Sex differences in the gastrointestinal tract of rats and the implications for oral drug delivery

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Key words

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ABSTRACT

Pre-clinical research often uses rodents as animal models to guide the selection of appropriate oral drug and dose selection in humans. However, traditionally, such research fails to consider the gastrointestinal differences between the sexes of rats and the impact on oral drug delivery. This study aimed to identify and characterise the potential sex-related differences in the gastrointestinal environment of sacrificed male and female Wistar rats. Their gastrointestinal tracts were excised and segmented into the stomach, duodenum, jejunum, ileum, caecum and colon. The respective contents and tissue sections were collected and analysed for pH, buffer capacity, surface tension, osmolality and relative P-glycoprotein (P-gp) expression. The pH in the stomach of females was found to be lower than in males. Female rats also exhibited a higher buffer capacity in the caecum and colon when compared with their male counterparts. Males were found to have a higher osmolality than females in the duodenum, ileum and colon. Significant sex differences ($p<0.05$) in surface tension were observed in the ileum, where females exhibited a higher surface tension. Interestingly, female rats displayed significantly higher relative P-gp expression levels ($p<0.05$) when compared with male rats in the duodenum (1.24 ± 0.85 vs. 0.36 ± 0.26), jejunum (1.45 ± 0.88 vs. 0.38 ± 0.26) and ileum (0.92 ± 0.43 vs. 0.40 ± 0.18) but not in the colon (0.5 ± 0.32 vs. 0.33 ± 0.16) segments. The work reported has demonstrated the stark physiological differences between male and female rats at a physiological level, indicating how the ‘sex of the gut’ could influence oral drug delivery. These findings, therefore, are of critical importance in pre-clinical research and drug development.

1. INTRODUCTION

It is widely understood that females and males respond to medicines differently. In clinical practice, negating the impact of sex on treatment could have severe

consequences; related to unexpected side effects or inadequate therapeutic efficacy. Despite this, early phase drug development traditionally fails to evaluate the differences between the sexes [1]. In particular, pre-clinical research has demonstrated a tendency to focus on males in both cell and animal studies, which may be obscuring key sex differences, as well as affecting important decisions during pre-clinical development [2, 3].

In an attempt to reduce the male-orientated bias in pre-clinical research, the US National Institute of Health (NIH) required applicants to incorporate a sex-balanced group of animals in pre-clinical studies [1, 3]. However, despite this call to action, there has not yet been a corresponding revolution or an agreed standardisation amongst the entire pre-clinical research community. To date, publications often continue to neglect the need for sex-based evaluation in pre-clinical studies [4, 5].

Laboratory animals are routinely used during pre-clinical research to act as intermediary models to identify compounds with promising biopharmaceutical properties. The majority of oral medicines are tested pre-clinically on rats due to their inexpensiveness and ease of handling [6]. Therefore, having an understanding of the gastrointestinal (GI) physiological differences between male and female rats is of utmost importance. However, basic information about their GI physiology, especially between the sexes, is not completely understood.

Previous studies from our group have evaluated the impact of ageing on the GI environment in rats [7], as well as the assessment of the GI environment in different animal models, ranging from the guinea pig, rabbit and pig [8]. With regards to sex differences, Oltra-Noguera. *et al.* [9] evaluated the differences in intestinal drug

absorption between male and female rats, as well as within different rat strains. Results found that there were significant differences in verapamil permeability between the sexes in several strains, demonstrating how potential sex-related differences may affect pre-clinical results.

As such, this present study aimed to identify and characterise the physiological differences in GI fluid (pH, buffer capacity, surface tension and osmolality) and tissue composition (relative P-gp expression) between male and female rats, and to further understand their implications on oral drug delivery.

2. MATERIALS AND METHODS

2.1. Materials

HPLC-grade water, methanol, peroxide-free tetrahydrofuran and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK). NaOH and HCl (0.1M standards) were used for buffer capacity determinations and were procured from Sigma Aldrich (Dorset, UK). Krebs-Bicarbonate Ringer's solution (KBR), pH 7.4, was composed of 10mM D-glucose, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 115mM NaCl, 25mM $NaHCO_3$, 0.4Mm KH_2PO_4 and 2.4mM K_2HPO_4 (Clarke, 2009). Lysis buffer was freshly prepared with 50mM Tris, 250mM NaCl, 5mM EDTA, 1mM Na_3VO_4, 1mM PMSF, 1% Nonidet P40 and protease inhibitor cocktail in PBS (phosphate-buffered saline). All other chemicals and kits are noted individually in the following methods.

2.2. Gastrointestinal tissues

8-week-old Wistar Hanover Outbred rats (8 males and 6 females), weighing 180g and 235g respectively, were purchased from Envigo Ltd. (Oxfordshire, UK). All animals

were fed *ad libitum* with Teklad Global 18% Protein Rodent Diet (Harlan Ltd., Oxfordshire, UK) and given free access to tap water. All procedures were approved by the Home Office (PPL No.70/6421) and were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK.

2.3. Gastrointestinal fluids

All measurements were performed on the supernatant obtained from the gastrointestinal fluids of the rats, except pH which was also measured *in situ*. The animals were sacrificed on the morning of the same day, in the same conditions, by CO_2 asphyxiation and the GI tract was promptly excised. This was divided into the stomach (antrum; glandular stomach, and fundus; forestomach), duodenum, jejunum, ileum, caecum and colon (within 10 minutes). The gastrointestinal sections were emptied into 1.5mL Eppendorf tubes and centrifuged (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 13000rpm for 20min. The supernatant obtained was kept at -80°C until analysed as followed:

Osmolality was measured with a Digital Micro-Osmometer (Type 5R), Hermann Roebling MESSTECHNIK, Berlin, Germany.

Surface tension was measured using a Delta 8 Tensiometer (Kibron Inc), controlled by the Delta-8 manager software (version 3.8). The measurement was performed using a DynePlates (96-well plate designed for tensiometer) with 50µL of sample placed in each well.

The pH was first measured *in situ* in the GI tract section by introducing the pH probe into the opening created by sectioning parts of the GI tract. For each GI segment, two *in situ* measurements were taken; one at the proximal opening (A) and the second at

the distal region (B). Due to the limited volume of fluids available from some of the intestinal segments, however, some tests were run in pooled samples, in which fluids from the same segment of different animals were mixed to increase the available volume to perform the tests. This includes the further measurement of pH and buffer capacity of the GI supernatants using a pH meter (HI99161) equipped with an FC202 electrode, designed for measurements of viscous and semi-solid materials (Hannah Instruments, Bedfordshire, UK).

Buffer capacity in pooled rodent samples, was measured at a pH change of 1.0 unit by adding aliquots (10µL) of 0.1M HCl (for intestinal fluids) or 0.1M NaOH (for gastric fluids) to a 300µL supernatant pooled GI fluid sample to achieve the desired pH change. Buffer capacity was then calculated using the following equation:

$$\beta\ (mmol/L/\Delta\mathrm{pH}) = \frac{M_a \times V_a}{\Delta pH} \times \frac{1000}{V_b}$$

Equation 1. β denotes the buffer capacity, M_a is the molarity of the acid, V_a is the volume of acid in mL, V_b is the volume of buffer in mL and ΔpH is the change in pH.

2.4. Western-blotting Studies

2.4.1 Total protein extraction and quantification

The whole intestine, including the duodenum, jejunum, ileum and colon, was removed from the sacrificed rats and stored in KBR solution. 1L of KBR solution (pH 7.4) was prepared freshly before the experiment at room temperature (25^{o}C). 1cm segments were cut from the duodenum, jejunum, ileum and colon. The mucosal tissue was then obtained by placing the tissue segments on an ice-cold glass plate and the serosa layer was gently squeezed out.

To extract the tissue protein, each segment was placed into a glass vial containing 3mL of lysis buffer and homogenised for 20 seconds at 10,000 rpm (T18 digital ULTRA-TURRAX®; IKA). The homogenised segment solutions were then incubated at 4^{o}C for 2 hours and transferred to 1.5mL Eppendorf tubes and centrifuged at 10,000 rpm at 4^{o}C for 10min. The supernatants, as the final extracted protein samples, were transferred to micro-tubes and then stored at -20^{o}C until analysis. Protein quantification was assessed by adapting the instructions from the Pierce BCA Protein Assay Kit (ThermoFisher), detailed in section 2.4.2.

2.4.2 Determination of relative P-gp expression in rat intestine

Protein samples (25μg) were suspended in NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA) and then kept in a 70^{o}C incubator for 10min denaturation. The denatured protein sample was then loaded on a NuPAGETM NovexTM 4–12% Bis-Tris gel (Invitrogen). 5μL Sharp Pre-Stained protein standard (Invitrogen) was also loaded as molecular weight marker. Protein gel electrophoresis was then undertaken according to the protein gel electrophoresis protocol from Invitrogen. The separated protein samples were then transferred to a nitrocellulose membrane with XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) according to the manufacturer's instructions. Membranes were blocked with 3% bovine serum albumin (BSA) in TBS-T； mouse monoclonal anti-P-gp (C-219 3:200; Enzo Life Science, Exeter, UK) and anti-β-actin mouse monoclonal antibody (1:2000; Sigma-Aldrich, Poole, UK). Samples were incubated for 1h at room temperature (25℃). For the detection of P-gp and reference protein (β-actin), blots were incubated for 1h at room temperature with the respective primary antibodies being diluted in a 3% BSA in TBS-T. The detection of bound antibodies was completed with affinity-purified rabbit anti-mouse IgG coupled to peroxidase (secondary antibody; Sigma) diluted to 1:5000 in

3% BSA in TBS-T. After a 1h incubation with the secondary antibody conjugated with horseradish peroxidase, protein bands were visualised by chem-iluminescence detection method with Pierce™ ECL Western Blotting Substrate (ThermoFisher). The blots were then photographed with a ChemiDoc XRS camera (Bio-Rad). The detection of P-gp and the reference protein bands were performed with the Image Lab™ software (Bio-Rad). For a calculation of the relative P-gp expression in selected groups, the reference protein was individually set to 1. The intensity of P-gp was consequently set relative to it.

2.5 Statistical analysis

The data was analysed by one-way ANOVA, followed by a post-hoc Tukey test with a 95% confidence interval using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A Univariate General Linear Model tool was used with a Tukey post-hoc analysis, taking species and locations as fixed factors.

3. RESULTS AND DISCUSSION

3.1. Gastrointestinal fluid pH

Figure 1 represents how the pH measured *in situ* changes along the GI tract in both male and female Wistar rats. For both sexes, the GI fluid pH profile followed a similar trend and such findings were comparable to trends published in the literature [7, 10]. In both males and females, pH was lowest in the stomach, with the antrum (also known as the glandular stomach) having a lower pH value than the fundus (also known as the forestomach).

Interestingly, females were found to exhibit a significantly ($p<0.05$) lower pH in the fundus than males (pH values of 3.6 vs. 4.6, respectively). As the fundus does not contain acid-secreting glands, this in part could be due to the different feeding behaviours of male and female rats. In this present study, rats had free access to food and water which, therefore, may have contributed to the differences seen. Another reason could be due to the differences in male and female rat physiology; previous research has shown that oestrogens are inhibitors of the gastric acid secretion whilst testosterone is an inducer [11, 12]. However, in a recent study, it was found that males have a higher gastric blood flow than their female counterparts [13]. Oestrogen administration was able to reduce the mean blood flow in the gastric mucosa by 31% in males, however, remained largely unchanged in females. The thickening of the mucus layer was also demonstrated at a faster rate in females than males. This suggests that females may be more “resistant” to feminine hormones and may be more effective in repairing damage to the gastric wall. If the mucus-producing rate of females is higher, it may suggest an evolutionary biological adaptation to higher stomach acidity.

In both sexes, a sharp rise in pH was observed from the antrum to the duodenum, which remained stable until the distal ileum where a small pH increase was observed. Such findings were also reported in previous studies [7]. This finding can be explained due to the presence of bicarbonate ions, bile and other species that neutralise the stomach acid. The pH then reduced markedly in the caecum and the colon, which was expected as it is a common site for fermentation and acid species production [14]. GI tract pH values were similar in both sexes between the antrum and the proximal colon. However, there were statistically significant ($p<0.05$) differences in the proximal

duodenum, distal jejunum and distal colon. The differences observed were likely due to the small standard deviation and are not considered to be relevant.

The standard deviation was highest in the stomach (both fundus and antrum) and in the distal portion of the colon, whilst remaining quite low throughout the small intestine. This suggests that inter-individual variability was higher in these regions, which may be due to the animals having free access to water and food. Following rodent sacrifice, it was observed the rats had different volumes of gastric contents, and thus, may have contributed to the variability observed. As for the colon, the higher variability may be due to the differences in microbial flora of each animal.

GI fluid pH is widely known to affect drug ionisation by influencing drug solubility, stability, absorption and, ultimately, bioavailability. As such, the observed differences in GI fluid pH between male and female rats may have implications for the *in vivo* testing of oral dosage forms. For example, the differences in pH between the sexes could affect the behaviour of pH-responsive formulations, potentially leading to incorrect pharmacokinetic extrapolation [10, 15].

3.2. Gastrointestinal fluid buffer capacity

The overall trend of buffer capacity was found to be relatively similar in male and female Wistar rats and followed the expected trend for both sexes (Figure 2) [15, 16]. In particular, buffer capacity was found to be higher in the stomach, lower in the small intestine (where it remained constant) and higher again in the caecum and colon. A higher buffer capacity in the stomach may be explained by the large amount of chyme

that is gradually released into the duodenum. As the chyme was converted into chyle, the buffer capacity reduced by approximately 20mmol.L^{-1}△pH^{-1}.

Females were found to have a higher buffer capacity than males across the whole GI tract. This is especially apparent in the caecum and in the colon, where 2-fold and 1.8-fold differences were observed between females and males, respectively. This could be explained by one of two reasons. Firstly, females may be producing a higher amount of buffering species in the caecum or colon. In particular, short-chain fatty acids (SCFAs) have been found to contribute to the overall buffer capacity of the luminal fluids [17]. SCFAs are mainly produced by certain bacteria taxa [18]. For example, the total faecal SCFA propionate concentration is linked to the abundance of Bacteroidetes [19], whereas SCFA butyrate is mainly produced by certain Coprococcus [20]. Both taxa have been reported to be more abundant in female rats [21] [22]. The higher amounts of SCFA-producing bacteria in the female distal gut may explain why the buffer capacity was higher in the female rats. Here, innate differences between the sexes may contribute to the microbiota distribution in rats, and thus, affect the buffer capacity of the luminal environment. A second theory could relate to the differences of the presence of food in female and male rats. In this study, the animals had free access to food and water prior to their sacrifice. When sacrificed, the rats were found to have different amounts of food in their GI tract. Co-ingestion of food and liquids have been known to impact buffer capacity and, as such, may have contributed towards the differences observed [23].

The buffer capacity of the GI luminal fluids plays a major role in the dissolution of ionisable drugs. In particular, buffer capacity determines the microclimate pH in the diffusion boundary layer adjacent to the dissolving surface [24]. As such, the large

differences between male and female rats buffer capacity may again pose implications for the *in vivo* testing of oral dosage forms. For example, colon-targeted formulations may exhibit different dissolution rates between male and female rat models due to the variation in buffer capacity.

3.3. Gastrointestinal fluid osmolality

The osmolality of GI fluids in male and female rats showed similar profiles across the whole GI tract (Figure 3), and followed a trend that was similar to other studies [7]. In both cases, osmolality was found to be higher in the stomach and reduced distally. Males were found to have a higher osmolality ($p<0.05$) than females in the duodenum, ileum and colon compared to females. In male rats, osmolality of the gastric contents was not statistically different from that in the proximal and mid small intestine due to high variability in gastric osmolality. This may be justified by the different gastric contents found in the animals, which was also observed to occur in humans [25]. Osmolality in the distal small intestine was also statistically similar to that of the caecum and the proximal colon, in spite of the mean values decreasing considerably.

GI fluid osmolality may contribute to oral drug absorption by affecting drug solubility; it has been reported that the “salting-out” and “salting-in” effect can change solubility [26]. Osmolality and fluid volume have been investigated in relation to buffer capacity and pH, given that the alteration of salt concentration and ingested fluids can affect both parameters through stimulating the secretion of gastric acid, bile and pancreatic juices [23, 27]. The continuous digestion and absorption of osmotically-active species may contribute to a distal reduction of the osmolality of the luminal environment. This finding is important due to the alteration of ionic content may further influence drug ionisation, and hence, limit drug absorption.

3.4. Gastrointestinal fluid surface tension

As shown in Figure 4, the surface tension values of GI fluids were similar amongst male and female rats. In both cases, the lowest surface tension value was recorded in the duodenum, which then increased distally. The surface tension of gastric acid in humans was previously reported to lie in the range of 35-45mNm^{-1} [28], which is also reflected in the results here. Significant sex differences were observed in the ileum, where females had a higher surface tension compared to males ($p<0.05$). The higher surface tension in the ileum of females suggests that they may be more efficient in the removal of bile salts from the luminal environment, leading to an increase in surface tension. The reason behind this is unclear, however, and may be related to a combined effect of higher reabsorption rates and a more efficient metabolism of bile salts. In the caecum and colon, the surface tension in females stabilised (equal to that of the ileum), whereas an increase was demonstrated in males.

Surface tension of the GI fluids from rats is significantly lower than that of water (which is approximately 72mN.m^{-1} [29]). This can be easily understood by the presence of a myriad of compounds that act as surfactants in the GI tract, of which bile salts are the most widely known [30]. Bile salts are released in the upper duodenum and, as a result, the surface tension of the chyme is reduced. Rats lack a gall bladder which signify that their bile is not concentrated and stored, but released continuously in the small intestine [31]. As a result, rat bile is reported to be more dilute, although secreted in relatively higher volumes, when compared with humans [31, 32]. This might lead to a constantly higher level of bile salts in the rat GI tract during the fasted state, allowing for a lower surface tension.

The standard deviation of these mean surface tension values were very low in the duodenum and jejunum, suggesting that the luminal environments in the upper GI tract were homogeneous with little inter-subject variability. However, in the ileum, caecum and colon, the standard deviation was very high, suggesting that the mechanism behind the distal increase of the surface tension is more variable. This supports the hypothesis of a variable, albeit more efficient, degradation or scavenging mechanism of retrieving bile salts in female rats compared to males.

For oral drug delivery, surface tension of GI fluids may impact drug absorption. Surface tension has been found to contribute to the degree of solvation of drug particles and respective wettability [33], as well as being inversely related to the dissolution rate of some active substances [34].

3.5. P-glycoprotein expression

Figure 5 shows that there was a significant sex difference ($p<0.05$) in the relative P-gp expression of rats under the aforementioned conditions. P-gp is an efflux transporter located at the apical side of epithelial cells. As a result, the absorption of xenobiotics, toxins or any other harmful substances is reduced due to the efflux transportation function of P-gp [35]. It is shown that female rats had a higher relative P-gp expression, while male rats displayed a lower expression level along the intestine. According to the Western-blotting results, shown in Figure 5, the relative expression of P-gp increased from the proximal to distal intestine in male rats were 0.36 ± 0.26 in the duodenum, 0.38 ± 0.26 in the jejunum, 0.40 ± 0.18 in the ileum and 0.33 ± 0.16 in the colon. Contrastingly, female rats displayed a relatively higher P-gp

expression level, especially in the duodenum, jejunum and ileum (1.24±0.85, 1.45±0.88 and 0.92±0.43, respectively). There was a statistically significant sex difference ($p<0.05$) in relative P-gp expression demonstrated along the whole intestine, except for the colon.

Interestingly, our results reported here contrast to the work of previous studies that have evaluated P-gp expression in rats. A study conducted by MacLean *et al.* [36] showed that the relative expression of P-gp in six male rats increased up to 5-fold from the proximal to distal intestine. In their study, there were no sex differences in P-gp expression along the intestine. The difference here may be due to the different rat strains, the housekeeping protein and potentially due to the fact that the previous study only investigated fasted-state rats. In this current study, Wistar male and female rats were used whereas Han-Wistar rats were used in the previous study. Moreover, the housekeeping protein used in the current study was beta-actin, whereas villin protein was used to normalise the P-gp expression in the previous study. More work is required to understand the difference between the current studies and previous studies; however, the work reported here provides an insight on the significant differences between the sexes.

In humans, previous studies regarding the sex differences in intestinal P-gp function have been inconsistent. A study conducted by Mouly *et al.* [37] investigated the P-gp expression level in the duodenum, the jejunum and the ileum obtained from four humans but did not mention sex differences as a variable. Following the re-analysis of the data reported by Schuetz *et al.* [38], Potter. *et al.* [39] reported that P-gp expression was higher in males when compared with females. Consequently, two studies were

later published and claimed that there were no sex difference exhibited in P-gp intestinal expression. In one study, Paine. *et al.* [40] investigated the P-gp expression in the proximal intestine (duodenum) in men and women. Their results revealed that there were no significant sex differences, (mean P-gp expression was 0.66 vs. 0.73 in males and females, respectively). Another study explored the P-gp expression in the stomach, the jejunum and the ileum regions of the intestine [41]. In all three portions, sex differences in P-gp expression were not observed.

The data presented here clearly shows the physiological differences between male and female rat models, which may in turn lead to differences in drug pharmacokinetics or efficacy. As P-gp is a biological membrane efflux transporter, which is capable of modulating the transmembrane activities of drugs in different organs [42], this could be particularly consequential if evaluating a novel drug that is a P-gp substrate. For example, previous studies have evaluated the permeability of of rhodamine 123 (Rho123; a known P-gp substrate) at different segmental regions of the rat small intestine. In fact, this study observed that the segmental differences in Rho123 permeability significantly correlated with P-gp activity, with permeability being highest in the middle ileum [43, 44]. With regards to sex differences, Oltra-Noguera. *et al.* [9] observed that the permeability of verapamil, which is a P-gp substrate, was significantly lower in females compared to male rats of the Wistar Unilever, CD*IGS and Long Evans strains. Our findings here could explain the reason behind this reduction in permeability could be due to a higher relative P-gp expression within female rats. However, it is worth noting that a different strain of rats was used in the current study (Wistar Hanover Outbred rats). Furthermore, the Western-blotting technique may have some limitations as it calculates only the relative expression of P-

gp as opposed to absolute expression. As such, it may be worth investigating absolute P-gp expression in future work.

5. CONCLUSION

Pre-clinical research often fails to evaluate the impact of sex differences on oral drug delivery. Here, the GI fluid and tissue environment (relative P-gp expression) of male and female rats were evaluated. GI fluid pH, buffer capacity, surface tension and osmolality were found to be similar in the sexes. Differences were predominantly identified in the stomach and in the distal portion of the GI tract (in the ileum, caecum and colon). In terms of relative P-gp expression, marked sex differences were observed along the intestinal tract. From the duodenum to the ileum segments, female rats expressed a significantly higher P-gp level when compared with their male counterparts ($p<0.05$). In the colon, however, no significant differences in relative P-gp expression were observed, with both male and female rats exhibiting low levels. Relative P-gp expression along the whole intestine in females was consistently high in the small intestine and dramatically reduced in the colon. Male rats, however, exhibited a consistently low level of P-gp along the whole intestine tract. The results presented in this study contribute towards an increased understanding of how the GI environment is innately affected by sex. Therefore, the work reported is especially important to consider during pre-clinical development, and during translation into humans.

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COMPETING INTERESTS

The authors declare no completing interests.

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Figure Captions

Figure 1. pH of the luminal environment of sections of the GI tract of male and female Wistar rats. A – Denotes proximal portion of the segment; B – denotes distal portion of the segment. The results are measured *in situ.* *denotes a statistical significance ($p<0.05$) between males and females.

Figure 2. Buffer capacity ($\Delta pH=1.0$) of pooled fluids of sections of the GI tract of male and female Wistar rats. The values are the mean of the repeated measurements.

Figure 3. Osmolality of the GI fluids in male and female Wistar rats. *denotes a statistically significant ($p<0.05$) between males and females.

Figure 4. Surface tension of the fluids of sections of the GI tract of male and female Wistar rats. *denotes a statistically significant ($p<0.05$) between males and females.

Figure 5. P-gp relative expression along the intestinal tract of male and female rats ($n = 6$). *denotes a statistically significant ($p<0.05$) between males and females.