An animal's sex influences the effects of the excipient PEG 400 on the intestinal P-gp protein and mRNA levels, which has implications for oral drug absorption.

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Abstract

There is a growing body of evidence which suggests that formerly regarded 'inert' pharmaceutical excipients have the potential to influence oral drug bioavailability. The solubilizing agent polyethylene glycol 400 (PEG 400), for instance, has a sex-specific effect on P-glycoprotein (P-gp)-mediated drug bioavailability. We hypothesized that such an effect could be via PEG-induced alteration of P-gp activity and/or expression to different extents in males and females. To test this hypothesis *in vivo*, we investigated the influence of orally administered PEG 400 on the protein content and mRNA expression of P-gp in different regions of the gastrointestinal tract in male and female rats. Fasted rats received an oral dose of PEG 400 and at different time intervals, rats were killed and their intestines were collected. The P-gp protein and mRNA expression in different intestinal segments (duodenum, jejunum, ileum and colon) were measured by Western blotting and PCR, respectively. It was found that P-gp protein and mRNA levels increased along the gastrointestinal tract in control animals (i.e. without PEG administration), and was higher in males compared to the female rats. The oral administration of PEG 400 decreased the P-gp expression in the jejunum, ileum and colon of males but not in the corresponding segments in females. This sex-dependent influence of PEG 400 on P-gp levels reflects and explains the sex-related effect of PEG 400 on oral absorption of certain drugs. The data further adds to the growing literature on the importance of taking into consideration an individual's sex for optimal drug administration.

Key words: sex difference, multi drug resistance protein 1 (MDR1), mRNA expression, protein abundance, polyethylene glycol 400, excipients, region-dependent.

1. INTRODUCTION

P-glycoprotein (P-gp), an ATP-binding cassette (ABC) protein, is often considered to be the most important efflux membrane transporter responsible for secreting passively

absorbed drugs and xenobiotics out of cells (Kobori et al., 2013). P-gp is widely expressed throughout the body, including in the luminal surfaces of epithelial cells of the small and large intestine, liver hepatocytes, proximal tubules of the kidney and blood-tissue barriers (Schinkel et al., 1994). P-gp has numerous substrates, including antibiotics, calcium channel blockers and anticancer agents, and its action can be inhibited by a wide range of chemicals and drugs (Sauna et al., 2004, Goole et al., 2010). It was based on this knowledge that the polypharmacy strategy to increase the bioavailability of P-gp substrate drugs was devised, by co-administering the drugs with a P-gp inhibitor.

At the same time, it has been found that the activity and expression of membrane transporters, including P-gp, can be directly and indirectly modified by pharmaceutical excipients that were formerly considered to be inert (Sauna et al., 2004, Goole et al., 2010). On the one hand, some of these excipients inhibit the **activity** of P-gp via a range of mechanisms. For example, Pluronic P85 reduces P-gp ATPase activity (Batrakova et al., 2004), Cremophor EL and Tween 80 enhance intestinal membrane fluidity (Regev et al., 1999) and PEGs induce mitochondrial toxicity and deplete the amount of intracellular ATP in male rat intestines (Johnson et al., 2002). On the other hand, certain excipients can alter the **expression** of P-gp. Lipid-based excipients (Peceol and Gelucire 44/14) decrease the P-gp protein level in Caco-2 cell lines (Sachs-Barrable et al., 2007), while Tween 20 and Eudragit S100 cause a reduction in the efflux of Rhodamine 123 via their inhibition on P-gp expression (Hodaei et al., 2015, Mohammadzadeh et al., 2014, Hodaee et al., 2013).

These emerging data demonstrate that the interaction between P-gp and assorted pharmaceutical excipients can be exploited in the formulation of oral dosage forms for the purpose of improving the bioavailability of drugs which are P-gp substrates. However, these improvements in drug bioavailability may differ in men and women, given that the intestinal P-gp abundance is greater in males than in females (Gerrard et al., 2004). Indeed, sex-related influence of the solubilizing agent PEG 400 on the bioavailability of ranitidine was reported, with up to a 63% and 47% increase in males

(human and rats, respectively), but with no change in the corresponding females (Ashiru et al., 2008, Afonso-Pereira et al., 2016). This sex-specific effect of PEG 400 on drug bioavailability also extends to another P-gp substrate, ampicillin, but not to metformin whose absorption is not controlled by P-gp. Furthermore, blocking P-gp with cyclosporine A (a P-gp inhibitor) eliminated the effect of PEG 400 on the bioavailability of the P-gp substrates ampicillin and ranitidine (Mai et al., 2017).

In light of the above and of other literatures regarding the modulation of P-gp activity and expression by PEG 400 (Ballent et al., 2012, Mariana et al., 2011), we proposed that the interaction between PEG 400 and P-gp is sex-dependent. Thus, the aim of the work reported here was to investigate the influence of PEG 400 and of an animal's sex on intestinal P-gp expression, with the purpose of ascertaining the reasons behind the aforementioned sex differences in the effect of PEG 400 on the bioavailability of ranitidine and ampicillin (Ashiru et al., 2008, Afonso-Pereira et al., 2016). Thus, we determined the following: i) the influence of an animal's sex on the protein abundance and mRNA expression of P-gp along the gastrointestinal tract; and ii) the effect of PEG 400 on these measurements, with the methods Western blotting and real-time reverse-transcription polymerase chain reaction (real time RT-PCR).

2. MATERIALS AND METHODS

2.1 Materials

Polyethylene glycol 400 (PEG 400) was purchased from Sigma-Aldrich (Poole, UK). Krebs-Bicarbonate Ringer's solution (KBR), pH 7.4, 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 phosphate-buffered saline (PBS). All other chemicals and kits are noted individually in the following methods.

2.2 Preparation of Intestinal Segments

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. Healthy 8-13 week old male and female Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250 g were used for excised rat intestine. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, provided with food and water, and were acclimatized for 7 days. One day before the experiments, the rats were fasted overnight and housed individually in metabolic cages.

On the day of the experiment, each rat was dosed with 26mg/kg PEG 400 using an oral gavage syringe. PEG 400 used at a dose of 26mg/kg was due to its greatest enhancement in P-gp substrates (ranitidine and ampicillin) bioavailability in rats (Afonso-Pereira et al., 2016, Mai et al., 2017). Subsequently, rats were killed after 15min, 30min, 60min, 90min, 120min or 180min using a CO_2 euthanasia chamber, and their intestines were immediately collected and kept in ice-cold KBR solution.

The intestines were cut into 4 segments: duodenum (1cm from the ligament of Treitz); jejunum (10cm from the ligament of Treitz); ileum (1cm from the cecum) and colon, which were then washed with ice-cold KBR solution. Roughly 2cm long pieces from the mid part of the duodenum (2 pieces), proximal part of the jejunum (3 pieces), the mid to distal part of ileum (1 piece) and the descending colon (2 pieces) were isolated and opened along their mesenteric border. The tissues were washed gently with KBR solution to remove the intestinal contents. To obtain the mucosal tissue, tissue pieces were placed on an ice-cold glass plate and the serosa layer was gently squeezed out with a cover slip, and divided into aliquots for determination of P-gp protein content and mRNA expression as detailed below.

2.3 Measurement of P-gp Protein Levels in Intestinal Segments by Western Blotting

The mucosal tissues (about 60mg) of each intestinal segment (duodenum, jejunum, ileum and colon) of 6 male and female rats were cut into small pieces and homogenized in 3mL lysis buffer at 10,00rpm for 20s on ice with a T18 digital ULTRA-TURRAX® (IKA, Wilmington, USA). The tissue homogenates were incubated at 4°C for 2h, then centrifuged at 10,000rpm for 10min. The total tissue protein was collected in the supernatants, and its concentration was subsequently determined with the Pierce™ BCA Assay Protein kit (ThermoFisher, Loughborough, UK) according to the manufacturer's instructions.

To measure the targeted protein (i.e P-gp) level, samples containing 25µg total protein were suspended in LDS (lithium dodecyl sulfate) sample loading buffer (Invitrogen, Carlsbad, CA) and denatured for 10 min at 70°C. As a molecular weight marker, 5µL of Sharp Pre-Stained protein standard (Invitrogen) was loaded on each gel.

Proteins in the samples were separated by electrophoresis in a NuPAGE™ Novex™ 4–12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane with XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) according to the manufacturer's instructions. Nitrocellulose membranes were blocked with 3% BSA (bovine serum albumin) in TBS-T (0.1% Tween 20 in tris-buffered saline) and incubated for 1h at room temperature. For detection of P-gp and reference protein (anti-β-actin), blots were incubated for 1h at room temperature with the respective primary antibodies diluted in 3% 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). Bound antibodies were detected with affinity-purified rabbit anti-mouse IgG coupled to horseradish peroxidase (secondary antibody; Sigma) diluted 1:5000 in 3% BSA in TBS-T.

After 1h incubation with the secondary antibody conjugated with horseradish peroxidase, protein bands were visualized by chemiluminescence detection with Pierce™ ECL Western Blotting Substrate (ThermoFisher), and subsequently photographed with a ChemiDoc XRS camera (Bio-Rad, Hertfordshire, UK). P-gp and

reference protein bands were qualified using the Image Lab™ software (Bio-Rad). To calculate the relative P-gp contents in the different samples, the reference protein band in each sample was set to 1, and the intensity of the P-gp band was measured relative to it.

2.4 Measurement of P-gp mRNA expression in Intestinal Segments by Real-Time Reverse-Transcription Polymerase Chain Reaction

Following collection of mucosal tissues (as described in Section 2.2), the tissue were kept in RNA later® Stabilization Solution (Thermofisher). Total RNA in each intestinal sample was isolated and purified with PureLink® RNA Mini Kit (Thermofisher), and RNA concentration was measured with Nanodrop 2000 (Thermofisher) according to the manufacturer's instructions.

Subsequently, the quantification of the target RNA was conducted as followed: 1 mg total RNA of each sample was reverse transcribed using the iScript™ cDNA Sythesis Kit (Bio-Rad). To quantify the amount of P-gp mRNA (*mdr1a* and *mdr1b)*, real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems, Thermofisher) using the method described in MacLean's study (MacLean et al., 2008). Briefly, 50μL PCR reaction contained 25μL of PowerUp™ SYBR Green PCR Master Mix (Thermofisher), 500nM each of forward and reverse primers, and 1μg of cDNA. Anti-beta actin was used for normalization and amplification of 1μg cDNA, respectively. Real-time PCR was carried out in 96 well PCR plates (Thermofisher). The amplification program for all genes consisted of one pre-incubation cycle at 95°C with a 10min hold, followed by 45 amplification cycles with denaturation at 95°C with a 10s hold, an annealing temperature of 50°C with a 10s hold, and an extension at 72°C with a 10s hold. Amplification was followed by a melting curve analysis, which ran for one cycle with denaturation at 95°C with a 1s hold, annealing at 65°C with a 15s hold, and melting at 95°C with a 1s hold. Distilled water was included as a negative control in each run to access specificity of primers and possible contaminants.

Primers (shown in Table 1) were designed by primer-BLAST searching with publicly available sequence information of the GeneBank of the National Center for Biotechnology Information (NCBI) and purchased from Eurofins (Eurofins Genomics, Germany).

Relative expression of *mdr1a* and *mdr1b* mRNA in different intestinal segments were calculated using 7500 software (version 2.0.6, Thermofisher). The average of the threshold cycle (Ct) values for tested genes (*mdr1a* and *mdr1b*) and the internal control (anti-beta actin) was taken, and then the differences between Ct values for tested genes and internal control (ΔCt) were calculated for all the experimental samples.

2.5 Statistical Analysis

The experiments were performed at least six times and data were expressed as mean ± standard deviation (S.D.). Significant differences among groups were analyzed by one-way ANOVA and three-way ANOVA using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A minimum p value of 0.05 was used as a significance level for the tests. Also, the relationship between P-gp protein levels and mRNA expression (both *mdr1a* and *mdr1b*) was investigated using Pearson product-moment correlation coefficient (r).

3. RESULTS AND DISCUSSION

3.1 P-gp Protein Level in Different Intestinal Segments

To obtain a general picture of the expression profile of P-gp along the intestinal tract, the amount of P-gp protein was quantified in the duodenum, jejunum, ileum and colon in male and female rats. As can be seen from Figure 1, there is a constant increase in the P-gp content along the intestine from proximal to distal parts (duodenum < jejunum < ileum < colon), with the trend being more obvious in males compared to female rats.

A progressive increase in P-gp protein abundance and activity from the proximal to the distal small intestine (Yumoto et al., 1999, Tian et al., 2002, Dahan and Amidon, 2009, Kagan et al., 2010), with the highest P-gp levels appearing in the colon (Fojo et al., 1987, Fricker et al., 1996) has previously been reported in rodents. In contrast to rodents, the P-gp contents in the intestine of human subjects is different. A recent study where the absolute P-gp content from six organ donors was quantified using LC-MC/MS (Drozdzik et al., 2014) showed a 3-fold higher P-gp level in the distal ileum compared to the duodenum or the proximal jejunum, alongside notably lower P-gp levels in the colon. Regardless of the different techniques used in the above studies, the influence of location on the protein content was similar in rats and humans for the small intestine, but not the colon. The lower P-gp level and related efflux in the proximal (in contrast to the distal) small intestine explains why the proximal end is the ideal absorption site for drugs which are P-gp substrates. It also explains how compounds which reduce the gastrointestinal motility, such as sodium acid pyrophosphate, mannitol and sorbitol, can significantly increase the oral absorption of concomitantly administered P-gp substrate drugs (Adkin et al., 1995a, Adkin et al., 1995b, Chen et al., 2007).

Figure 1 also shows a considerable influence of an animal's sex on the intestinal P-gp level. While the P-gp abundance was similar in male and female duodenums ($p>0.05$), it differed markedly in other intestinal segments. Specifically, abundance was about 40% higher in the male jejunum, ileum and colon ($p<0.05$). This reflects previous reports of higher enterocyte P-gp content in men's small intestine compared to women's (Schuetz et al., 1995, Potter et al., 2004), and a lack of difference in the upper duodenum (Ungell et al., 1992, Tozaki et al., 1997). As far as the colon is concerned, there is limited literature on sex-related P-gp protein abundance or mRNA expression in rodents and humans. In contrast to our studies, MacLean et al (2008) reported no significant sex-related difference in the intestinal P-gp expression along the whole intestine of male and female rats (MacLean et al., 2008). Such a difference between our studies and those of MacLean et al could have been due to the fasted/fed status of the rats, which were fasted in our studies but fed in MacLean's study, as well as high inter- and intra-

variability.

3.2 Influence of PEG 400 on the P-gp Protein Contents

The influence of orally administered PEG 400 on P-gp protein level in the jejunum is shown in Figure 2. Similar effects were found in the duodenum, ileum and colon (supplementary Figures A-C). It can be seen that PEG 400 administration reduced P-gp protein levels in a time- and sex- dependent manner. In male rats, the reduction in P-gp protein levels peaked at 90 min post PEG dosing, after which, P-gp levels seem to recover to their original values. In contrast, in female rats, the reduction in P-gp levels occurred to a much lower extent compared to the control values (i.e. when PEG was not administered), such that no statistical differences were found. There was a general trend of decreasing P-gp levels, however, this trend continued for a longer time, i.e. until the final measurement at 180 min.

Reduction of P-gp levels by PEG 400 in the rats reflects a previous report where PEG 400 reduced P-gp protein abundance and enhanced the uptake of Rho-123 (a P-gp substrate) into Caco-2 cells (Hodaei et al., 2015). The mechanisms underlying the reduction in P-gp levels was unclear, and the authors suggested that the alteration of fluidity of the epithelial membranes by PEG 400 was the principal reason for the inhibition of P-gp activity and enhancement of Rho-123 uptake. An earlier report showed that PEG 400 and other excipients such as Tween-80 and Pluronic F-68 blocked the binding sites of P-gp and altered the membrane fluidity (Li et al., 2011). Although, there is still controversy over whether P-gp-inhibiting excipients increase or decrease the fluidity of the epithelial membranes (Dudeja et al., 1995, Rege et al., 2002), it is known that P-gp is highly sensitive to the lipid environment of the cell membrane (Regev et al., 1999, Ferte, 2000). Therefore, any disturbance in this environment by excipients can possibly result in changes in the secondary and tertiary structures of the P-gp protein. It is feasible that PEG 400, containing oxyethylene groups, can intercalate in the lipid phase of the membrane, thereby changing the latter's fluidity (Hugger et al., 2002). It has also been suggested that the alkyl and unsaturated C-C bonds in the

chemical structure of PEG derivatives may influence the function of P-gp (Shen et al., 2006). On the other hand, the modulation of excipients on the nuclear receptors was also considered as another possible reason behind the phenomenon, given that transporter protein abundance is generally regulated by these nuclear receptors, such as pregnane receptor in rodents (PXR), retinoic acid receptor (RAR) and farnesoid receptor (FXR) (Yoshikawa et al., 2002, Lee et al., 2006). For instance, mRNA expression of P-gp was strongly predicted by the mRNA levels of PXR, whose signaling pathway for intestinal efflux transporter was likely in a constantly activated physiological state (Drozdzik et al., 2014). In addition to P-gp, other transporters were reportedly altered *in vivo* temporarily. Indoxyl sulfate (IS) demonstrated a significantly increased expression of the glucose transporter-1 (GLUT1) and S6 protein kinase (S6K) in vascular smooth muscle cells in Wistar male rats 3h after treatment. Protein Kinase B (Akt) and (tuberous sclerosis 2 protein) TSC2, however, were suppressed after 6h and 12h treatment (Lin et al., 2013).

While the above studies shows that P-gp modulation by excipients is well-known, we report, for the first time, an influence of an animal's sex. The influence of PEG 400 on the P-gp abundance in female rats was quite different to that in the males. Firstly, PEG 400 administration had a longer-lasting effect such that the P-gp protein content continuously decreased over the 3hr duration. Secondly, the magnitude of reduction in P-gp levels was lower in females. The reason behind this sex-based phenomenon is unknown, but the rat intestinal P-gp seems to be less sensitive to PEG 400 in females than in males. It is possible that the intestinal membrane was more stable to changes in fluidity and/or the epithelial renewal was slower in females compared to males. To further understand the time- and sex- dependent influence of PEG 400 on P-gp levels, P-gp related mRNA expression was measured and is discussed below.

3.3 P-gp mRNA (*mdr1a* and *mdr1b*) expression in the absence and presence of PEG 400 and correlation with protein levels

The control (i.e. without PEG administration) gene expression of P-gp, namely *mdr1a*

and *mdr1b*, is shown in Figure 3. In both male and female rats, the *mdr1a* expression of P-gp increased from the proximal to the distal end of the intestinal tract, whereas no obvious trend was observed for the *mdr1b* expression along the intestine. When an animal's sex was considered, *mdr1a* expression was consistently lower in females except in the duodenal segment. Once more, *mdr1b* expression did not seem to follow any trend.

Following oral administration of PEG 400, the *mdr1a* expression in the male rat intestine decreased in the first 1 to 2 h and subsequently constantly increased, returning to the control levels within 3h of PEG administration. In contrast, *mdr1a* expression in female intestine kept decreasing following the PEG 400 administration and *mdr1a* contents had not returned to control levels by the end of the study (shown in Figure 4 for the jejunum). Similar results were found in the duodenum, ileum and colon (shown in supplementary Figure D-F). The intestinal *mdr1b* expression in both males and females fluctuated with time after PEG 400 dosing, and no trend could be seen (shown in supplementary Figure G-J).

It can be seen from Figure 5 and supplementary Table A that there is a strong positive correlation between *mdr1a* (but not *mdr1b*) expression and P-gp protein abundance, $r=0.8$, $n=336$, $p<0.001$, with high levels of P-gp protein associated with high levels of *mdr1a* expression. This positive correlation in *mdr1a* expression and P-gp protein level in the rats reflects similar positive correlation between gene expression and absolute intestinal P-gp protein level in humans (Drozdzik et al., 2014). Having said that, the literature about the relationship between P-gp and its mRNA levels is contradictory (Guo et al., 2008, Koussounadis et al., 2015). It has been suggested that the predictive power of transcript analysis must be investigated on a gene-by-gene a basis, and that mRNA expression data should only be used as supportive information regarding protein levels. Nevertheless, from our work, we can conclude a greater usefulness of *mdr1a* compared to *mdr1b* for predicting P-gp protein levels.

4. Conclusions and implications of this study

This work has highlighted several new aspects regarding the excipients PEG 400 and its influence on oral drug absorption. To begin with, the P-gp protein abundance in the colon, in relation to the other intestinal segments, is different in rats and humans. Such differential P-gp levels can have considerable implications when the rat model is used in drug development, for example, of colon-targeted drug carriers. This shows that greater characterization of membrane transporters in animal models in relation to human parameters is needed to judge the value and limitations of the rat model. Our work also shows that fast-release oral dosage forms would be a better choice for drugs which are P-gp substrates, owing to the lower P-gp content in the proximal small intestine in both sexes. Furthermore, inclusion of "active" pharmaceutical excipients, such as PEG 400, is likely to benefit oral formulations containing P-gp substrates that are BCS Class IV drugs (poor solubility and poor permeability), as PEG 400 could improve both the solubility and the permeability of co-formulated drugs. Finally, the unexpected sex differences demonstrated in this study highlight a potential safety concern, requiring greater care in the selection and use of pharmaceutical excipients for oral drug development, and the inclusion of females in drug pharmacokinetics and clinical studies.

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Figure Captions:

Figure 1. Relative quantitation of P-gp protein abundance in selected intestinal segments (duodenum, jejunum, ileum and colon) (n=6). The levels of protein are normalized to anti-β actin. * Values are statistically different between the male and female groups at $p<0.05$.

Figure 2. The effect of PEG 400 on relative quantitation of P-gp protein expression in jejunum over 3h (n=6). The levels of protein are normalized to anti-β actin. * Values are statistically different between the control (i.e. no PEG administration) and PEG groups at $p<0.05$.

Figure 3. Comparison of *mdr1a* (A) and *mdr1b* (B) mRNA expression in different intestinal segments of male and female rats (n=6). The level of mRNA is normalized to anti-β actin. * Values are statistically different between the male and female groups at $p<0.05$.

Figure 4. Fold changes in *mdr1a* expression in jejunum of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Figure 5. Correlation coefficients between P-gp protein levels and its related *mdr1a* (A) and *mdr1b* (B) mRNA expression.

Supplementary Figure A. The effect of PEG 400 on relative quantitation of P-gp protein expression in duodenum over 3h (n=6). The levels of protein are normalized to anti-β actin. * Values are statistically different between the control (i.e. no PEG administration) and PEG groups at $p<0.05$.

Supplementary Figure B. The effect of PEG 400 on relative quantitation of P-gp protein expression in ileum over 3h (n=6). The levels of protein are normalized to anti-β actin. * Values are statistically different between the control (i.e. no PEG administration) and PEG groups at $p<0.05$.

Supplementary Figure C. The effect of PEG 400 on relative quantitation of P-gp protein expression in colon over 3 hours (n=6). The levels of protein are normalized to anti-β actin. * Values are statistically different between the control (i.e. no PEG administration) and PEG groups at $p<0.05$.

Supplementary Figure D. Fold changes in *mdr1a* expression in duodenum of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure E. Fold changes in *mdr1a* expression in ileum of rats after 3h administration with

PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure F. Fold changes in *mdr1a* expression in colon of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure G. Fold changes in *mdr1b* expression in duodenum of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure H. Fold changes in *mdr1b* expression in jejunum of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure I. Fold changes in *mdr1b* expression in ileum of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Supplementary Figure J. Fold changes in *mdr1b* expression in colon of rats after 3h administration with PEG 400 (n=6). * Values are statistically different between the control (rats which are not dosed with PEG 400) and PEG groups at $p<0.05$.

Table. 1. Primers used for the analysis of P-gp gene expression by real-time qPCR

Gene		**Primer (5' – 3')**	**Amplicon**	**Genebank**
mdr1a	Forward	CACCATCCAGAACGCAGACT	139	NM_133401
	Reverse	ACATCTCGCATGGTCACAGTT		
mdr1b	Forward	AACGCAGACTTGATCGTGGT	144	NM_012623
	Reverse	AGCACCTCAAATACTCCCAGC		
anti-beta actin	Forward	GCAGGAGTACGATGAGTCCG	74	NM_031144
	Reverse	ACGCAGCTCAGTAACAGTCC		

Supplementary Table A. Correlation coefficient (r) from Pearson correlation analysis between P-gp protein levels and its related mRNA expression (*mdr1a* and *mdr1b*).

Groups	P-gp protein level	*mdr1a* expression	*mdr1b* expression
P-gp protein level	——	0.785 **	0.095

mdr1a expression	——	0.083
mdr1b expression		——

** p < 0.001 (2-tailed).

Supplementary Table B. Probability value (p) between jejunal P-gp protein levels at different time points after PEG 400 treatment.

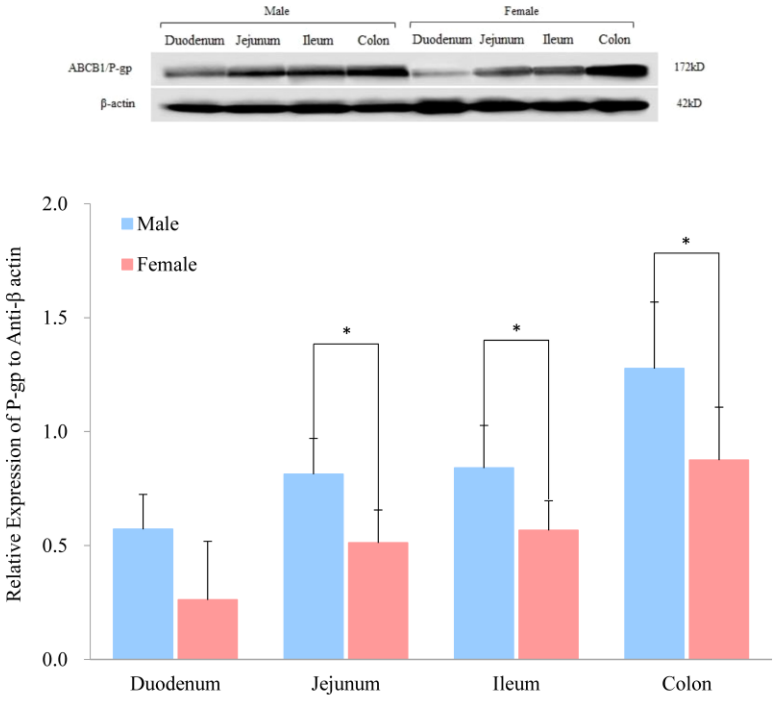
Time (min)	0	15	30	60	90	120	180
Male							
0	——	0.064	0.112	0.014 *	0.007 *	0.009 *	0.093
15		——	0.482	0.117	0.096	0.083	0.072
30			——	0.323	0.127	0.076	0.089
60				——	0.644	0.542	0.048 *
90					——	0.612	0.05 *
120						——	0.058
180			——				——
Female							
0	——	0.392	0.069	0.236	0.462	0.938	0.029 *
15		——	0.504	0.111	0.086	0.129	0.332
30			——	0.216	0.429	0.108	0.09
60				——	0.123	0.063	0.139
90					——	0.24	0.255
120						——	0.084
180							——

* p < 0.05.

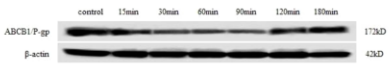
Supplementary Table C. Probability value (p) between jejunal *mdr1a* expression at different time points after PEG 400 treatment.

Time (min)	0	30	60	120	180
Male					
0	——	0.016 *	0.004 *	0.009 *	0.273
30		——	0.382	0.466	0.172
60			——	0.308	0.054
120				——	0.118
180		—			——
Female					
0	——	0.373	0.531	0.103	0.006 *
30		——	0.25	0.399	0.043 *
60			——	0.492	0.106
120				——	0.334

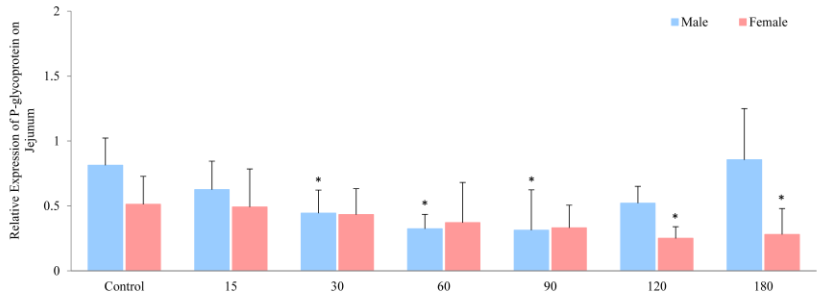
* $p < 0.05$.


Male
Female
Duodenum
Jejunum
Ileum
Colon
Duodenum
Jejunum
Ileum
Colon
ABCB1/P-gp
β-actin
172kD
42kD
Relative Expression of P-gp to Anti-β actin
2.0
1.5
1.0
0.5
0.0
Male
Female
*
*
*
Duodenum
Jejunum
Ileum
Colon

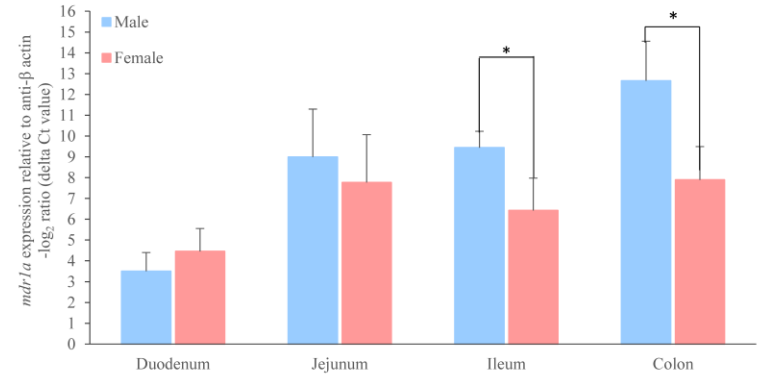
Male-Jejunum



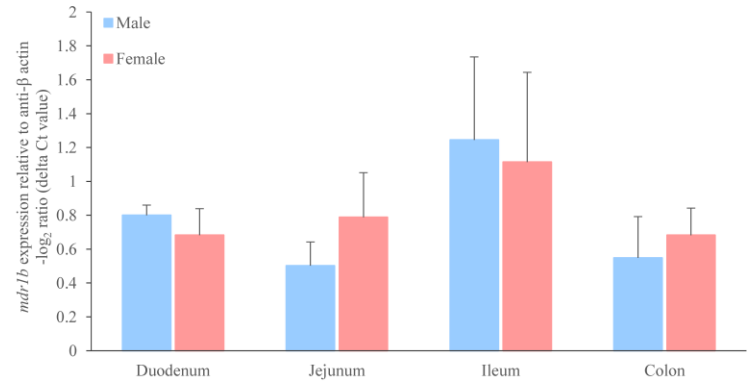
Female-Jejunum

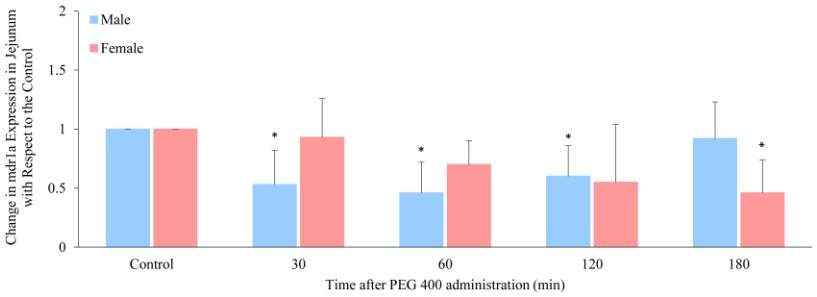


A


Male
Female
*
*
mdr1a expression relative to anti-β actin
-log2 ratio (delta Ct value)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Duodenum
Jejunum
Ileum
Colon

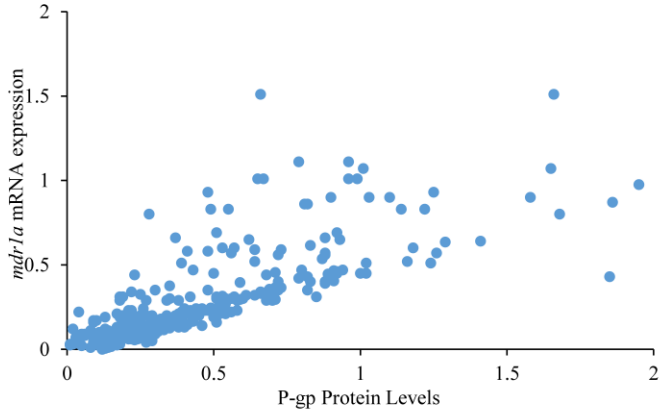
B


Male
Female
mdr1b expression relative to anti-β actin
-log2 ratio (delta Ct value)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Duodenum
Jejunum
Ileum
Colon

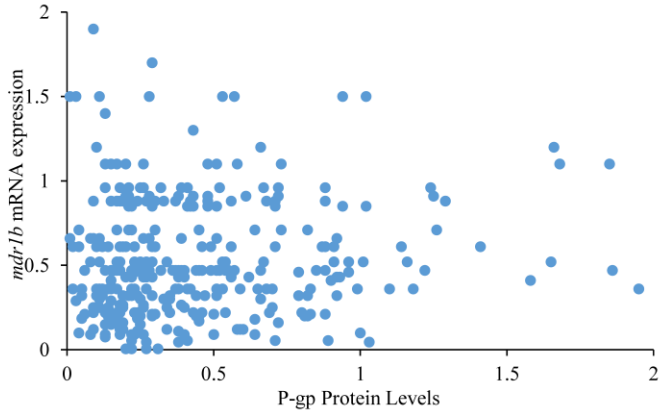

Male
Female
Change in mdr1a Expression in Jejunum with Respect to the Control
2
1.5
1
0.5
0
*
*
*
*
Control
30
60
120
180
Time after PEG 400 administration (min)

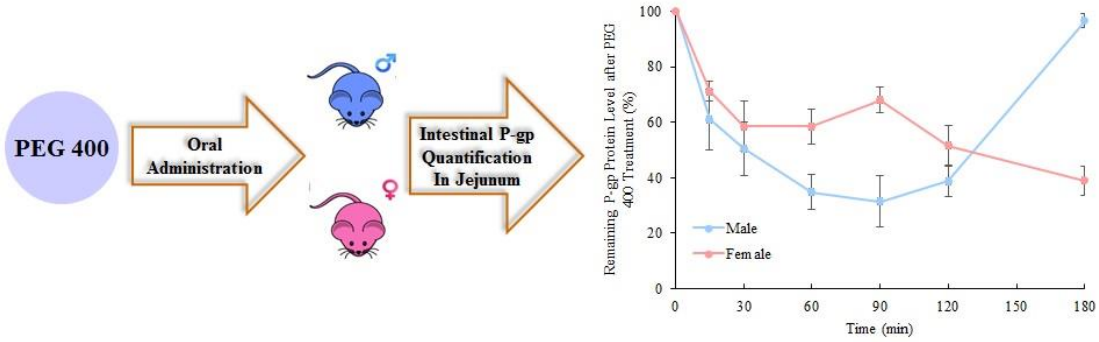
4

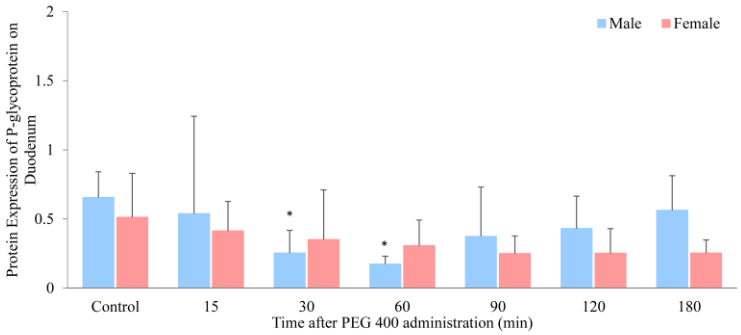
A


2
1.5
1
0.5
0
mdr1a mRNA expression
0
0.5
1
1.5
2
P-gp Protein Levels

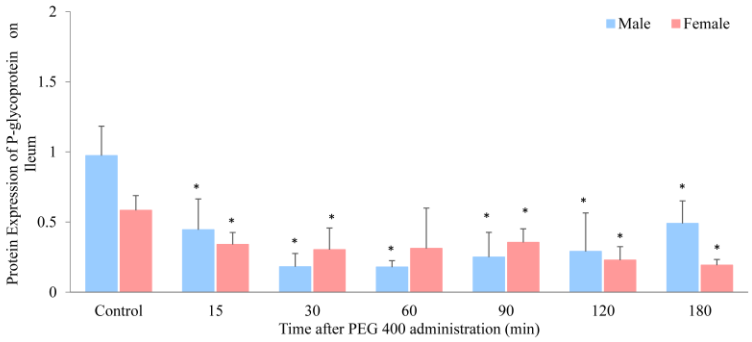
B


2
1.5
1
0.5
0
mdr1b mRNA expression
0
0.5
1
1.5
2
P-gp Protein Levels

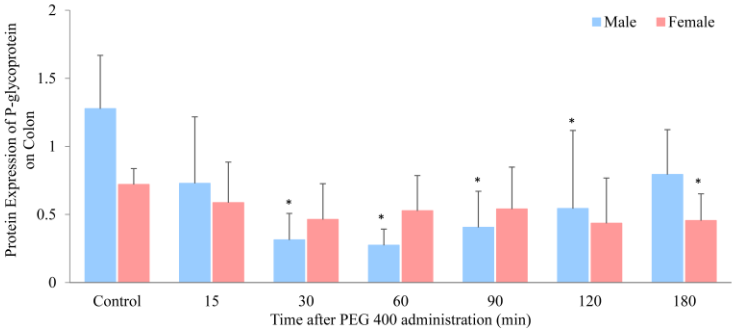

PEG 400
Oral Administration
Intestinal P-gp Quantification In Jejunum
♂
♀
Remaining P-gp Protein Level after PEG 400 Treatment (%)
100
80
60
40
20
0
0
30
60
90
120
150
180
Time (min)
Male
Female


Protein Expression of P-glycoprotein on Duodenum
2
1.5
1
0.5
0
Male
Female
*
*
Control
15
30
60
90
120
180
Time after PEG 400 administration (min)

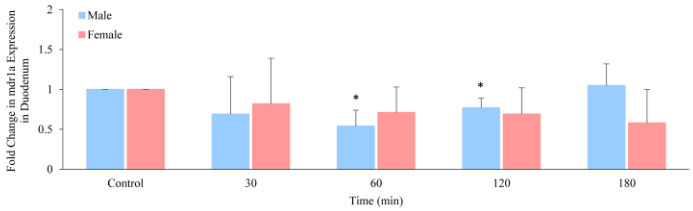
6


Male
Female
Protein Expression of P-glycoprotein on Ileum
2
1.5
1
0.5
0
Control
15
30
60
90
120
180
Time after PEG 400 administration (min)

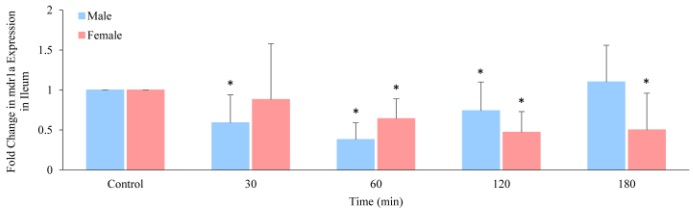
7


Male
Female
Protein Expression of P-glycoprotein on Colon
2
1.5
1
0.5
0
Control
15
30
60
90
120
180
Time after PEG 400 administration (min)

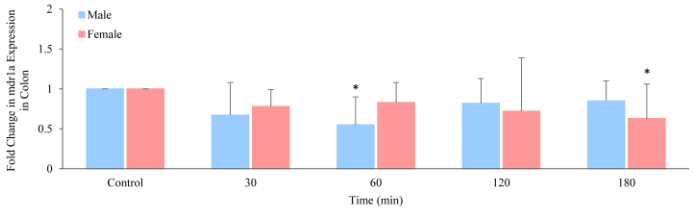
8


Male
Female
Fold Change in mdr1a Expression in Duodenum
2
1.5
1
0.5
0
*
*
Control
30
60
120
180
Time (min)

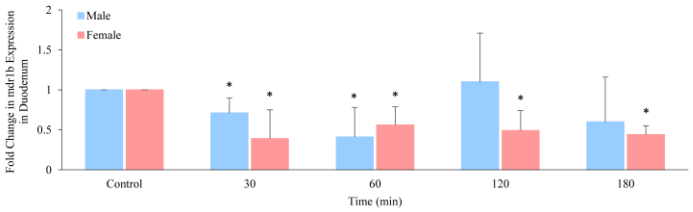
9


Male
Female
Fold Change in mdr1a Expression in Ileum
2
1.5
1
0.5
0
*
*
*
*
*
Control
30
60
120
180
Time (min)

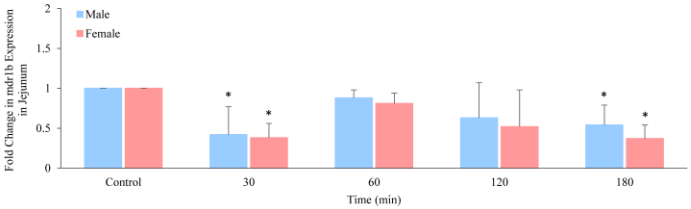
10


Male
Female
Fold Change in mdr1a Expression in Colon
2
1.5
1
0.5
0
*
*
Control
30
60
120
180
Time (min)

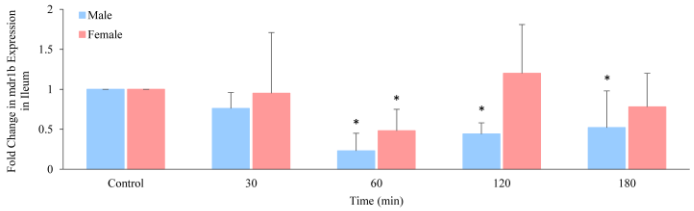
11


Male
Female
Fold Change in mdr1b Expression in Duodenum
2
1.5
1
0.5
0
*
*
*
*
*
*
Control
30
60
120
180
Time (min)

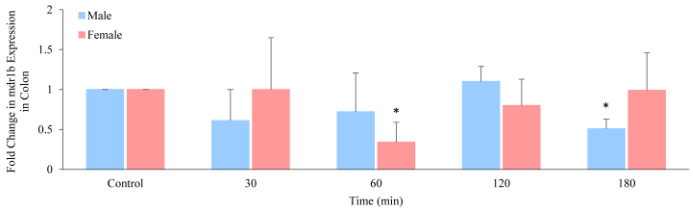
12


Male
Female
Fold Change in mdr1b Expression in Jejunum
2
1.5
1
0.5
0
*
*
*
*
Control
30
60
120
180
Time (min)

13


Male
Female
Fold Change in mdr1b Expression in Ileum
2
1.5
1
0.5
0
*
*
*
*
Control
30
60
120
180
Time (min)

14


Male
Female
Fold Change in mdr1b Expression in Colon
2
1.5
1
0.5
0
*
*
Control
30
60
120
180
Time (min)

15