Establishing an *in vitro* permeation model to predict the *in vivo* sex-related influence of PEG 400 on oral drug absorption

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Abstract

The notion that certain formerly regarded "inert" pharmaceutical excipients are capable of modifying the bioavailability of oral drugs has gained increasing attention in recent years. For instance, the commonly-used solubilizing agent polyethylene glycol 400 (PEG 400) exhibits a sex-specific effect on the bioavailability of ranitidine in both humans and rats, mediated by the efflux transporter P-glycoprotein (P-gp). To determine whether such *in vivo* effect could be predicted by *in vitro* tests, an *in vitro/ex vivo* model was established using tissues from male and female rats to characterize the influence of PEG 400 on the intestinal transport of ranitidine in the absence and/or presence of a P-gp inhibitor, cyclosporine A (CsA). We found the absorptive permeability of ranitidine in the small intestine (duodenum, jejunum, ileum) and colon was higher in females compared with males. PEG 400 significantly increased the absorption and decreased the secretion of ranitidine in the intestine of male rats ($p<0.05$), but no such effects were observed in female intestines. In addition, while the P-gp inhibitor CsA increased the intestinal uptake of ranitidine in both male and female rats, a greater extent of intestinal transport modulation was observed in males compared to females. These *in vitro* data on the influence of PEG 400 on the intestinal transport of ranitidine in a sex-dependent manner are in agreement with previously published *in vivo* data. This good *in vivo-in vitro* correlation means that the *in vitro* method will be quicker, cheaper and easier to investigate any sex-related influence of pharmaceutical excipients on oral drug bioavailability.

Key words: polyethylene glycol 400; sex difference; cyclosporin ; permeability; ranitidine; intestine; bidirectional transport; Ussing chamber.

1. INTRODUCTION

There is a long and storied tradition of ignoring sex when it comes to health research. Female subjects have historically been excluded from toxicological or biomedical research for several reasons, such as the complexity of their menstrual cycles and risks of teratogenicity. However, between 1977 and 1995, twenty-six studies were submitted to the FDA demonstrating sex-dependent differences in drug bioavailability (Chen et al., 2000, Coker, 2008, Nicolas et al., 2009). Subsequently, progress has been made since 2000, when the U.S Food and Drug Administration (FDA) stated that both sexes should be represented in all clinical trial phases to avoid any sex-related differences in drug efficacy and side effects remaining undetected (Kando., 1995, Beierle et al., 1999, Soldin et al., 2011). This major change was largely owed to antipyrine, which was the first drug reported to exhibit different hepatic metabolism in men and women (Omalley et al., 1971). Despite such progress, there is still a long way to go. A 2014 report found that only one-third of cardiovascular clinical-trial subjects are women (Nowogrodzki et al., 2017).

Sex-related differences in drug pharmacokinetics, especially regarding absorption and metabolism, are in part caused by a differential expression and/or activity of membrane transporters between males and females (Soldin and Mattison, 2009, Morris et al., 2013). Certain transporters are known to be influenced by formerly considered "inert" pharmaceutical excipients with which drugs are co-formulated. Indeed, excipients have shown a sex-specific influence on drug bioavailability via their interaction with transporters. A study on the influence of polyethylene glycol 400 (PEG 400) on the bioavailability of ranitidine in men and women showed that PEG 400 increased ranitidine absorption in men but not in women (Ashiru et al., 2008). In order to determine the mechanism underlying this unexpected sex-specific phenomenon, an animal model was used in our laboratory (Afonso-Pereira et al., 2016), and we found that the sex-related influence of PEG 400 occurred for drugs whose absorption is controlled by the efflux transporter P-gp, but not for the drugs

which are not P-gp substrates. Additionally, the PEG 400 sex-based effect on the oral bioavailability of P-gp substrates could be eliminated by the inhibition of P-gp transporters using cyclosporine A (CsA), a P-gp inhibitor. The reason behind this phenomenon was hypothesized to be a sex-dependent impact of PEG 400 on P-gp (Mai et al., 2017).

In order to test this hypothesis, an *in vitro* study was set up using an Ussing chamber system. The aim was to determine whether sex of the organism was the reason for the differences seen in the *in vivo* studies, in the absence of potentially complicating *in vivo* factors, such as differential GI transit time as well as the activity and composition of intestinal bacteria. Thus, the impact of PEG 400 on the permeation of ranitidine in the absence or presence of the P-gp inhibitor CsA, in four intestinal segments of male and female rats was determined. This study also allowed us to determine whether the *in vitro* Ussing chamber system would show a good correlation with *in vivo* studies, and if such a system could in future be used to test any sex-dependent influence of other excipients on oral drug absorption.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Ranitidine hydrochloride and polyethylene glycol 400 (PEG 400) were purchased from Sigma-Aldrich (Poole, UK). Cyclosporine A (CsA) was purchased from Cambridge Bioscience (Cambridge, UK). HPLC-grade reagents, such as acetonitrile and glacial acetic acid, were supplied from Fisher Scientific (Loughborough, UK). Krebs-Bicarbonate Ringer's buffer (KBR) was prepared with 10mM D-glucose, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 115mM NaCl, 25mM $NaHCO_3$, 0.4mM KH_2PO_4, 2.4mM K_2HPO_4 in distilled water, and the pH was adjusted to 7.4 with NaOH/HCl (Clarke, 2009).

2.2 Investigation of Chemical Stability in the Ussing Chamber System

The stability of ranitidine (3mM) and CsA (8 μM) in KBR medium was assessed by incubating the drug KBR solutions at 37℃ for 180 min. The drug concentration at 180 min was compared with that measured at the beginning of the incubation period.

2.3 Mucosal Tissue Preparation

Intestinal tissue was obtained from healthy male and female, 8-13 week old Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250g. The rats had been housed at controlled temperature (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, provided with food and water, and acclimatized for 7 days. One day before the collection of the intestinal tissue, the rats had been fasted overnight and housed individually in metabolic cages.

On the day of experiment, the rats were killed in a CO_2 euthanasia chamber and their intestines were immediately removed. Each intestine was cut into four segments (duodenum, jejunum, ileum and colon), which were then placed in beakers containing ice-cold KBR buffer. The intestinal segments were allowed to rest for approximately 20 min after lowering the tissue temperature in order to minimize tissue damage during preparation. Subsequently, approximately 2-3cm long pieces from the mid part of the duodenum, proximal part of the jejunum, distal to mid part of ileum and mid part of ascending colon were taken and opened along their mesenteric border. The intestinal segments were gently washed with KBR buffer to remove their contents. To obtain the mucosal tissue, sections were placed on an ice-cold glass plate and the serosa was gently squeezed out with tweezers.

2.4 Ussing Chamber Set-up

Once the mucosal tissues were prepared, they were mounted in the vertical Ussing Chamber (Harvard Apparatus Inc., Holliston, MA, U.S.A) as flat sheets on a 0.28 cm^2 segment holder with needles for stabilization. The mucosal surface of the tissue faced the apical chamber, while the endothelial surface faced the basolateral chamber. 5 mL blank KBR buffer was placed into each chamber, and a gas mixture of O_2/CO_2

(95%/5%) was bubbled through to oxygenate the liquid contents and ensure complete convection. The chambers were tightly screwed with high spring-tension retaining rings and the entire assembly was kept at 37℃ with a circulating water bath for an equilibrium period of 20-30 min.

To evaluate the integrity of the mucosal tissue during experiments, tissue transepithelial electrical resistance (TEER) was measured using an EVOMX meter (World Precision Instruments Inc., WPI, Hertfordshire, United Kingdom) at half-hourly intervals. Any duodenum, jejunum, ileum and colon segments that presented a TEER value below $20\Omega{\cdot}cm^2$, $40\Omega{\cdot}cm^2$, $50\Omega{\cdot}cm^2$ and $70\Omega{\cdot}cm^2$, respectively, at the beginning of the experiment was regarded as poorly viable and excluded. Whenever TEER values decreased by more than 15% from the original value (measured at the end of equilibration period), the tissue was considered not viable and eliminated from the investigation (Polentarutti et al., 1999).

2.5 Measurement of Bidirectional Transport of Ranitidine

After an equilibrium period of 20-30 min, the experiment was started by replacing the blank KBR buffer in the donor compartment with pre-warmed 3mM ranitidine in KBR solution. For the absorptive transport (mucosa to serosa, M-S), the apical chamber was the donor; while for the excretive transport (serosa to mucosa, S-M), the basolateral chamber was donor. The permeation experiment lasted for 3 h, and 100 µL of the receiver solution was withdrawn every 30 min in order to measure drug transport from donor to receiver. Drug concentration in the receiver phase samples was measured by HPLC as described in section 2.6. The receiver phase sample withdrawn was immediately replaced with an equal volume of pre-warmed KBR buffer.

2.5.1 Effect of PEG 400 Concentration on the Intestinal Permeability of Ranitidine

This experiment was conducted to determine the dose of PEG 400 which had the highest impact on the absorptive (i.e. M-S) transport of ranitidine. That PEG 400 dose

was subsequently used to assess the influence of PEG 400 on the secretive (i.e. S-M) ranitidine transport, and to assess the influence of the P-gp inhibitor, CsA.

This study was conducted as described in section 2.5, except for the addition of PEG 400 at 0.25%, 0.5%, 1%, 1.5%, 2% or 3% PEG 400 in the apical chamber.

2.5.2 Effect of a P-gp Inhibitor on the Modulation of Ranitidine Permeability by PEG 400

The effect of a validated P-gp inhibitor (CsA) on the absorptive (M-S) and secretive (S-M) intestinal transport of ranitidine was investigated. The experiment was conducted as described in section 2.5 except that CsA was placed into the donor chamber 15min prior to the addition of ranitidine $\pm$ PEG 400.

A 15 min pre-incubation with CsA before the addition of the drug solution was chosen following a study on the influence of pre-incubation duration, which was tested for 0, 15, 30 or 60 min. The results showed that a pre-incubation duration of 15 min had the greatest inhibitory influence on ranitidine permeability (shown in supplementary Figure A).

2.6 Quantification of Drug Transport

The amount of drug in the receiver samples was quantified using a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B), a diode-array UV detector (model G1314B) and a 5 μm Luna SCX column (Phenomenex, UK). A mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) was used as the mobile phase for elution, with a flow rate of 2 mL/min (Ashiru et al., 2008, Xu et al., 2013).

2.7 Calculation

The apparent permeability coefficient (P_{app}) in each experiment, in cm/s, was calculated using the following equation:

$$Papp = \frac{Q}{C \cdot A \cdot t}$$

where Q (μmol) is the total amount of drug that permeated to the receiver compartment throughout the incubation time, C (μmol/mL) is the initial drug concentration in the donor side, A (cm^2) is the diffusion area of the Ussing Chamber, and t (s) is the duration time of experiment.

To quantitate ranitidine efflux, an efflux ratio was calculated from the mean serosal-to-mucosal (S-M) P_{app} and mucosal-to-serosal (M-S) P_{app} values, as follows:

$$\text{Efflux Ratio} = \frac{Papp\ (S-M)}{Papp\ (M-S)}$$

2.8 Statistical Analysis

The experiments were performed with at least six replicates and data were expressed as mean ± standard deviation (S.D.). Differences between groups were analysed 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 significance level for the tests.

3. RESULTS

3.1 Influence of Animal's Sex on the Intestinal Transport of Ranitidine

The absorptive and secretive permeability of ranitidine in the different intestinal segments of male and female rats, presented as the apparent permeability coefficient (P_{app}), are shown in Figure 1a and Figure 1b, respectively. Sex differences were observed for both absorption and secretion of ranitidine. In all the intestinal segments, except for the duodenum, absorption (P_{app} mucosa-serosa, M-S) was higher while secretion (P_{app} serosa-mucosa, S-M) was lower in female rats, compared to males. Interestingly, no sex-related differences were observed for ranitidine transport in the duodenum.

3.2 Influence of Intestinal Region on the Transport of Ranitidine

Figure 2 and Figure 3 represent the time course of absorptive and secretive permeability (M-S and S-M) transport of ranitidine in the different intestinal segments

of male and female rats. It can be seen that absorptive transport of ranitidine in the small intestine (duodenum, jejunum and ileum) were higher than in the colon (p<0.05). Its absorption in male rats did not exhibit a regional difference, and was similar in the duodenum, jejunum and ileum. However, significant differences were found in ranitidine absorption in different small intestine segments of female rats. In contrast, ranitidine secretion in both male and female rats was found to differ between intestinal segments, being greatest in the jejunum and ileum, and lowest in the duodenum and colon.

3.3 Influence of PEG 400 and its Dose on Ranitidine Absorption

The presence of PEG 400 increased the intestinal absorption of ranitidine in males but not in females, in a dose- and region- dependent manner. As shown in Figure 4 for the jejunum and supplementary Figure B-C for the duodenum and ileum, increasing the dose of PEG 400 led to increasing and then decreasing ranitidine absorption in male rats, such that a bell-shaped effect (in relation to PEG concentration) was observed. However, a rising curve was found in the male colon, as shown in Figure 5.

In contrast to the males, PEG 400 did not cause any change to ranitidine absorption in any of the female intestinal segments. The greatest enhancement in ranitidine absorption in the small intestinal segments was with a PEG 400 dose of 0.5%. Therefore, 0.5% PEG 400 was selected as the appropriate dose for the subsequent studies discussed in the following sections.

3.4 The Influence of PEG 400 on Ranitidine Secretion

PEG 400 (0.5%) decreased the secretion of ranitidine in the male jejunum, ileum, and colon (but not duodenum) (p<0.05), but not in any of the female intestinal segments (p>0.05), as shown in Figure 6. That is, at this low dose, PEG 400 influences the intestinal efflux of ranitidine in a sex-dependent manner. Since P-gp is the major efflux transporter involved in the intestinal permeation of ranitidine (Cook and Hirst, 1994, Collett et al., 1999) and PEG 400 is known to inhibit P-gp (Hugger et al., 2002, Shen et al., 2006), we come to the conclusion that intestinal P-gp may be involved in

this sex-related phenomenon. To test this, we further investigated the role of P-gp by using a P-gp inhibitor (CsA) as discussed in the following section.

3.5 The Effect of PEG 400 on Ranitidine Permeability in the Presence of CsA

The effect of PEG 400 on the absorptive transport of ranitidine in the CsA pre-treated rat intestine is shown in Figure 7. Pre-incubation with CsA, considerably and significantly ($p<0.05$) increased ranitidine absorption in the male intestinal segments, while a smaller and statistically insignificant increase ($p>0.05$) was seen in the female segments. At the same time, pre-incubation with CsA decreased ranitidine secretion in the male intestinal segments, while causing no change in female ones (shown in Figure 8). The overall impact of CsA is that a net increase in ranitidine absorption (i.e. a decrease in efflux ratio) is seen in male intestinal segments, while no change is observed in the females (Table 1).

Addition of PEG 400 to CsA pre-treated male or female rat intestines had no significant effect on ranitidine permeability ($p>0.05$) (shown in Figure 7, Figure 8 and Table 1), i.e. once P-gp has been blocked by CsA, PEG 400 had no influence as its site of action was not available.

4. DISCUSSION

The *in vitro* results reported here mostly reflect and further explain the previously published *in vivo* work (Mai et al., 2017), where 1) PEG 400 dose-dependently enhanced the ranitidine bioavailability in males but not in females; 2) the most pronounced effect in males was noted with PEG 400 dose of 0.5%; 3) blocking of P-gp by CsA (a P-gp inhibitor) eliminated PEG 400's impact on the bioavailability of ranitidine.

This good *in vitro-in vivo* correlation stands that the *in vitro* model developed in this study could be a more accessible and economical method to investigate any other influences of pharmaceutical excipients and other drugs (especially BCS Class III

drugs which are P-gp substrates) on oral drug absorption. The findings herein also highlight the importance of excipients selection in formulation development, emphasize the sex equality in clinical studies and raise several new aspects that could be following discussed.

The influence of PEG 400 on ranitidine permeability was region-related, which was observed in the jejunum, ileum and colon, but not in the duodenum. These findings, especially in the jejunum, correlates with our earlier *in vivo* investigations in humans and animals (summarized in Table 2) (Ashiru et al., 2008, Afonso-Pereira et al., 2016). It stands that the jejunum should therefore be the optimal intestinal segment in further studies on the influence of excipients on ranitidine absorption.

To be specific, low doses of PEG 400 (<0.75g, corresponding to a dose of 0.5% *w/v*) increased ranitidine bioavailability potentially owing to the induction of its intestinal permeation. However, a controversy was found over the effect of high-dose PEG 400 on ranitidine absorption. In the previous *in vivo* studies, high doses of PEG 400 (>1g, corresponding to dose of 0.7% *w/v*) decreased the bioavailability of ranitidine, and the reason behind this was suggested to be PEG 400's effect in reducing transit time in the small intestine. However, our new findings indicate that the lower ranitidine bioavailability in the presence of high-dose PEG 400 is attributed to the latter's modulation on the absorptive permeability of ranitidine, possibly via the alteration of membrane fluidity and/or inhibition of certain membrane uptake transporters. For example, it was reported that mannitol and sorbitol reduce the intestinal uptake of ranitidine and cimetidine due to high pressure on the membrane (Adkin et al., 1995a, Adkin et al., 1995b, Chen et al., 2007). Further, the inhibition of OATPs by PEG 400 in the absorption process of estrone-3-sulfate and taurocholate from the gut lumen suggest that PEG 400 might be a selective modulator of other intestinal uptake transporters (Engel et al., 2012).

Of considerable interest is the emerging data demonstrating that certain formerly regarded "inert" excipients, such as PEG 400, could directly or indirectly influence the membrane transporters, thereby altering the bioavailability of an incorporated

drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects.

More surprisingly, these interactions between excipients and drugs have shown a sex-related manner. For instance, PEG 400's impact on the intestinal absorption of ranitidine was only shown in male rats, but not in females. The reason behind this phenomenon was attributed to the inhibitory effect of PEG 400 (0.1 to 20% *w/v*) on P-gp on Caco-2 cells (Shen et al., 2006), and sex differences in the P-gp activity on the rat intestine. For example, a study by Ballent et al., provided results that corroborated with a study conducted by Mariana et al., whereby a greater P-gp activity was found in male rats compared to females (Ballent et al., 2012, Mariana et al., 2011). The reason for greater P-gp activity in males compared to females is likely owing to the sex differences in its own modifying-mechanisms, such as higher P-gp ATPase or ATP levels in the cells of males, and also due to the higher P-gp expression in males' intestine (Gerrard et al., 2004).

The importance of studying sex-based differences has been supported by an increasing body of evidence demonstrating that sex can alter drug efficacy and toxicity profiles. It has been stated that "every cell in our bodies has a sex" (Wizemann and Pardue, 2001), and this was currently endorsed in this study. Higher P-gp activity in males was exhibited, which highlights the influence of sex within clinical research and the selection of excipients in the development of oral formulation for drugs, particularly for P-gp substrates. As some excipients alter the expression of membrane transporters (Goole et al., 2010), better efficacy and safety outcomes for the use of pharmaceutical excipients could also be potentially obtained with further research on this topic.

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Figure Captions

Fig. 1. Absorptive (A) and secretive (B) transport of ranitidine across the intestinal segments of male and female Wistar rats (Mean ± S.D., n=6). * Values are statistically different between the male and female groups at $p<0.05$.

Fig. 2. Time course of the apparent permeability coefficients for ranitidine absorption (mucosal-to-serosal, M-S) in male (A) and female (B) Wistar rat intestinal segments (Mean ± S.D., n=6).

Fig. 3. Time course of the apparent permeability coefficients for ranitidine secretion (serosal-to-mucosal, S-M) in male (A) and female (B) Wistar rat intestinal segments (Mean ± S.D., n=6).

Fig. 4. Absorptive transport of ranitidine across the jejunum of male and female Wistar rats (Mean ± S.D., n=6) in the presence of increasing concentrations of PEG 400. * Values are statistically different between the control (ranitidine only) and tested groups (with PEG 400) at $p<0.05$.

Fig. 5. Absorptive transport of ranitidine across the colon of male and female Wistar rats (Mean ± S.D., n=6) in the presence of increasing concentrations of PEG 400. * Values are statistically different between the control (ranitidine only) and tested groups (with PEG 400) at $p<0.05$.

Fig. 6. Secretive permeability of ranitidine in the absence or presence of PEG 400 in male (A) and female (B) Wistar rat intestinal segments (Mean ± S.D., n=6). Ranitidine (R) 3mM, PEG 400 0.5%. * Values are statistically different between the control (R, ranitidine only) and tested group (R+PEG 400) at $p<0.05$.

Fig. 7. Absorptive permeability of ranitidine in the absence and presence of PEG 400 or CsA in male (A) and female (B) Wistar rat intestinal segments (Mean ± S.D., n=6). Ranitidine (R) 3mM, PEG 400 0.5%, CsA 8μM. * Values are statistically different between the control (R, ranitidine only) and tested groups (R+PEG 400, R+CsA, R+CsA+PEG 400) at $p<0.05$.

Fig. 8. Secretive permeability of ranitidine in the absence and presence of PEG 400 or CsA in male (A) and female (B) Wistar rat intestinal segments (Mean ± S.D., n=6). Ranitidine (R) 3mM, PEG 400 0.5%, CsA 8μM. * Values are statistically different between the control (R, ranitidine only) and tested groups (R+CsA, R+CsA+PEG 400) at $p<0.05$.

Supplementary Figure A. Absorptive transport of ranitidine in the absence or presence of CsA at 0, 15, 30 and 60min pre-incubated to ranitidine in male and female Wistar rat jejunum (Mean ± S.D., n=6). Ranitidine 3mM, CsA 8µM. *Values are statistically different between the control (ranitidine only) and tested groups at $p<0.05$.

Supplementary Figure B. Absorptive transport of ranitidine across the duodenum of male and female Wistar rats (Mean ± S.D., n=6) in the presence of increasing concentrations of PEG 400. * Values are statistically different between the control (ranitidine only) and tested groups (with PEG 400) at $p<0.05$.

Supplementary Figure C. Absorptive transport of ranitidine across the ileum of male and female Wistar rats (Mean ± S.D., n=6) in the presence of increasing concentrations of PEG 400. * Values are statistically different between the control (ranitidine only) and tested groups (with PEG 400) at $p<0.05$.

A
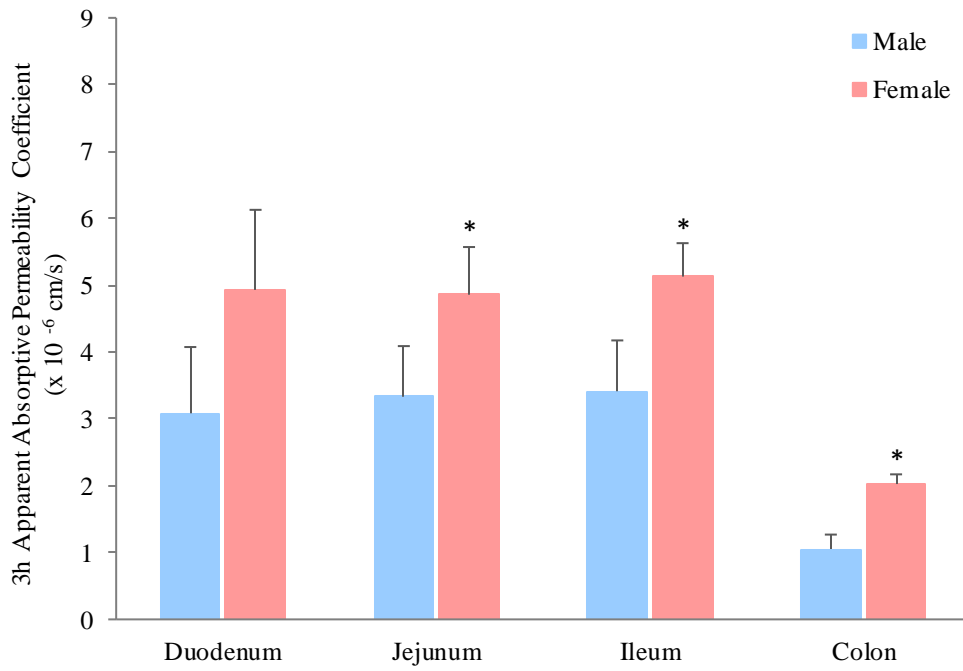
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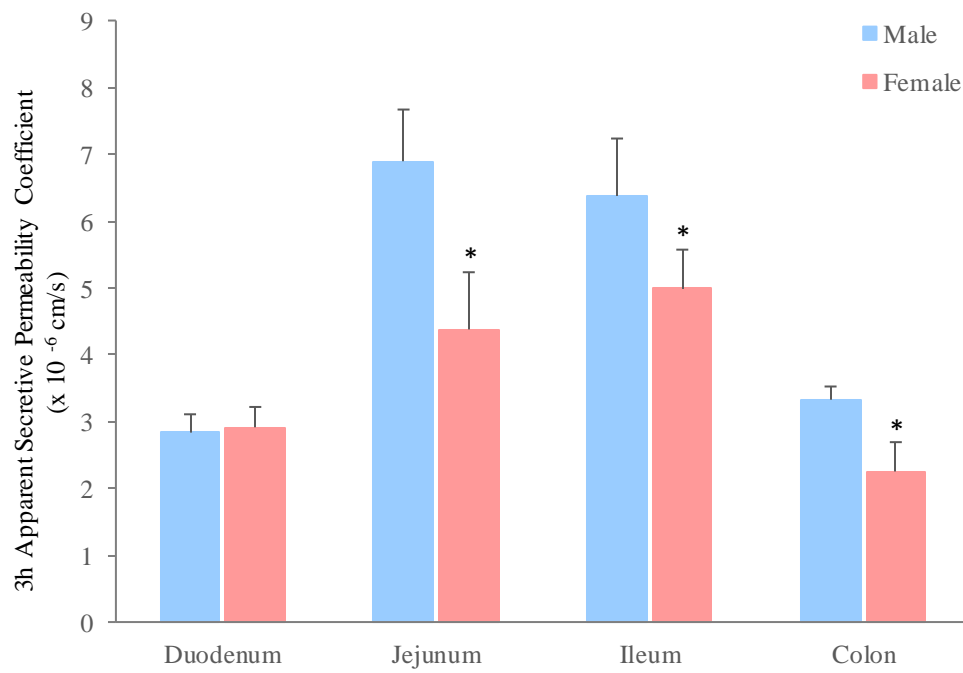
Fig.1.

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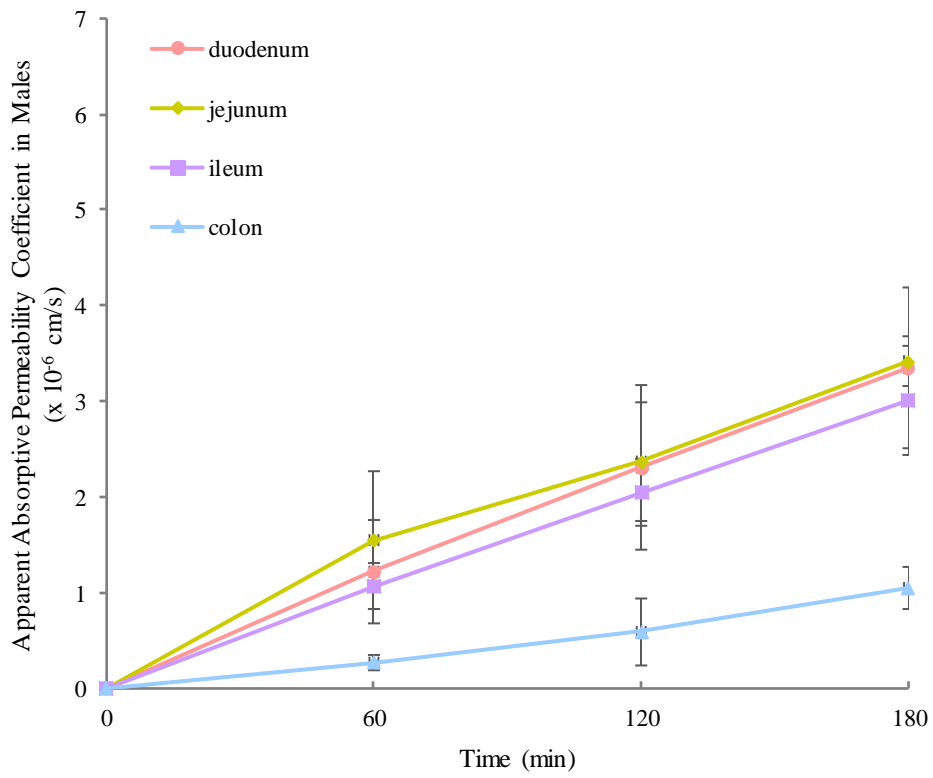
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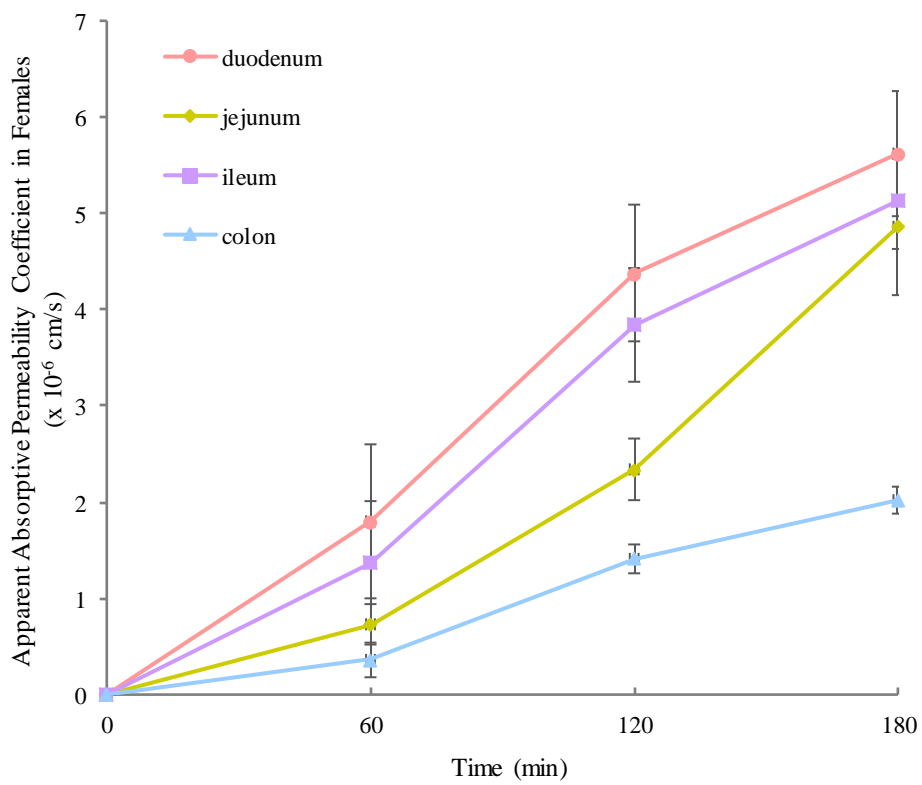
Fig.2.

A

B

Fig.3.

Fig.4.
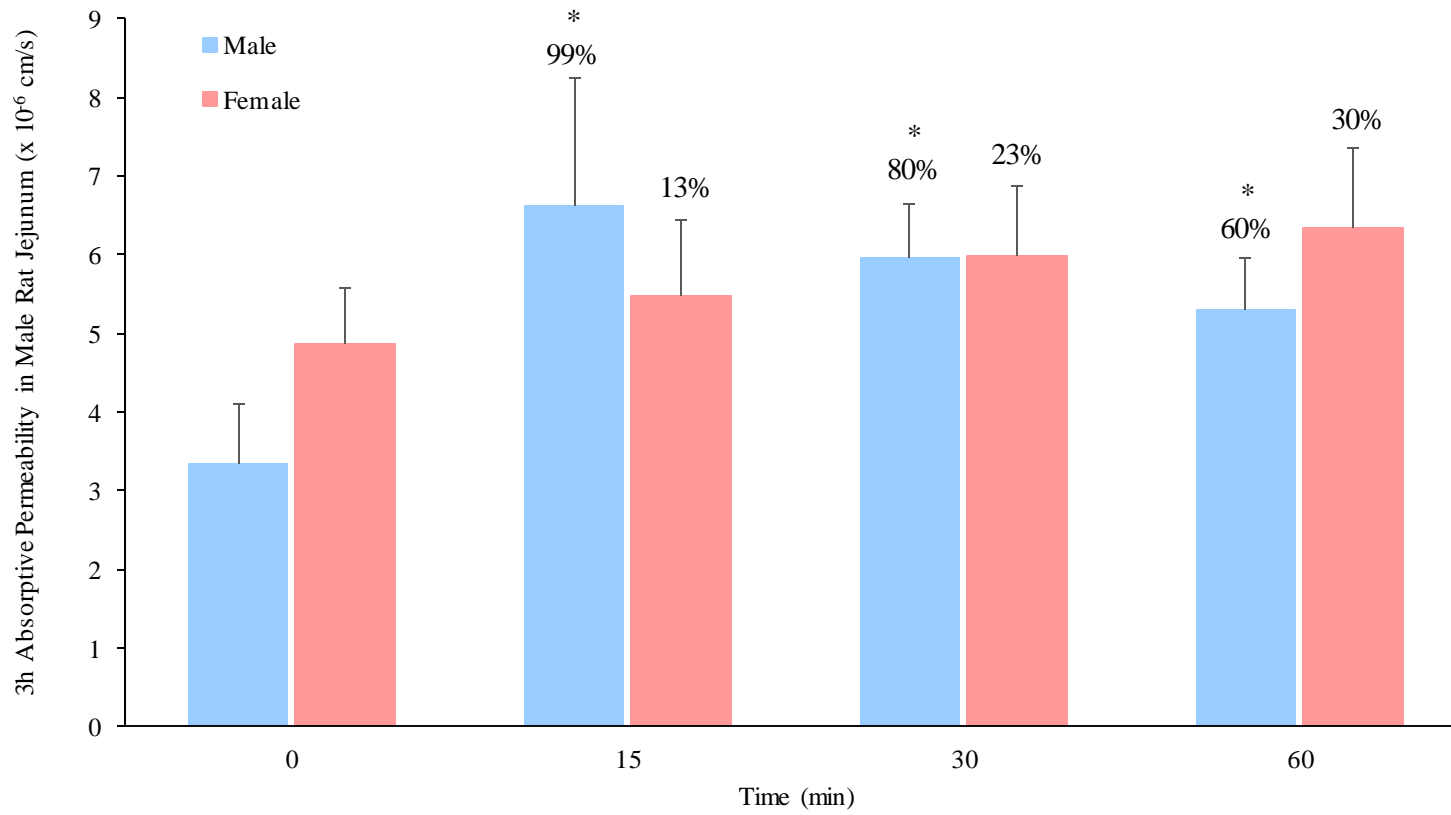
Fig.5.

A

B

Fig.6.

A

B

Fig. 7.

A

B
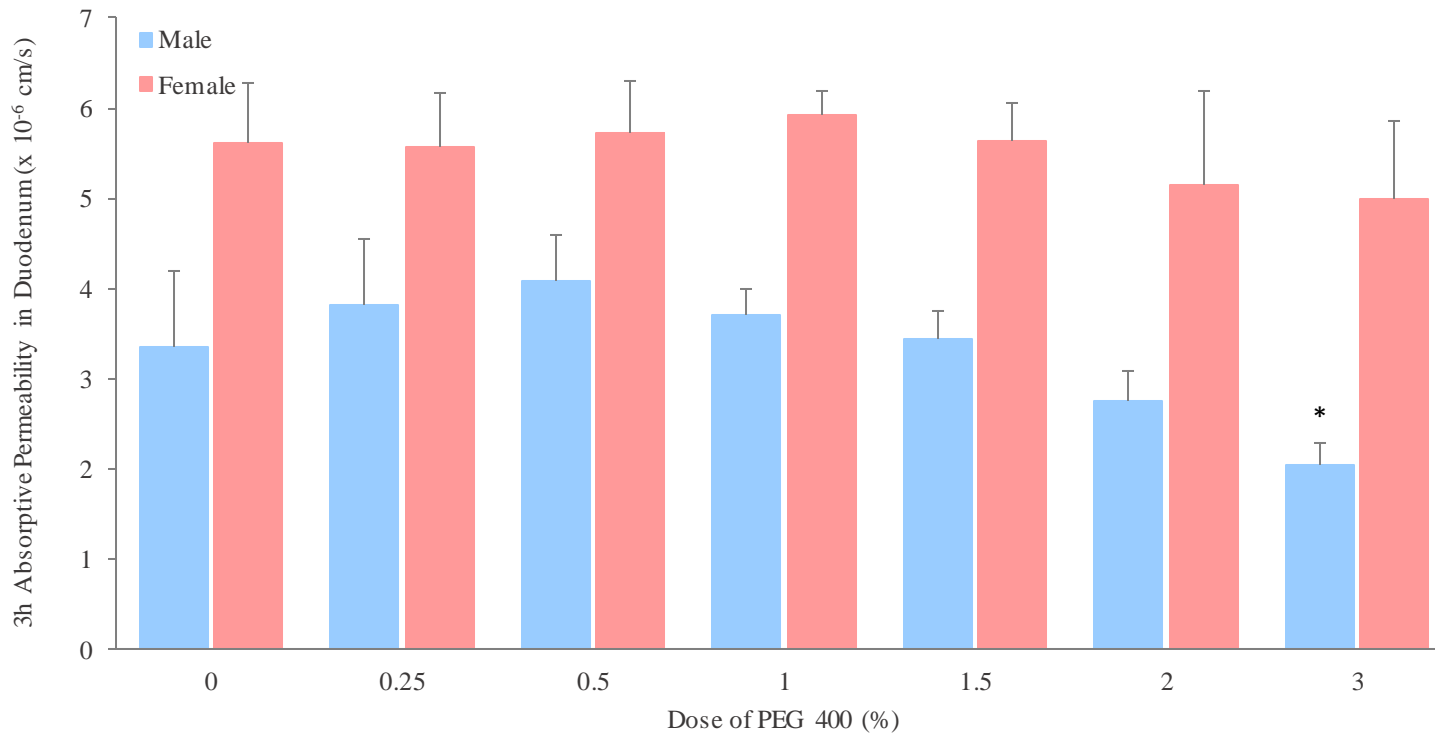
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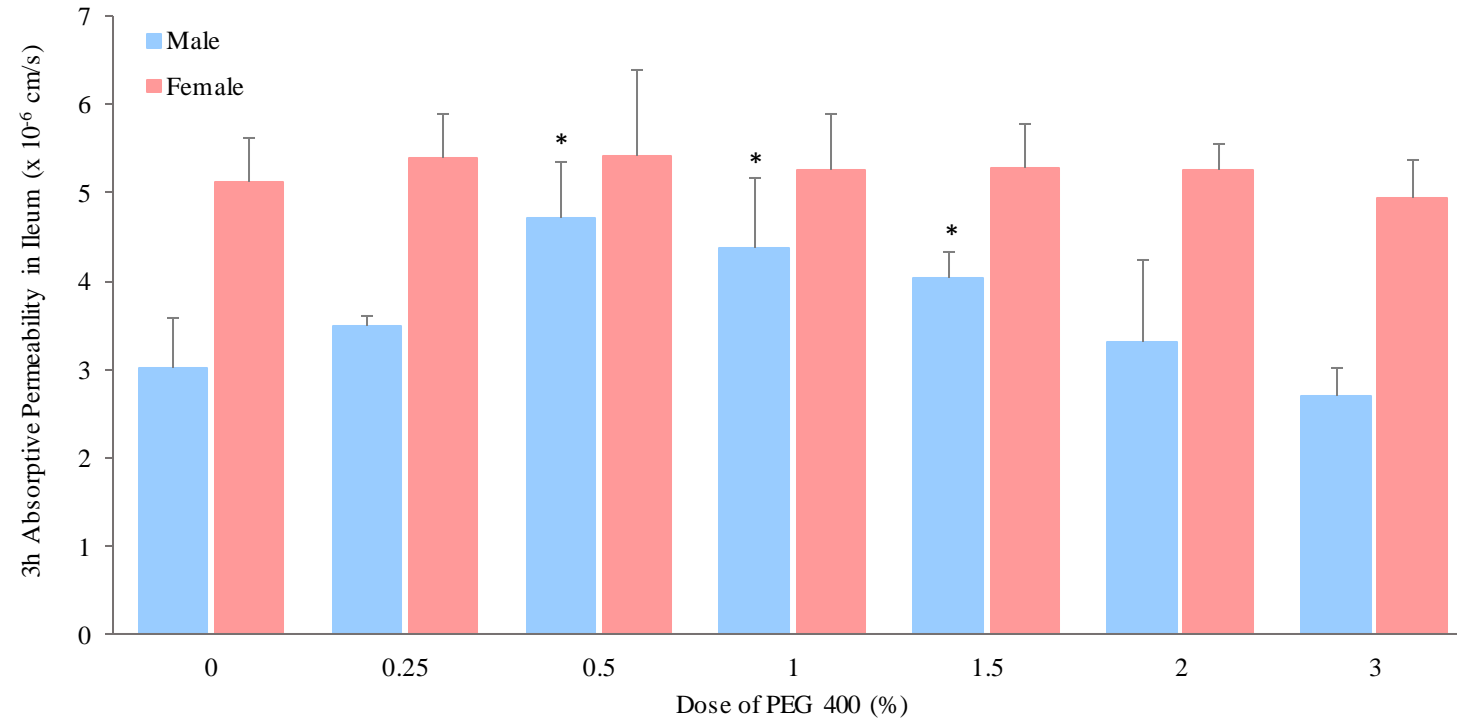

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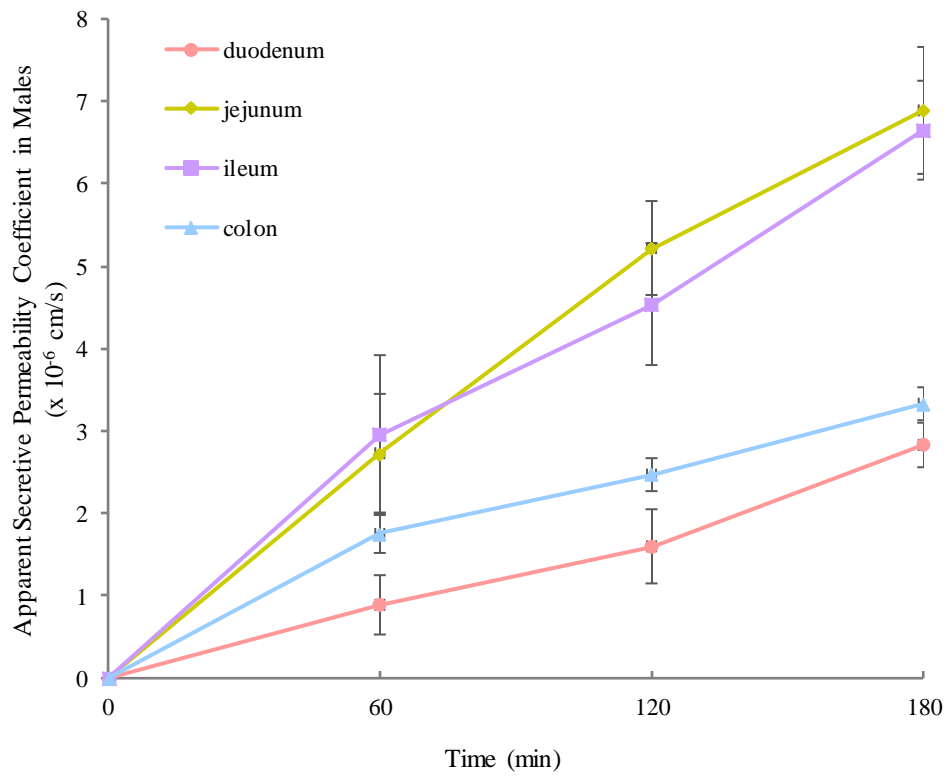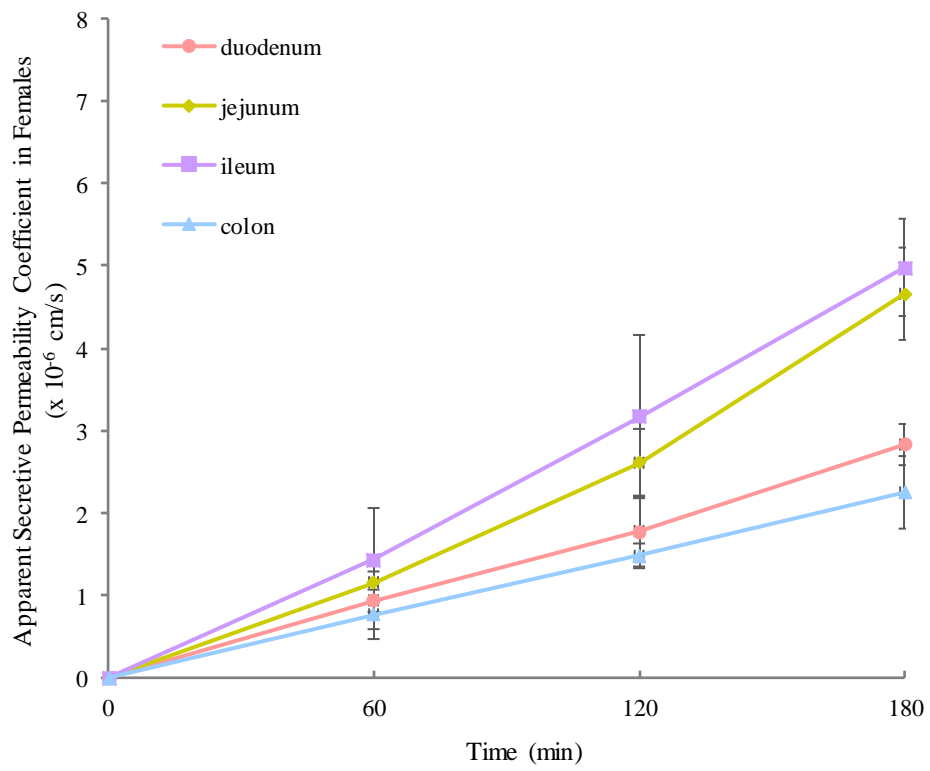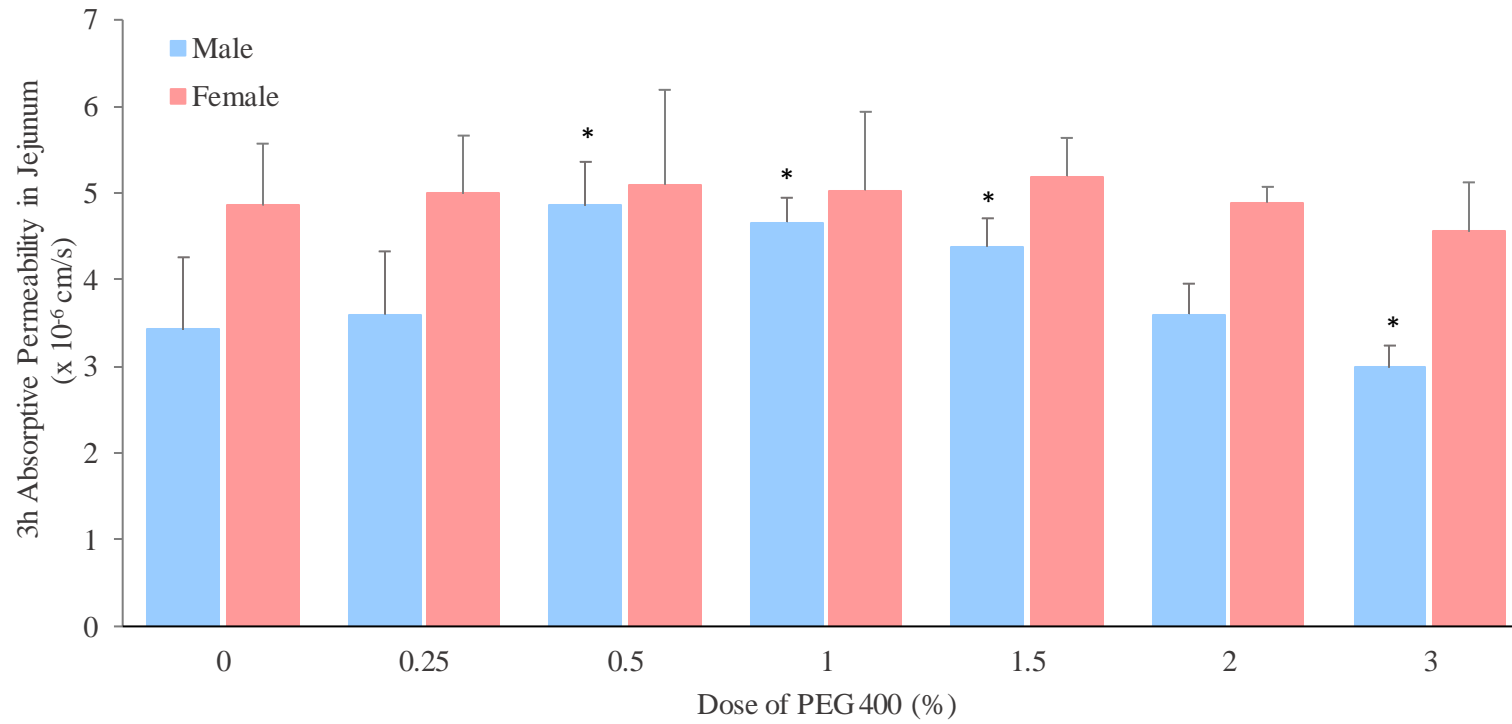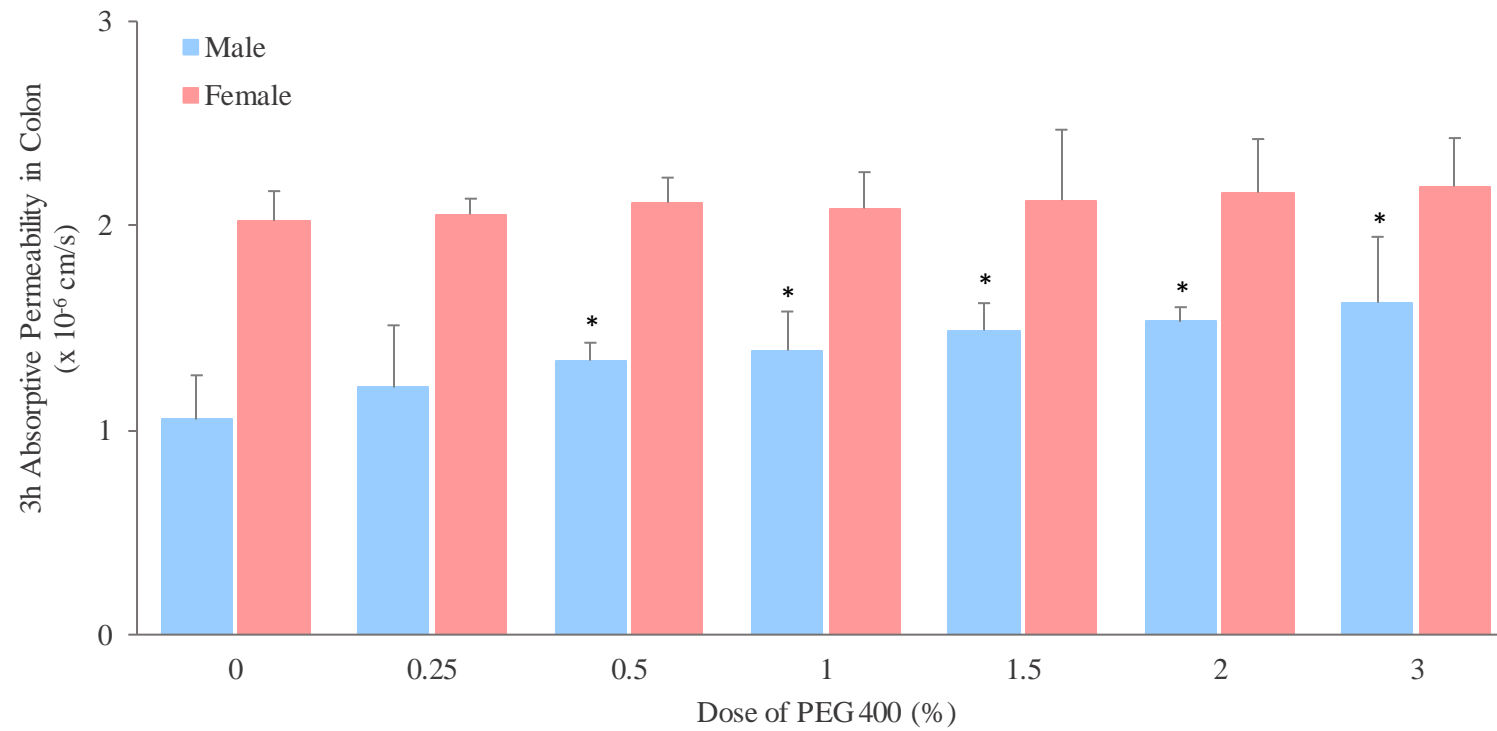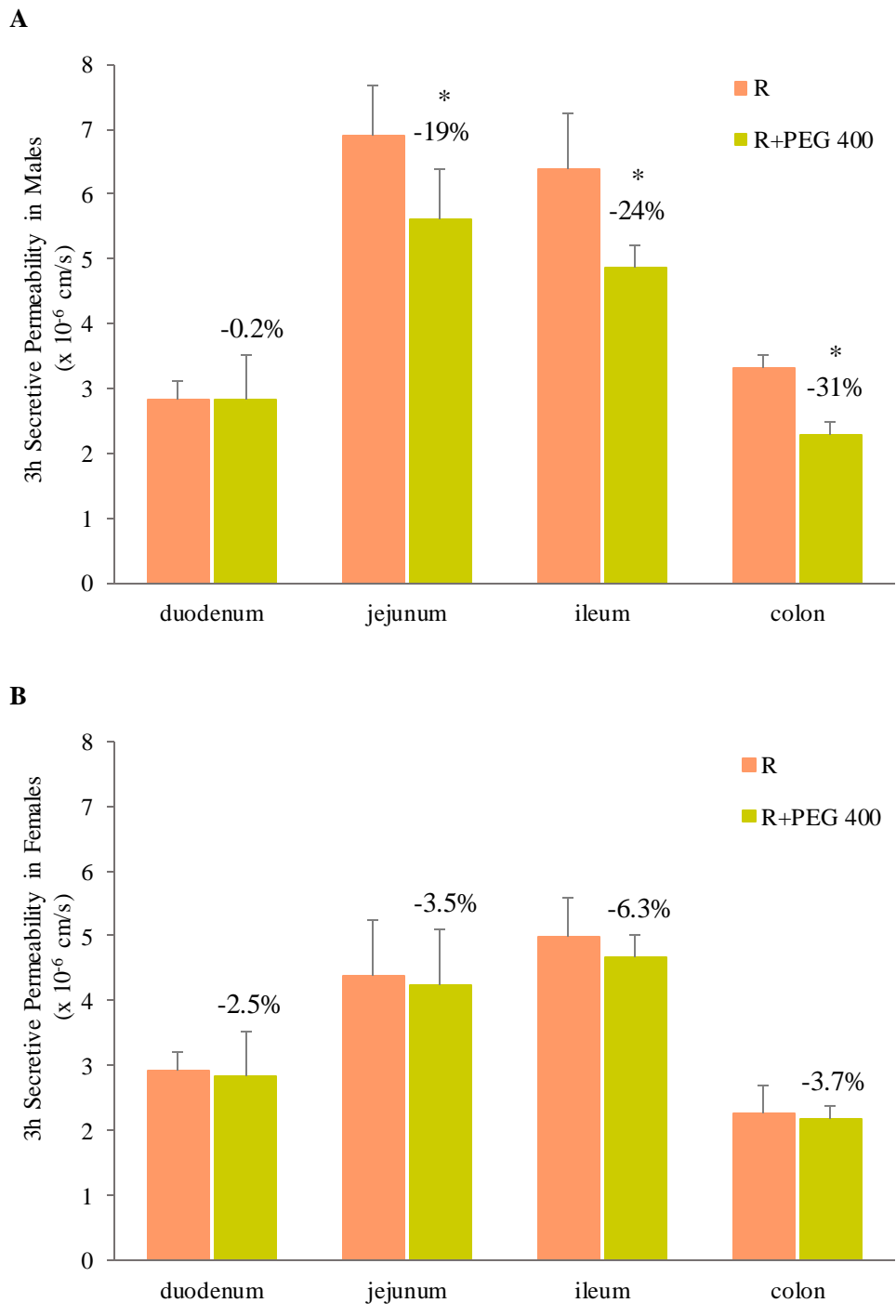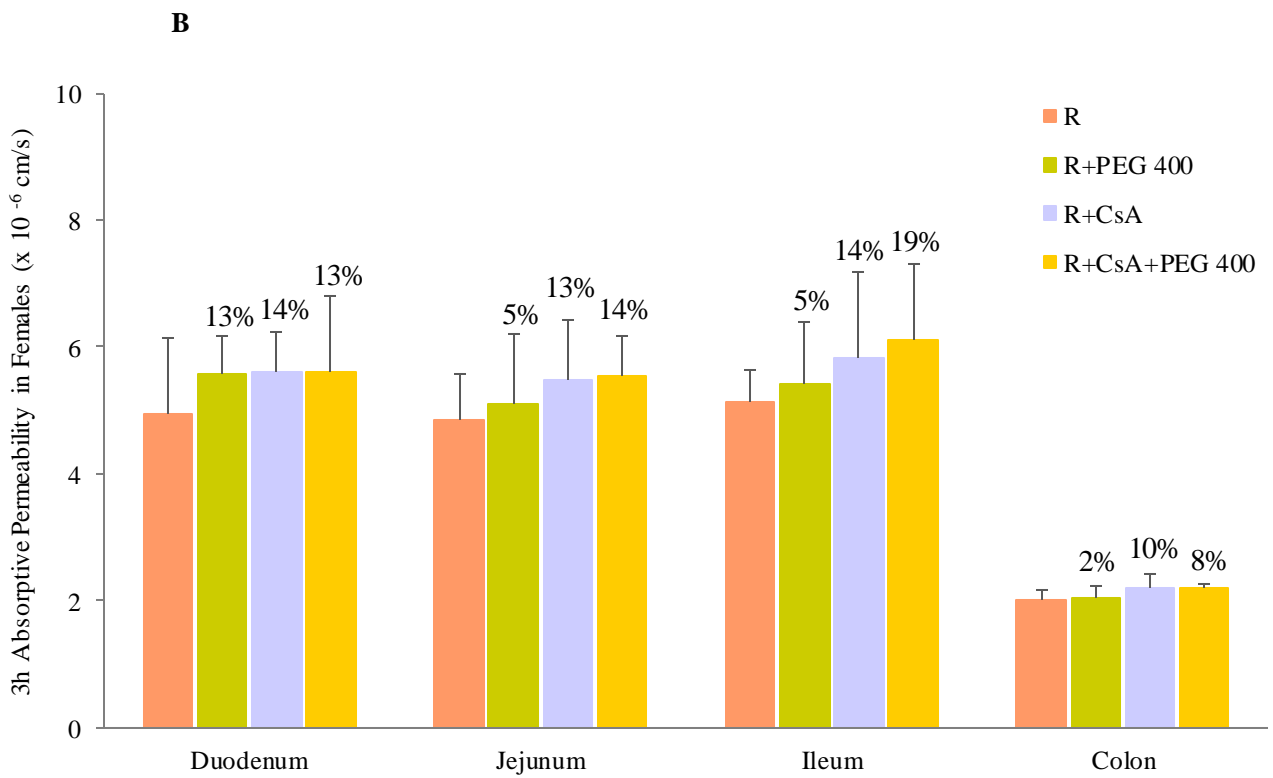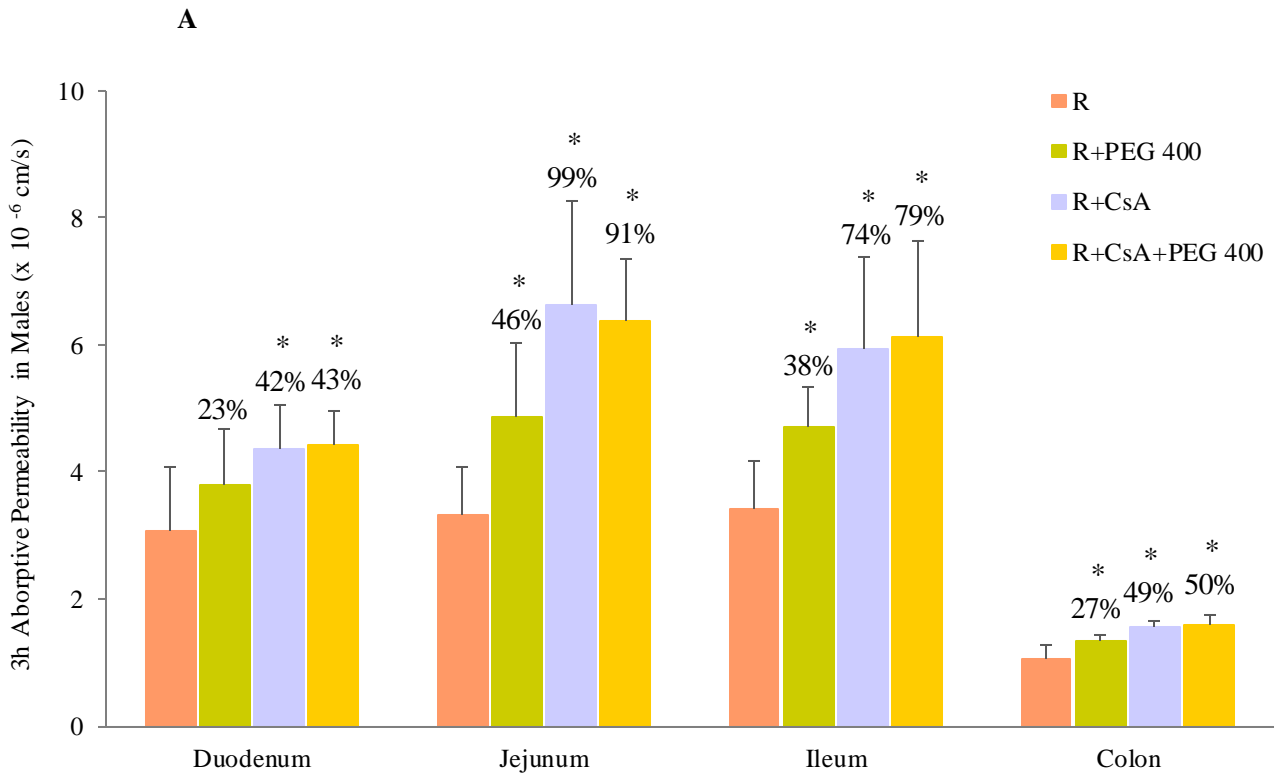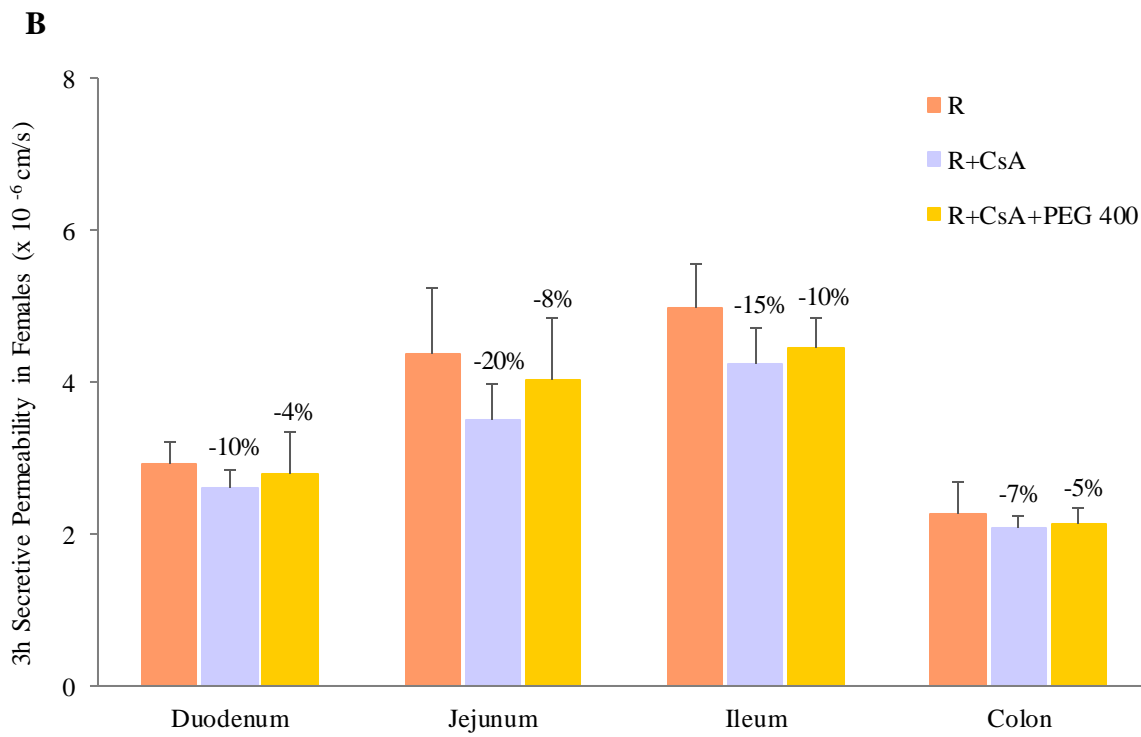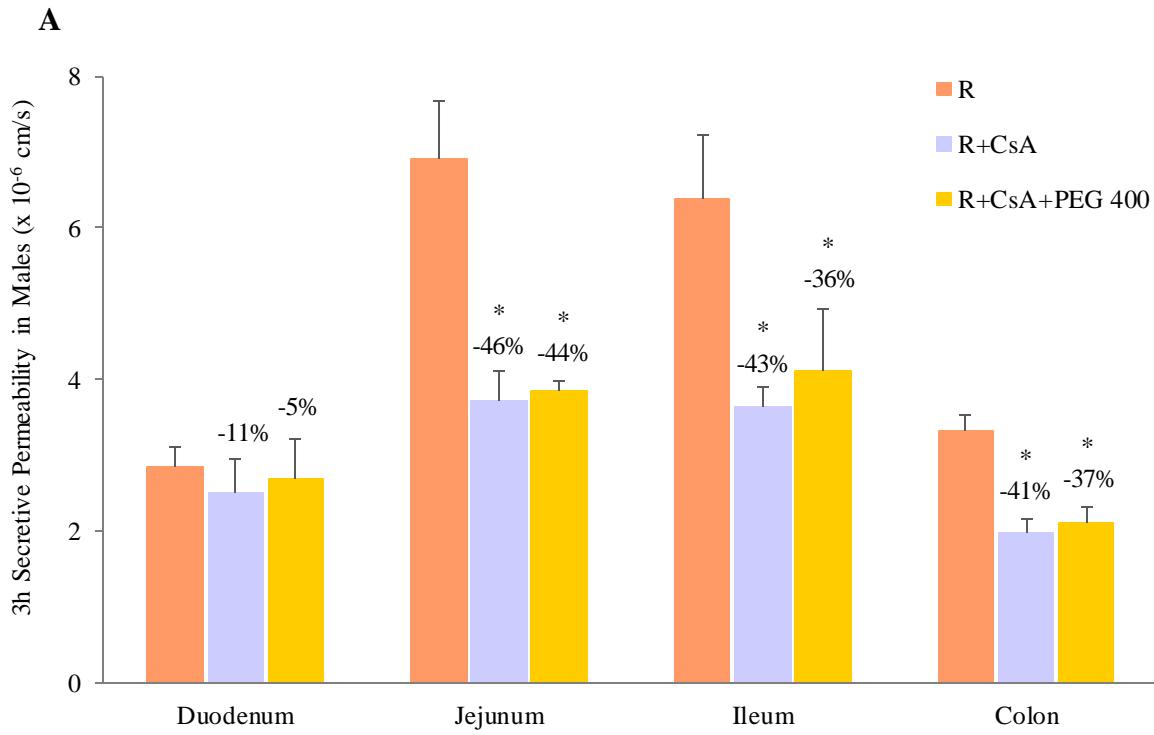
Table 1. Efflux ratio of ranitidine in the absence and presence of PEG 400 or CsA in the duodenum, jejunum, ileum and colon of male and female rats (Mean ± S.D., n=6). Ranitidine 3mM, PEG 400 0.5%, CsA 8μM. * Values are statistically different between the control (R, ranitidine only) and tested groups (R+PEG, R+CsA, R+CsA+PEG) at p<0.5.

		duodenum	jejunum	ileum	Colon
Males	Ranitidine	1.01±0.36	2.20±0.70	1.96±0.58	3.29±0.83
	Ranitidine + PEG	0.64±0.03 *	1.14±0.22 *	1.05±0.15 *	1.75±0.13 *
	Ranitidine + CsA	0.56±0.11 *	0.64±0.16 *	0.65±0.13 *	1.30±0.12 *
	Ranitidine + PEG + CsA	0.61±0.09 *	0.60±0.11 *	0.67±0.21 *	1.33±0.25 *
Females	Ranitidine	0.55±0.18	0.92±0.25	0.98±0.18	1.36±0.48
	Ranitidine + PEG	0.49±0.14	0.84±0.19	0.88±0.13	1.06±0.09
	Ranitidine + CsA	0.47±0.09	0.71±0.09	0.76±0.17	0.95±0.09
	Ranitidine + PEG + CsA	0.5±0.12	0.73±0.12	0.73±0.15	0.98±0.14

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Table 2. Sex differences in the effect of PEG 400 on ranitidine bioavailability in rat study and Ussing chamber study

Dose of PEG 400 in Ussing chamber Study (%)	Sex Differences in the Segments of Intestine				Sex Differences in Wistar rats study	Dose of PEG 400 in Wistar rats study (mg/kg)
	duodenum	jejunum	Ileum	colon		
0.25						12.9
0.5		√	√	√	√	25.7
0.1		√	√	√	√	51.4
1.5		√	√	√	√	77.1
2				√		102.8
3	√	√		√	√	154.2

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('√'=obvious sex differences; blank=no sex differences)

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