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Measuring intestinal fluid transport *in vitro*: Gravimetric method *versus* non-absorbable marker

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ABSTRACT

The gut sac is a long-standing, widely used *in vitro* preparation for studying solute and water transport, and calculation of these fluxes requires an accurate assessment of volume. This is commonly determined gravimetrically by measuring the change in mass over time. While convenient this likely under-estimates actual net water flux (J_v) due to tissue edema. We evaluated whether the popular *in vivo* volume marker [^{14}C]-PEG 4000, offers a more representative measure of J_v *in vitro*. We directly compared these two methods in five teleost species (toadfish, flounder, rainbow trout, killifish and tilapia). Net fluid absorption by the toadfish intestine based on PEG was significantly higher, by almost 4-fold, compared to gravimetric measurements, compatible with the latter underestimating J_v. Despite this, PEG proved inconsistent for all of the other species frequently resulting in calculation of net secretion, in contrast to absorption seen gravimetrically. Such poor parallelism could not be explained by the absorption of [^{14}C]-PEG (typically < 1%). We identified a number of factors impacting the effectiveness of PEG. One was adsorption to the surface of sample tubes. While it was possible to circumvent this using unlabelled PEG 4000, this had a deleterious effect on PEG-based J_v. We also found sequestration of PEG within the intestinal mucus. In conclusion, the short-comings associated with the accurate representation of J_v by gut sac preparations are not overcome by [^{14}C]-PEG. The gravimetric method therefore remains the most reliable measure of J_v and we urge caution in the use of PEG as a volume marker.

1. Introduction

The *in vitro* gut sac preparation, involving longitudinal sections of the intestine ligated at one end and filled and emptied at the other *via* a catheter, is a long standing and popular method for the study of intestinal transport that is still used to address current physiological questions (Hamilton, 2014). With relative technical ease gut sacs permit the simultaneous, straightforward measurement of intestinal solute and water fluxes, and can also be modified to enable the monitoring of electrophysiological parameters (Grosell et al., 2005). Introduced in its everted form by Wilson and Wiseman (1954) using segments of the mammalian small intestine, the first published investigations employing this methodology with teleost models appeared 10 years later (House and Green, 1963, 1965; Smith, 1964). This approach has since been used extensively to investigate various aspects of fish physiology, finding particular application in the study of osmoregulation (Collie and Bern, 1982; Veillette et al., 1995; Grosell et al., 2005) and ecotoxicology (Grosell et al., 1999; Nadella et al., 2006; Ojo and Wood, 2007).

Over the course of an experiment the concentrations of solutes within the gut sac will change, not only as they are transported by the epithelium, but also as a result of water movement. Calculating the net flux of a solute therefore requires a sufficiently accurate measurement of fluid volume within the sac. This change in volume is most commonly obtained gravimetrically and involves lightly blotting the outer surface of the sac before weighing. Subsequently, the change in overall mass during the experiment is representative of the change in mucosal fluid volume and used to calculate fluid transport rate, J_v (Collie and Bern, 1982; Grosell et al., 1999, 2005; Marshall et al., 2002).

One shortcoming associated with the gravimetric method is that the blotting process alone can change the mass of the gut sac (House and Green, 1965), which can substantially influence the recorded volume. Irregularities may therefore arise depending on the consistency of the blotting method used. Secondly, one of the most significant issues is tissue edema (Parsons and Wingate, 1961; Smith, 1964; Jackson and Cassidy, 1970), due to the absence of functioning vasculature leading

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to the consequent accumulation of fluid (Lee, 1961, 1963; LeFevre et al., 1970; Levine et al., 1970). The gravimetric method is therefore likely to underestimate actual J_v since only fluid that has actually traversed the serosal layer is being measured. These issues have therefore led to the consideration of non-absorbable volume markers as an alternate method of calculating volume change that should, in theory, provide a more accurate representation of fluid transport.

Polyethylene glycol (PEG) is an inert, synthetic polymer commonly used as a volume marker. High molecular weight PEGs (3350–4000) are considered to be poorly absorbed by the intestine (Schedl, 1966; Krag et al., 1975; Winne and Gorig, 1982; Furuichi et al., 1984; Schiller et al., 1997; Grosell and Genz, 2006), and easily determined in biological fluids by direct assay (Malawer and Powell, 1967) or labelling the PEG molecule with a radioisotope such as ^{3}H or ^{14}C (Wingate et al., 1972; Krag et al., 1975). The latter offers the greatest sensitivity and practical advantage, hence radiolabelled PEG has become a common choice in studies of gastrointestinal physiology, such as gut transit and digestibility in ruminants (Till and Downes, 1965; Pickard and Stevens, 1972), drinking rates in fish (Shehadeh and Gordon, 1969; Scott et al., 2006; Genz et al., 2008), and is widely regarded as a suitable volume marker for studies of intestinal absorption *in vivo* (Jacobson et al., 1963; Schedl, 1966; Maddrey et al., 1967; Schiller et al., 1997).

Using a volume marker such as PEG in gut sacs should eliminate errors concerning edema and inconsistencies associated with blotting. However, examples of volume markers being successfully used in gut sac preparations are scarce and appear limited to [^{14}C]-inulin (Aull, 1966) and more recently [^{14}C]-PEG 4000 (Grosell and Genz, 2006; Grosell and Taylor, 2007; Genz and Grosell, 2011; Genz et al., 2011). Although considered reliable *in vivo*, to the best of our knowledge there has not been any prior, direct validations of PEG as a volume marker for the gut sac technique. Furthermore, despite a large number of published studies using PEG as a reference marker some investigators have raised doubts about its reliability compared to more direct, gravimetric methods suggesting that volume markers such as PEG may not distribute homogeneously (Worning and Amdrup, 1965; Bunce and Spraggs, 1982; Ando et al., 1986), becoming trapped in the overlying mucus layer (Kirsch and Meister, 1982), and may also be appreciably absorbed (Sutton et al., 2001; Wood and Grosell, 2012). However, because two to three water molecules can be bound per PEG repeat unit (Antonsen and Hoffman, 1992), the effective molecular size of PEG is increased in aqueous solution, and this is suggested to reduce potential interactions with the mucus layer, as well as absorption across the epithelium (Schiller et al., 1997). In contrast to this view, the lone pair of electrons on the oxygen atom of the PEG repeat unit (CH_2CH_2O) offers numerous potential hydrogen bond acceptors for adhesion to the ionised sialic acid residues of mucus (Shojaei and Li, 1997). Furthermore, PEG adsorbs to glass and polypropylene (Crouthamel and Van Dyke, 1975; Gulliford et al., 1987) thus introducing another potential source of error.

The present study sets out to directly compare the performance of [^{14}C]-PEG 4000 alongside the traditional gravimetric method of measuring fluid transport by gut sacs from five different species of marine teleost; Gulf toadfish (*Opsanus beta*), European flounder (*Platichthys flesus*), rainbow trout (*Oncorhynchus mykiss*), mummichog killifish (*Fundulus heteroclitus*) and blue tilapia (*Oreochromis aureus*). All of these species are popular comparative models that have been frequently used for a variety of *in vivo* and *in vitro* investigations, including gut sacs. We hypothesised that the gravimetric method would under-estimate fluid transport relative to PEG. Furthermore, with the apparent lack of consensus in the literature on the performance of PEG, experiments were designed to evaluate PEG as a non-absorbable volume marker. If PEG could be validated as reliable this could lead to a standardised methodology for gut sacs, and the promise of more accurate and representative measurements of fluid transport *in vitro*.

2. Materials and methods

2.1. Experimental animals

Toadfish (n = 15, body mass 27–95 g) were obtained as by-catch from local shrimp fishermen around Biscayne Bay, Florida, U.S.A. and transferred to the aquarium facilities at the University of Miami, Rosenstiel School of Marine and Atmospheric Sciences. Upon arrival fish received a prophylactic treatment against ectoparasites, as previously described (McDonald et al., 2003), and were subsequently held in tanks receiving a continuous flow of filtered sea water (34–37 ppt, temperature 22–26 °C; from Bear Cut, Florida). Killifish (n = 6) were supplied by Aquatic Research Organisms Ltd. (Hampton, NH, U.S.A.) and shipped to the aquarium facilities at the University of Miami where they were maintained under similar seawater conditions to the toadfish for at least 2 weeks prior to use. Toadfish were fed frozen squid twice weekly but food was withheld for 48 h prior to experiments. Killifish were fed a daily ration of AquaMax pellets (Purina, MN, U.S.A.) and food withheld for at least 24 h before experiments.

European flounder (n = 15, mean body mass 390 ± 30 g) were obtained from local fishermen in Flookburgh, Cumbria, U.K. and transported to the marine aquarium facilities at the Department of Biosciences, University of Exeter, where they were held in flowing, aerated artificial seawater, made with commercial marine salts (Tropic Marin), as part of a recirculating seawater system maintained at 33–35 ppt and 11–13 °C. Food was typically withheld for at least 72 h prior to experimentation, otherwise flounder were maintained on a diet of fresh ragworm (*Nereis virens*) fed once per week. Freshwater rainbow trout were obtained from Houghton Springs Fish Farm (Dorset, U.K.) and 6 of these fish (210 ± 8 g body mass) were acclimated to seawater (~28 ppt) over the course of 5 days by the addition of marine salts. The fish were then maintained at this salinity for a further 7 days prior to experimentation. During this 12 day period water temperature was 13.2 ± 0.3 °C and food withheld throughout. Tilapia (n = 8, mean body mass 255 ± 15 g) were obtained from the Institute of Aquaculture, University of Stirling (Stirling, U.K.) and transported to the marine aquarium facilities at the University of Exeter, where they were held in flowing, aerated artificial seawater maintained at 29.5 ± 0.2 ppt and 22.3 ± 0.2 °C. Food was withheld for one week prior to experimentation, otherwise tilapia were maintained on a commercial pellet diet (Skretting, Cheshire, U.K.) fed 3 times per week.

All of the experimental procedures were approved by the Institutional Animal Care and Use Committee (Miami, U.S.A.) and the University of Exeter Ethical Committee under a U.K. Home Office licence in accordance with the Animals (Scientific Procedures) Act 1986 (Exeter, U.K.).

2.2. General experimental approach

Fish were euthanized by an overdose (300 mg l^{-1}) of buffered tricaine methane sulfonate (MS-222; Pharmaq Ltd.) followed by destruction of the brain. The abdominal cavity was opened and the entire intestine, from the stomach to the rectum was removed and bathed in serosal saline (Table 1), while the sacs were constructed. After carefully removing the attached vasculature and connective tissue, a short, 5 cm length of polyethylene tubing (1.19 mm ID; 1.70 mm OD), heat-flared at one end, was tied into the anterior section with a double silk ligature (US 2/0 or 3/0) immediately distal to the pyloric cecae (where present) or pyloric sphincter. This catheter was then used to flush the entire intestine through with mucosal saline (Table 1).

For the toadfish and flounder intestine, sacs were made from 2 to 3 cm lengths of the anterior, mid and posterior segments of the intestine. With little in the way of regional distinctions, the mid portion of the intestine was arbitrarily taken as the middle third of the intestine, with the posterior section taken as 2–3 cm proximal to the rectal sphincter. In contrast, the intestine of the rainbow trout, being a farmed