JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Mucus thickness in the gastrointestinal tract of laboratory animals

Felipe J. O. Varum[a,b], Francisco Veiga[a], João S. Sousa[a] and Abdul W. Basit[b]

[a]Center for Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal and [b]The School of Pharmacy, University of London, London, UK

Keywords
gastrointestinal mucus; mucoadhesion; pig; rabbit; rat

Correspondence
Abdul W. Basit, The School of Pharmacy, University of London, 29/39 Brunswick Square, London, WC1N 1AX, UK.
E-mail: abdul.basit@pharmacy.ac.uk

Received August 5, 2011
Accepted October 11, 2011

doi: 10.1111/j.2042-7158.2011.01399.x

Abstract

Objectives The objective of this study was to systematically assess the mucus thickness in the gastrointestinal tract of laboratory animals commonly used in preclinical studies.

Methods Mucus thickness was studied post-mortem in the rat, rabbit and pig, using cryosections stained by the modified periodic acid Schiff/Alcian blue method.

Key findings The mucus thickness in the fundus region of the stomach was higher in the pig (190.7 ± 80.7 μm) than in the rabbit (155.1 ± 85.8 μm) and the rat (31.3 ± 11.4 μm). However, along the small intestine (ileum), mucus was thicker in the rabbit (147.8 ± 115.6 μm), followed by the pig (53.8 ± 22.1 μm) and the rat (34.1 ± 14.9 μm). This rank order was also observed in the ascending colon.

Conclusions Inter-species variability in mucus thickness along the gut was demonstrated and suggests that the pig resembles more closely the mucus pattern of humans. This may be highly relevant when preclinical animal models are used in drug absorption studies or in the development of oral mucoadhesive drug delivery systems.

Introduction

Mucus is ubiquitous in the gastrointestinal tract and constitutes a dynamic biophysical barrier between the lumen and the underlying epithelium. Mucus is a complex secretion composed of water (95%), glycoproteins (mainly O-linked oligosaccharides), lipids, electrolytes, sloughed epithelial cells, bile salts and other components available in the gut lumen.[1,2] It provides lubrication and protection of the underlying epithelium against mechanical damage from food, acid, digestive enzymes, commensal and pathological bacteria, toxins, carcinogens and oxygen-derived free radicals.[3–8] In the colon, the mucus layer also provides a hospitable environment for the microbiota, where the host and the bacteria benefit from a symbiotic relationship.[6,9]

The architecture of the mucus layer and the molecular mechanisms responsible for the protective and lubricant function have only recently been elucidated. Mucus has a two-layer structure – a loosely bound outer layer and an adherent layer. This double-layer concept is clearer in the stomach and in the colon, whereas in the small intestine, mucus discontinuity occurs, reflecting distinct physiological functions.[10] The adherent inner layer is insoluble and formed by tight sheets of mucin (MUC2), whereas the structure of the outer layer is wider mainly due to proteolytic breakdown, resulting in a network expansion.[6,11,12] In the colon, commensal bacteria inhabit the loosely bound outer layer, where they can bind to specific glycans and use mucins as an energy source. In contrast, bacteria are absent from the adherent layer.[11,13,14]

There are three types of mucins: secreted gel-forming mucins, cell-bound mucins and secreted non-gel-forming mucins.[13] The secreted mucus forms a viscoelastic 'adhesive' gel upon hydration, which is dependent on mucin glycolysation.[15,16]These viscoelastic properties can be compromised by reduction of disulfide bounds or proteolysis.[17]

The mucus layer acts as a primary barrier to drug absorption by two different mechanisms, through drug binding to mucins and drug diffusion through the mucus layer.[18] Some drugs have been shown to chemically interact with the glycoproteins from mucus, limiting the drug absorption rate and decreasing drug bioavailability. These mucus–drug interactions can occur by electrostatic interactions between positively charged drugs and negatively mucins, which are ionized at pH > 2.6 (due to the sialic acid component), by hydrophobic interactions with the protein core of mucins or through hydrogen bonding.[19–22]

The mucus blanket limits drug absorption by acting as a diffusion barrier.[23–25] The mucus layer is presented to the drug as a selective mesh restricting diffusion of molecules towards the epithelium, particularly those with higher molecular weight, such as peptides, proteins and DNA, which can become entrapped.[12,26–28] This is also relevant to the delivery of oral vaccines and other biotechnology products, formulated in the nanosize range, which can become trapped within the mucin network.[29] Furthermore, a range of different enzymes, both from host and microbial origin,[6] are present within the mucus layers, which can result in drug/peptide degradation. The extent of the diffusion barrier effect depends on the diffusion coefficient of the drug and on the mucus thickness. This is more relevant and more limiting in the stomach and in the large intestine, where a two-layer and thick mucus structure exists. In contrast, the patchy and discontinuous mucus layer in the small intestine facilitates absorption of nutrients and drugs.[10]

The properties of the mucus layer have been exploited in the development of mucoadhesive drug delivery platforms to increase and harmonize residence time in the mucosal surface to increase oral drug bioavailability.[30,31] The glycan component of the mucus provides a source for mucoadhesive interaction either by electrostatic (sialic acid and sulfated moieties), hydrophobic interactions (fucose residues), hydrogen bonding or covalent linkages (disulfide bridges).[32,33] The barrier function of the mucus layer is highly dependent on its rheological properties, dictated by glycoprotein composition,[34–36] mucus clearance or turnover[37] and its thickness.[2,10] The mucus thickness in the gut results from the balance between mucus secretion, by the goblet and Paneth cells, and mucus degradation by mechanical shear and enzymatic digestion by secreted enzymes or bacteria.[2,9,38,39]

The rat, rabbit and pig are the most commonly used animal models in drug research and development.[40] Differences in gastrointestinal physiology between animal models are not completely known, which presents some challenges when selecting the most appropriate model for pre-clinical studies.[41,42] Mucus thickness in the gastrointestinal tract has been reported for different animal models by means of in-vitro[43–46] and in-vivo[10,47–49] methods. Gastrointestinal mucus in humans has also been characterized using tissue resections.[50–52] Also, the distribution of mucins and patterns of glycosylation along the human gut has been recently reported.[35,36] However, the variety of methods employed to analyse mucus thickness has generated contradictory results[30] and a systematic comparison of mucus thickness along the complete gastrointestinal tract of different animal models is lacking.

The aim of this work was to systematically assess the mucus thickness in the gastrointestinal tract of sacrificed common laboratory animals, such as the rat, the rabbit and the pig, using the modified periodic acid Schiff/Alcian blue method. To the authors' knowledge, this is the first time that the mucus thickness in the entire gastrointestinal tract of common laboratory animals is reported using the same methodology.

Materials and Methods

Materials

O.C.T. compound (cryogel), glacial acetic acid and polyl-L-lysine slides were purchased from VWR International (Lutherworth, UK). Sodium metabisulfite and periodic acid were obtained from Sigma-Aldrich (Poole, UK) . Clearmount mounting solution was purchased from Invitrogen (Paisley, UK). Schiff's reagent, Alcian Blue 8GX and paraformaldehyde were obtained from Fisher Scientific (Loughborough, UK).

Animal models

Gastrointestinal mucus thickness was assessed in three different animal models, namely in rats, rabbits and pigs. The gastrointestinal tract of three different animals was used for each species. Rats (Wistar, 200–230 g, 8 weeks old) were purchased from Harlan UK Ltd (Biscester, UK). Rabbits (New Zealand white, 2.1–2.3 kg, 9–10 weeks old) were acquired from Harlan UK Ltd (Biscester, UK). The gastrointestinal tract of pigs (cross-breed of large white and landrace, 95–110 kg, 6 months old) were obtained from Cheale Meats Ltd. (Brentwood, UK). Rats were fed with Teklad Global 18% protein Rodent Diet (Harlan Olac, Biscester, UK). Rabbits were fed with Lab Diet 5322 with added vitamin C (IPS, London, UK). Pigs were fed with Eltabreed Bingle Sow Cake (ABN Ltd, Peterborough, UK). All animals were healthy males and allowed free access to food before the experiments. All experiments were conducted in accordance with the Home Office standards under the Animals Act (Scientific Procedures). The animals were sacrificed and immediately dissected and samples from the gastrointestinal tract were collected for further processing.

Preparation of blocks for cryosectioning

Small gastrointestinal sections (approximately 4×1 cm) were removed and placed in individual square-shaped aluminium foil moulds previously semi-filled with cryogel. A second layer of cryogel was added on top of the mucosa sample and the blocks were immediately snap-frozen in liquid nitrogen until a solid block was obtained. Prepared blocks were maintained at −80°C until use.

Cryosectioning procedure

Blocks containing gastrointestinal samples were removed from the aluminium foil and inserted into the sample holders using cryogel and kept inside the cryostat (Leica CM3050 S; Leica Microsystems, Nussloch, Germany) chamber (−25°C)

until a strong adhesion was achieved. The sample holder was placed into the cryostat head and the cryogel block was trimmed (30–40 μm slicing) to expose the gastrointestinal mucosa sample. Thin sections (10 μm) were then obtained and collected on poly-L-lysine-coated microscope slides to obtain a better adhesion. Slides were maintained at −20°C until the staining procedure.

Staining procedure

The method used here was developed by Jordan *et al*. to avoid damage or shrinkage when a conventional periodic acid Schiff/Alcian blue (PAS/AB) staining technique is used.[51] Briefly, microscope slides were defrosted and were given a pre-treatment in 100% ethanol for 10 min and rinsed in running tap water for another 10 min. Slides were then dipped in 3% acetic acid (2 min) and stained in 1% Alcian blue 8GX in 3% acetic acid (pH 2.5) for 2.5 h and washed again in 3% acetic acid and rinsed in running tap water. Next, slides were oxidized in 1% (v/v) periodic acid (aqueous) for 10 min and washed in running tap water. The second staining step was performed by immersing the samples in Schiff's reagent for 15 min and repeating the water-rinsing step. Slides were then immersed three times in 0.5% (w/v) sodium metabisulfite, rinsed in running tap water and post-fixed in paraformaldehyde vapour at 37°C for 45 min. Stained slides were mounted in aqueous medium (Clearmount; Invitrogen, Paisley, UK), protected with a cover slip.

Microscopic observation and mucus thickness measurements

For each gastrointestinal region, three different sections were obtained and mounted in slides. The glass slides were observed using an optical microscope with a camera (Nikon Microphot – FXA) and image acquisition software (Infinite 2) at 10 × and 40 × lens magnifications. Mucus thickness measurements were performed using image analysis software (Able Image analyser, Mu Labs) in three different images of each glass slide. At least 20 measurements were recorded for each image. The software was calibrated using images of a grid at different magnifications.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 for Windows. The Kruskal–Wallis test was used to assess differences in mucus thickness between different specimens from the same species. Statistically significant differences in mucus thickness between gastrointestinal sections, in each species, and between different animal species, were evaluated using one-way analysis of variance and the Tukey's test was used for comparisons between groups. Results were considered statistically significant when $P \leq 0.05$.

Results and Discussion

Mucus thickness in the gastrointestinal tract of pig

Mucus was thicker in the stomach than in the small and large intestines as can be seen in Table 1 and Figure 1 ($P \leq 0.05$). The stained sections of the gastrointestinal tract of pig, where the mucus layer is delimited are presented in Figure 1. Gastric mucus thickness values obtained in our study are in accordance with those obtained by Dixon *et al*. using the same method.[53] Along the small intestine, mucus thickness increases significantly ($P \leq 0.05$) from the duodenum (25.6 ± 12.2 μm) to the ileum (53.8 ± 22.1 μm) as reported in Table 1. In the caecum, mucus thickness is lower than in the ileum ($P \leq 0.05$). No differences were observed along the colon ($P > 0.05$); however, in the rectum mucus thickness was lower ($P \leq 0.05$). The pattern of mucus thickness along the gastrointestinal tract of pig observed in this study (Figure 2), mirrors the pattern that we recently reported using a simple and routine hematoxylin and eosin staining procedure.[46] However, the values reported in that publication are generally lower than those presented here. It has been previously proposed that mucus is composed of an adherent mucus layer, tightly bound to the epithelium and a loosely bound mucus layer which is less viscous and shreds off easier, due to enzymatic degradation and erosion.[6,10,12,54] One of the reasons behind the observed differences may be the lack of a fixation step in the hematoxylin and eosin staining method. This may result in the loss of the loosely bound mucus layer, which is easier to wash off during the washing steps involved in the staining procedure.[55] Therefore, only the adherent mucus layer remains closely bound to the mucosa, which can be visualized by the hematoxylin and eosin staining method. The modified PAS/AB staining method is a more complex and lengthier process; however, it involves a mild fixation step, allowing the full preservation of the mucus layer.[51,56]

Mucus thickness in the gastrointestinal tract of rabbit

The pattern of mucus thickness along the gastrointestinal tract of the rabbit (Table 1) was distinct from that of the pig (Figure 2). The measurements of the mucus layer are represented in the stained gastrointestinal sections as can be observed in Figure 3. Mucus was as thick in the stomach, particularly in the antrum, as in the ascending colon, as presented in Table 1 ($P \leq 0.05$). Surprisingly, a significantly thicker mucus layer was found in the appendix compared with all small intestine segments ($P \leq 0.05$). The appendix is well developed in rabbits, in contrast to humans.[42] Unlike the pig, mucus was thinner ($P \leq 0.05$) in the duodenum and distal regions of the large intestine, such as the descending colon and rectum. As observed in the pig, mucus thickness

Table 1 Mucus thickness in the gastrointestinal tract of pig, rat and rabbit

	Stomach			Small Intestine			Large Intestine					
	Fundus	Body	Antrum	Duodenum	Jejunum	Ileum	Caecum	Appendix	Asc. colon	Trans. colon	Desc. colon	Rectum
Pig	190.7^{a,d} (80.7)	213.9^{a,c} (87.9)	222.2^{b,d} (112.2)	25.6^{b,c} (12.2)	35.3^{b,d} (17.8)^{b,d}	53.8^{a,c,d} (22.1)	37.2^{a,c,d} (16.1)	–	68.1^{b,c,d} (36.5)	83.6^{c} (36.2)	76.3^{a,d} (56.7)	58.8^{a,c} (27.9)
Rabbit	155.1^{a,d} (85.8)	124.5^{a,c,d} (68.8)	277.6^{b,c,d} (129.4)	73.3^{a,c,d} (42.6)	94.6^{a,c,d} (67.9)	147.8^{a,c} (115.6)	134.4^{a,d} (88.4)	266.6^{c} (135.6)	265.1^{a,d} (125.6)	–	63.2^{a,c,d} (41.2)	111.5^{a,c} (99.6)
Rat	31.3^{a,d} (11.4)	57.9^{a,c,d} (34.8)	69.4^{a,c,d} (24.8)	30.6^{b,c,d} (8.8)	38.5^{b,c,d} (16.4)	34.1^{a,c,d} (14.9)	49.6^{a,c,d} (31.5)	–	65.2^{b,c,d} (39.8)	–	48.4^{a,c,d} (30.0)	–

Mucus thickness was determined by the modified periodic acid Schiff/Alcian blue (PAS/AB) staining procedure. Results are expressed as the mean ± standard deviation of multiple measurements as described in the methods section. The missing values in the empty cells means that tissue from the corresponding region was not collected due to technical difficulties or absence/limited size of the organ. [a]Statistically different across species. [b]Not statistically different between species. [c]Statistically different relative to the proximal gastrointestinal section. [d]Statistically different relative to the distal gastrointestinal section

seems to increase along the small intestine, but due to the large variability encountered, this was not statistically significant ($P > 0.05$). In contrast, mucus thickness decreases ($P \leq 0.05$) in the large intestine from the proximal to distal areas (Table 1).

Mucus thickness in the gastrointestinal tract of rat

For inter-species comparison, young adult rats were considered. As observed in the pig and the rabbit, mucus thickness was higher in the stomach, particularly in the antrum and in the ascending colon, as presented in Table 1 and Figure 2 ($P \leq 0.05$). The stained sections of the gastrointestinal tract of rat, where the mucus layer is indicated, are presented in Figure 4. Similar to the rabbit, a very thin layer of mucus was present in the duodenum (30.6 ± 8.8 μm). Contrary to the other animal models studied here, no differences in mucus thickness were noticed along the small intestine or along the large intestine using this method ($P > 0.05$). The values reported here for mucus thickness were higher than those found in rats using unfixed sections stained with Alcian blue visualized under inverted phase microscopy.[45] The washing step used in the previous method may have resulted in the loss of the loosely bound mucus layer. In contrast, gastric mucus thickness values reported here were generally lower than those observed by Jordan *et al.*[51] However, it must be noted that in that study, rats were fasted for 12 h before use, contrasting to our setup, where rats had free access to food and water. It has been demonstrated that a positive correlation exists between mucin and acid secretion in the stomach. The more acid secreted, the more mucus needs to be produced to provide the necessary protection to the underlying epithelium.[57]

Discussion

Animal models have been widely used in preclinical research and development. The choice of the animal model is highly dependent on the expected outcome and physiology differences are critical. The modified periodic acid Schiff/Alcian blue method, first proposed by Jordan *et al.*,[51] using rat and human gastric tissues, is able to preserve the mucus layer due to the elimination of dehydration steps involved in traditional staining methods.[51,56] Furthermore, this method provides similar mucus thickness measurements to those obtained *in vivo* in the stomach of rats.[47]

A thick layer of mucus (144 ± 52 μm) has been observed in the antrum of the human stomach for the group of subjects studied.[51] These values are close to those observed in our study in the antrum of pig (222.2 ± 112.2 μm). The mucus thickness in the rat gastric antrum was lower (69.4 ± 24.8 μm) than the values reported for pig and rabbit ($P \leq 0.05$). This thicker mucus present in the stomach provides a protective barrier to the diffusion of hydrogen ions

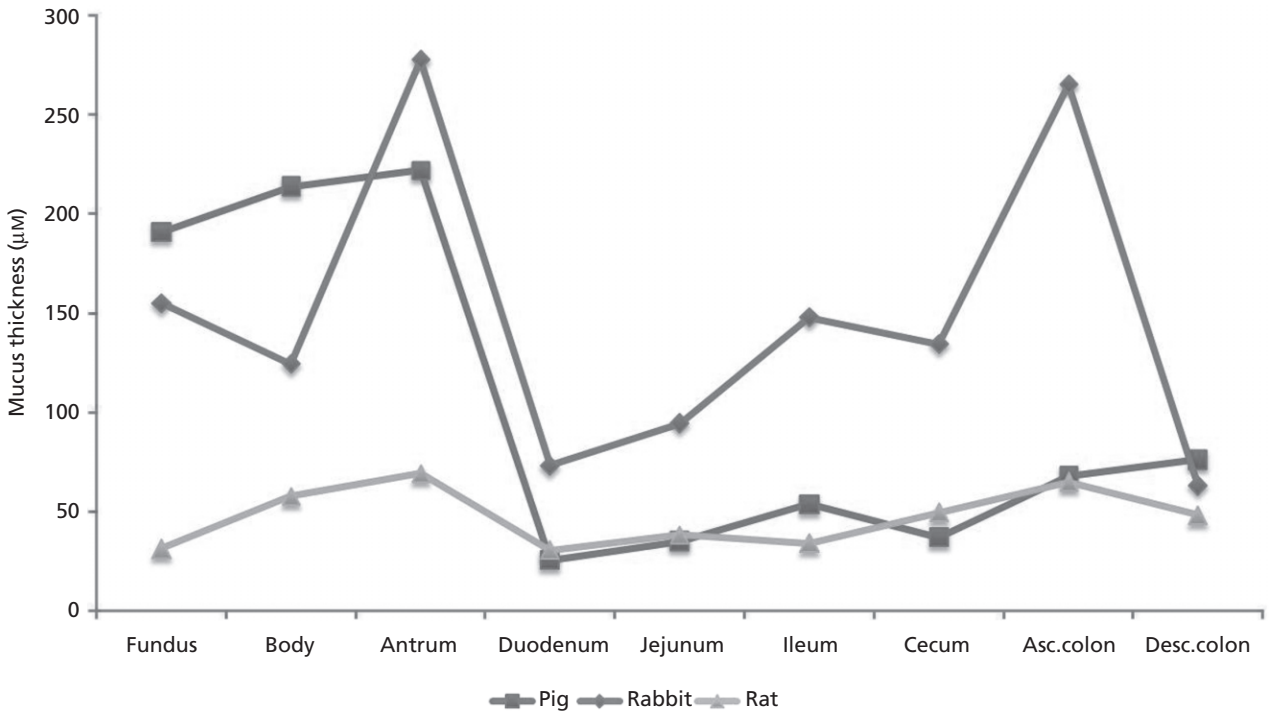

Figure 1 Mucus thickness patterns along the gastrointestinal tract of common laboratory animal models.

and pepsin, which in combination with bicarbonate secretion, maintains a neutral pH on the epithelium surface.[5,54,58] Studies by Atuma *et al*. demonstrated that the rat gastrointestinal mucus lining is composed of an adherent mucus layer and a loosely bound mucus layer.[10] The adherent mucus layer has been observed to be much thicker in the stomach, providing protection to the underlying epithelium, whereas the loosely bound mucus layer provides lubrication properties.[5] Infection by *Helicobacter pylori* can change the dynamics of the gastric mucus layer. It has been shown that *H. pylori* can be lodged deep in the mucus layer, protected from the acid contents. This is possible due to its flagella, which promote its movements through the mucus layer and the ability to increase the microenvironmental pH of the mucus, reducing its rheological properties.[59]

In the small intestine, particularly in the ileum, and large intestine, both the adherent and loosely bound mucus layers have also been reported to be thick. This serves two different purposes. The adherent mucus layer provides, along with the cell surface mucins,[60] protection to the mucosa from luminal contents, from macromolecules[61] and from the endogenous bacteria.[11,62] Colonization of the gastrointestinal tract by bacteria is mediated by binding to specific glycans in the mucus layer, particularly in the loosely bound layer, which is mainly composed of mucins degraded by proteolysis or oligosaccharide hydrolysis.[9,38,63] This loosely bound mucus layer is fundamental to provide lubrication to the luminal contents in the lower gastrointestinal tract and serves as a source for bacterial binding and feeding.[6,13]

Mucus thickness in the human colon, determined by the same method as employed in our study, has been reported to increase from the proximal (10–30 μm in the caecum) to the distal regions (30–85 μm in the rectum).[52] This pattern was also partially observed in the pig and comparable values of mucus thickness were observed in our study. The mucus thickness in the rabbit large intestine mucosa was shown here to be higher than in the human colon, particularly in the caecum (134.42 ± 88.4 μm) and ascending colon (265.1 ± 125.6 μm) (Table 1). This thicker secreted mucus layer in the large intestine of rabbit may be explained by the particular dietary cycle. Along with hard faecal pellets, mainly composed of excreted fibre, rabbits also excrete cecotropes, which are rich in nutrients but cannot be completely absorbed in the colon. These cecotropes are formed in the caecum and are covered with mucus to help bind them together, lending protection from the acid in the stomach during a second passage through the rabbit gut.[64,65]

Interestingly, gut microbiota and dietary composition have been shown to affect the secretory pattern of intestinal mucins (particularly in the jejunum and proximal colon) in rats. In the small intestine, the effect of diet was more pronounced, whereas in the large intestine the microbiota is the main modulator.[6,66,67] Therefore, interspecies variations in

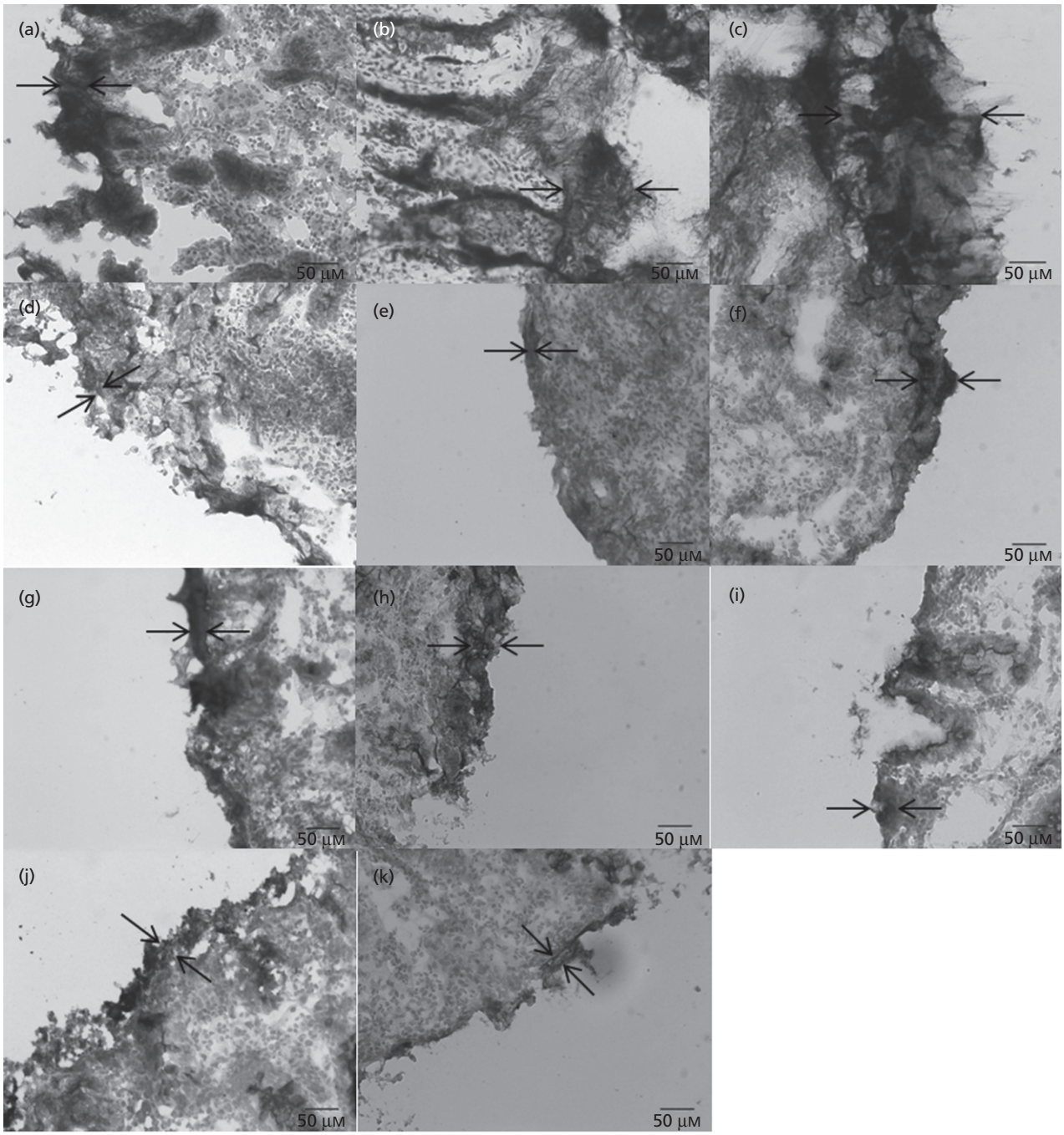

Figure 2 Microphotographs of gastrointestinal sections of pig stained by the periodic acid Schiff/Alcian blue (PAS/AB) method. (a) fundus, (b) body, (c) antrum, (d) duodenum, (e) jejunum, (f) ileum, (g) caecum, (h) ascending colon, (i) transverse colon, (j) descending colon, (k) rectum. The secreted mucus layer is limited by the arrows displayed. Total magnification (100×).

terms of mucus thickness may not be only linked to physiology but also to external factors.

These interspecies differences in terms of mucus thickness may contribute to the interspecies variability in terms of drug absorption and overall oral drug bioavailability. This can be extended to the situation in the diseased gut where changes in mucus thickness need to be factored in. For instance, a thinner mucus layer, dependent on disease severity, has been observed in patients with ulcerative colitis and Crohn's disease,[52] which has been linked with a depletion of goblet

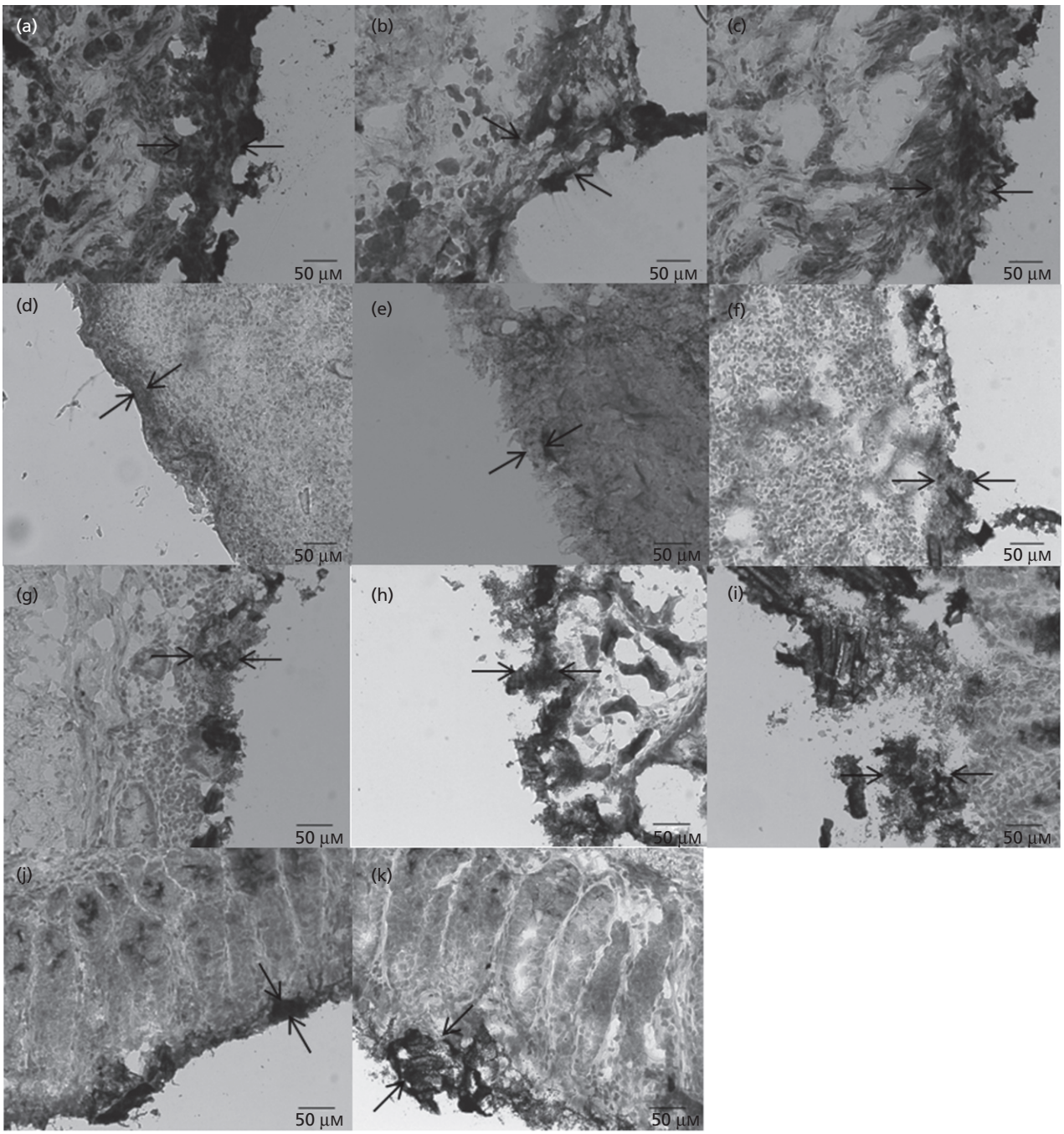

Figure 3 Microphotographs of gastrointestinal sections of rabbit stained by the periodic acid Schiff/Alcian blue (PAS/AB) method. (a) fundus, (b) body, (c) antrum, (d) duodenum, (e) jejunum, (f) ileum, (g) caecum, (h) appendix, (i) ascending colon, (j) descending colon, (k) rectum. The secreted mucus layer is limited by the arrows displayed. Total magnification (100×).

cells.[68] Therefore, variability in oral drug bioavailability would result from different extents of drug binding to mucus[19] and differences in drug diffusion rates through the mucus layer.[20] A thicker mucus layer offers a longer pathway through which a drug diffuses. This is particularly relevant for high-molecular-weight drugs or peptides and proteins.[18] Moreover, thicker mucus also provides more available groups to establish mucus–drug interactions. From the results presented here, the pig more closely resembles the human in terms of mucus thickness in the gastrointestinal tract.

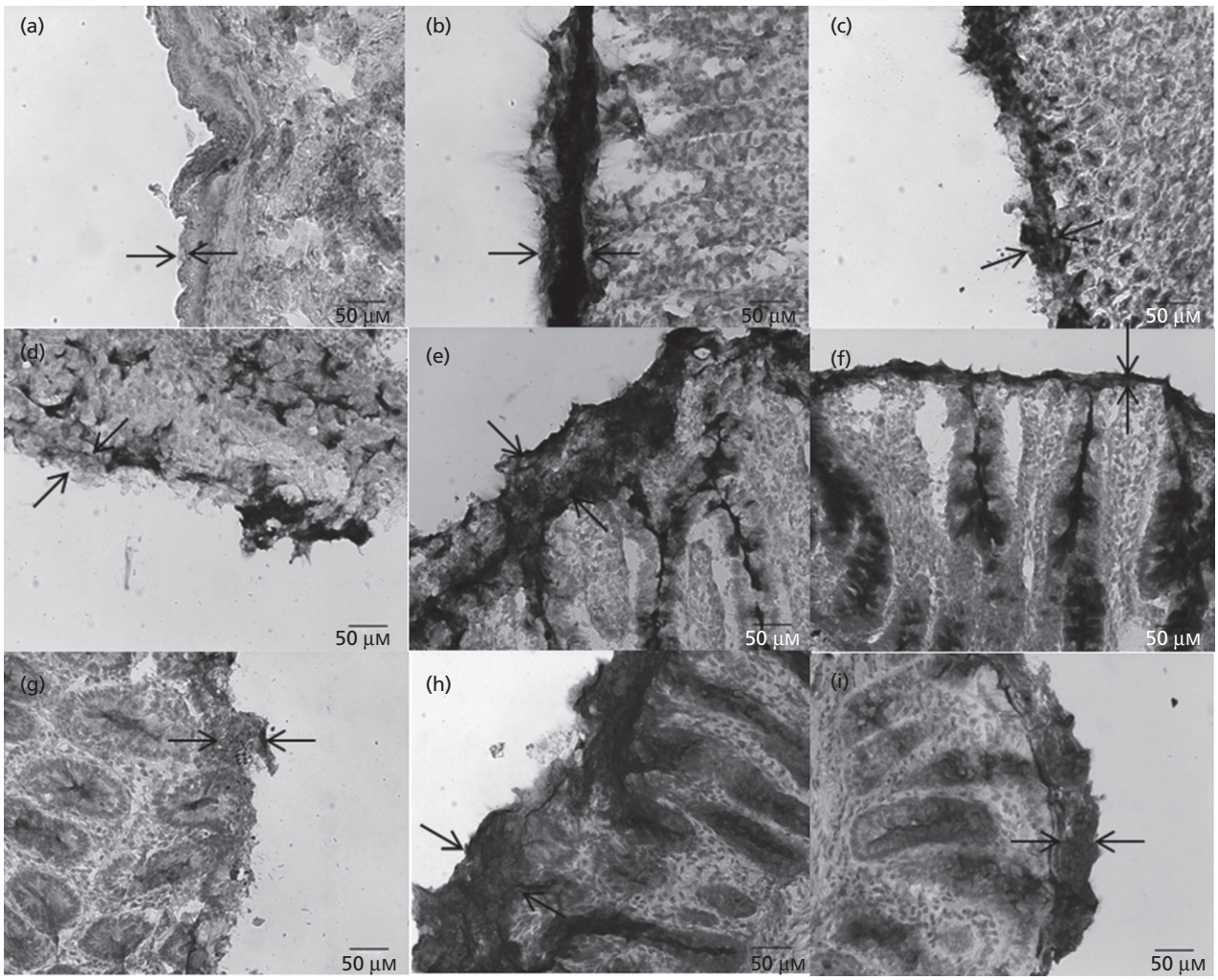

Figure 4 Microphotographs of gastrointestinal sections of rat stained by the periodic acid Schiff/Alcian blue (PAS/AB) method. (a) fundus, (b) body, (c) antrum, (d) duodenum, (e) jejunum, (f) ileum, (g) caecum, (h) appendix, (i) ascending colon. The secreted mucus layer is limited by the arrows displayed. Total magnification (100×).

Therefore, the pig may be a better animal model in the preclinical assessment of drug absorption, particularly for those drugs more susceptible to mucus binding or those for which absorption is rate-limited by the mucus layer. However, other factors need to be considered, such as the transit time, fluid volumes available, metabolism and transporters.

Conclusions

To our knowledge, this is the first study to provide a systematic assessment of the mucus thickness pattern along the gastrointestinal tract of common laboratory animals. Interspecies differences in the gastrointestinal mucus thickness were demonstrated. Small laboratory animal models, such as the rat differed most to man, whereas pig was shown to most closely resemble the mucus distribution along the gut of humans. These findings may have significant implications for the choice of the right animal model for preclinical studies, in particular if the mucus layer has a significant role in the expected outcome, such as in oral drug absorption studies or assessment of mucoadhesive drug delivery systems.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/30160/2006).

References

1. Forstner JF. Intestinal mucins in health and disease. *Digestion* 1978; 17: 234–263.
2. Allen A. Structure of gastrointestinal mucus glycoproteins and the viscous and gel-forming properties of mucus. *Br Med Bull* 1978; 34: 28–33.
3. Bell AE *et al*. Functional interactions of gastric mucus glycoproteins. *Int J Biol Macromol* 1984; 6: 309–315.
4. Allen A *et al*. Mucus glycoprotein structure, gel formation and gastrointestinal mucus function. In: Nugent J, O'Connor M, eds. *Ciba Foundation Symposium – Mucus and Mucosa*. London: Pitman Publishing Ltd, 1984: 137–156.
5. Phillipson M *et al*. The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G211–G2G9.
6. Johansson MEV *et al*. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *PNAS* 2011; 108(1): 4659–4665.
7. Linden SK *et al*. Mucin dynamics in intestinal bacterial infection. *PLoS ONE* 2008; 3: e3952.
8. McGuckin MA *et al*. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* 2011; 9: 265–278.
9. Hoskins LC, Boulding ET. Mucin degradation in human colon ecosystems: evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. *J Clin Invest* 1981; 67: 163–172.
10. Atuma C *et al*. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Gastrointest Liver Physiol* 2001; 280: 922–929.
11. Johansson MEV *et al*. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* 2008; 105: 15064–15069.
12. Gouyer V *et al*. The extraordinarily complex but highly structured organization of intestinal mucus-gel unveiled in multicolor images. *PLoS ONE* 2011; 6: e18761.
13. Linden SK *et al*. Mucins in the mucosal barrier to infection. *Mucosal Immunol* 2008; 1: 183–197.
14. Karlsson NG *et al*. The glycosylation of rat intestinal Muc2 mucin varies between rat strains and the small and large intestine. A study of O-linked oligosaccharides by a mass spectrometric approach. *J Biol Chem* 1997; 272: 27025–27034.
15. Tam PY, Verdugo P. Control of mucus hydration as a Donnan equilibrium process. *Nature* 1981; 292: 340–342.
16. Verdugo P. Mucin exocytosis. *Am Rev Respir Dis* 1991; 144: A33–AS7.
17. Bell AE *et al*. Properties of gastric and duodenal mucus: effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure. *Gastroenterology* 1985; 88: 269–280.
18. MacAdam A. The effect of gastrointestinal mucus on drug absorption. *Adv Drug Deliv Rev* 1993; 11: 201–220.
19. Bhat PG *et al*. Drug binding to gastric mucus glycoproteins. *Int J Pharm* 1996; 134: 15–25.
20. Braybrooks MP *et al*. The effect of mucin on the bioavailability of tetracycline from the gastrointestinal tract: in vitro and in vivo correlations. *J Pharm Pharmacol* 1975; 27: 508–515.
21. Kearney P, Marriott C. The effects of mucus glycoproteins on the bioavailability of tetracycline. II. Binding. *Int J Pharm* 1987; 35: 211–217.
22. Kearney P, Marriott C. The effects of mucus glycoproteins on the bioavailability of tetracycline. III. Everted gut studies. *Int J Pharm* 1987; 38: 211–220.
23. Harding SE. Trends in mucoadhesive analysis. *Trends Food Sci Techn* 2006; 17: 255–262.
24. Khanvilkar K *et al*. Drug transfer through mucus. *Adv Drug Deliv Rev* 2001; 48: 173–193.
25. Larhed AW *et al*. The influence of intestinal mucus componenents on the diffusion of drugs. *Pharm Res* 1998; 15: 66–71.
26. Flemstrom G *et al*. Adherent surface mucus gel restricts diffusion of macromolecules in rat duodenum in vivo. *Am J Physiol Gastrointest Liver Physiol* 1999; 277: 375–382.
27. Cone RA. Barrier properties of mucus. *Adv Drug Deliv Rev* 2009; 61: 75–85.
28. Sturroek N, Hopwood D. The effects of mucus on the binding of cationized ferritin by human and animal gastrointestinal epithelium. *Histochemistry* 1986; 85: 255–258.
29. Lai SK *et al*. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev* 2009; 61: 158–171.
30. Varum FJO *et al*. Mucoadhesion and the gastrointestinal tract. *Crit Rev Ther Drug Carrier Syst* 2008; 25: 207–258.
31. Peppas NA, Buri PA. Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues. *J Control Release* 1985; 2: 257–275.
32. Harding SE. Mucoadhesive interactions. *Biochem Soc Trans* 2003; 31: 1036–1041.
33. Shahnaz G *et al*. Synthesis, characterization, mucoadhesion and biocompatibility of thiolated carboxymethyl dextran-cysteine conjugate. *J Control Release* 2010; 144: 32–38.
34. Sellers LA *et al*. Mechanical characterization and properties of gastrointestinal mucus gel. *Biorheology* 1987; 24: 615–623.
35. Robbe C *et al*. Evidence of regiospecific glycosilation in human intestinal humans. Presence of an acidic gradient along the intestinal tract. *J Biol Chem* 2003; 278: 46337–46348.
36. Robbe C *et al*. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J* 2004; 384: 307–316.
37. Lehr C-M *et al*. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. *Int J Pharm* 1991; 70: 235–240.
38. Corfield AP *et al*. Mucin degradation in the human colon: production of sialidase, sialate O-acetyltransferase, N-acetylneuraminate lyase, Arylesterase, and Glycosulfatase activities by strains of feacal bacteria. *Infect Immun* 1992; 60: 3971–3978.
39. Corfield AP *et al*. Sialic acids in in human gastric aspirates: detection

of 9-O-lactyl- and 9-O-acetyl-N-acetylneuraminic acids and a decrease in total sialic acid concentration with age. *Clin Sci* 1993; 84: 573–579.
40. Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos* 1995; 16: 351–380.
41. McConnell EL *et al.* Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol* 2008; 60: 63–70.
42. Merchant HA *et al.* Assessment of gastrointestinal pH, fluid and lymphoid tissue in the guinea pig, rabbit and pig, and implications for their use in drug development. *Eur J Pharm Sci* 2010; 42: 3–10.
43. Bickel M, Kauffman GLJ. Gastric gel mucus thickness: effect of distention, 16,16-dimethyl prostaglandin e2, and carbenoxolone. *Gastroenterology* 1981; 80: 770–775.
44. Kerss S *et al.* A simple method for measuring thickness of the mucus gel layer adherent to rat, frog and human gastric mucosa: influence of feeding, prostaglandin, N-acetylcysteine and other agents. *Clin Sci* 1982; 63: 187–195.
45. Rubinstein A, Tirosh B. Mucus gel thickness and turnover in the gastrointestinal tract of the rat: response to cholinergic stimulus and implication for mucoadhesion. *Pharm Res* 1994; 11: 794–799.
46. Varum FJO *et al.* An investigation into the role of mucus thickness on mucoadhesion in the gastrointestinal tract of pig. *Eur J Pharm Sci* 2010; 40: 335–341.
47. Schade C *et al.* Hydrogen-ion concentration in the mucus layer on top of acid-stimulated and acid-inhibited rat gastric mucosa. *Gastroenterology* 1994; 107: 180–188.
48. Chu S *et al.* Dynamic regulation of gastric surface pH by luminal pH. *J Clin Invest* 1999; 103: 605–612.
49. Akiba Y *et al.* Dynamic regulation of mucus thickness in the rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 2000; 279: G437–GG47.
50. Matsuo K *et al.* Histochemistry of the surface mucous gel layer of the human colon. *Gut* 1997; 40: 782–789.
51. Jordan N *et al.* A novel method for the visualization of the in situ mucus layer in rat and man. *Clin Sci* 1998; 95: 97–106.
52. Strugala V *et al.* Thickness and continuity of the adherent colonic mucus barrier in active and quiescent ulcerative colitis and Crohn's disease. *Int J Clin Pract* 2008; 62: 762–769.
53. Dixon J *et al.* Esophageal mucin: an adherent mucus gel barrier is absent in the normal esophagus but present in columnar-lined Barrett's esophagus. *Am J Gastroenterol* 2001; 96: 2575–2583.
54. Allen A, Carrol NJH. Adherent and soluble mucus in the stomach and duodenum. *Dig Dis Sci* 1985; 30(11 Suppl.): 55S–62S.
55. Jubeh TT *et al.* Differential adhesion of normal and inflamed rat colonic mucosa by charged liposomes. *Pharm Res* 2004; 21: 447–453.
56. Strugala V *et al.* Colonic mucin: methods of measuring mucus thickness. *Proc Nutr Soc* 2003; 62: 237–243.
57. Niv Y *et al.* Association between gastric acid and mucin secretion in dyspeptic patients. *Digestion* 2002; 65: 141–148.
58. Sellers LA *et al.* Misoprostol-induced increases in adherent gastric mucus thickness and luminal mucus output. *Dig Dis Sci* 1986; 31(Suppl. 2): 91S–95S.
59. Celli JP *et al.* Helicobacter pylori moves through mucus by reducing mucin viscoelasticity. *PNAS* 2009; 106: 14321–14326.
60. McAuley JL *et al.* MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest* 2007; 117: 2313–2324.
61. Iiboshi Y *et al.* Developmental changes in distribution of the mucous gel layer and intestinal permeability in rat small intestine. *JPEN J Parenter Enteral Nutr* 1996; 20: 406–411.
62. Forstner JF *et al.* Production, structure, and biologic relevance of gastrointestinal mucins. In: Blaser MJ *et al*, ed. *Infections of the Gastrointestinal Tract*. New York: Raven Press, 1995: 71–88.
63. Kim YS, Ho SB. Intestinal Goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* 2010; 12: 319–330.
64. Irlbeck NA. How to feed the rabbit *(Oryctolagus cuniculus)* gastrointestinal tract. *J Anim Sci* 2001; 79: 343–346.
65. Harrenstien L. Gastrointestinal diseases of pet rabbits. *Sem Avian Exotic Pet Med* 1999; 8: 83–89.
66. Sharma R *et al.* Rat intestinal mucosal responses to a microbial flora and different diets. *Gut* 1995; 36: 209–214.
67. Sharma R, Schumacher U. Morphometric analysis of intestinal mucins under different dietary conditions and gut flora in rats. *Dig Dis Sci* 1995; 40: 2532–2539.
68. Longman RJ *et al.* Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. *J Histochem Cytochem* 2006; 54: 1335–1348.

