercellular space is similar to that seen with the 90 mM sodium ORS. With the 60 mM sodium ORS, dilation of the intercellular spaces on the villus mucosa and lamina propria is most pronounced in 2 mM L-arginine perfusions (B). The expansion of the extracellular spaces is then reduced when L-arginine is perfused at 20 mM (C). Originally ×450.

Discussion

The results indicate that the addition of relatively low concentrations of L-arginine (1–2 mM) to an ORS can induce positive increments in the rates of sodium and water absorption, when tested in an in vivo jejunal perfusion system in the rat. This effect was seen in ORS with 90 mM as well as 60 mM sodium. These physiological transport data were paralleled by morphological studies showing expansion of the intercellular spaces after perfusion with low concentrations of L-arginine, a finding consistent with increased water and electrolyte absorption.

By contrast with the present data, earlier studies with L-arginine indicated induction of water secretion in the rat small intestine. However, those secretory effects occurred at a relatively high (20 mM) concentration of the amino acid; lower L-arginine concentrations were apparently not tested. In the present study, increasing the concentration of L-arginine to 20 mM also produced a decrease in electrolyte absorption with the 90 mM sodium preparations. These results are comparable with those reported earlier with a glucose free solution containing 140 mM sodium. It should be noted that L-arginine retained its proabsorptive effect up to a 10 mM concentration when the ORS contained 60 mM sodium. The enhancement of sodium uptake from the 60 mM ORS was remarkable in that it greatly exceeded the equilibrium point for sodium transport into the jejunum, which is in the 60–65 mM range, as documented in rodent and human studies.

Amino acids generally tend to increase water and sodium absorption in the small intestine, as a consequence of the sodium cotransport mechanism involved in the translocation of many natural amino acids across epithelia. This explanation is not tenable for L-arginine for several reasons. In the present study the effect of this amino acid was not concentration dependent, whereas a dose-response relation with water and electrolyte absorption has been found in animal experiments with L-alanine and L-glutamine in animals and in humans. The sodium cotransport linkage is also not applicable, as the y-system involved in L-arginine transport is considered to be a facilitated diffusion process dependent on a differential in membrane potential. Amino acids are not uniformly proabsorptive. Glycine, initially postulated as a stimulatory additive, is ineffective at high concentrations; this is probably due to inappropriate sodium:amino acid stoichiometry and increased osmolality. Moreover, the transport enhancing effects of L-arginine shown in the experiments presented here took place at concentrations one order of magnitude below those attempted in trials with either L-alanine or L-glutamine. As L-arginine concentration increased to 20 mM, the reduction in absorptive capacity made evident by the decrease in [\(\gamma\)] and [\(\gamma\)] is consistent with vasoconstrictive effects linked to NO under certain circumstances.

Perfusion of ORS with L-arginine had a modest proabsorptive role in regard to glucose at concentrations of the amino acid generally higher than those altering water and sodium absorption, particularly in perfusions with 60 mM sodium. As both the 90 mM and 60 mM sodium ORS contained 111 mM glucose (20 g/l), the overriding proportion of the transport is likely to occur by solvent drag. Therefore, this result is compatible with glucose entry into the enterocyte after water absorption.
movement with rapid removal into the circulation. Calcium is normally secreted into the intestinal lumen during peristalsis with solutions which do not contain this element. Whereas the presence of citrate in the ORS in our study may impair calcium absorption, it does not alter secretion of the cation.34 In solutions which omit calcium, such as those used in these experiments, a leakage of this cation into the lumen as sodium enters the cell is therefore expected, and consistent with sodium–calcium exchange across membranes.35 36 This was clearly the case here, although no correlation was found between water or sodium absorption and calcium secretion. Osmolality may also play a part in this process, as secretory rates of calcium were higher in experiments with 60 mM sodium than with 90 mM sodium. In view of these results it cannot be argued that a calcium–sodium exchange is putatively modulated by NO.

The present experimental model has been extensively used in previous investigations intended to compare the effectiveness of ORS with variable concentrations of solutes, osmolality, or additives, with the ultimate goal of improving their effectiveness in sodium transport and water retention in humans.27 29 30 37 Positive ORS studies on rodents38 have subsequently been applied to clinical trials, especially in infantile diarrhea of various aetiologies. In a clinical study,39 the ORS which best reduced stool volume in infants and children with diarrhea was the same which produced the highest rates of water absorption in the rat. Similarly, the greatest decrease in sodium losses in the faeces paralleled maximal sodium experimental absorption rates in the experimental model.

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