Role of Gender in Absorption and Disposition of Soy Isoflavones in Rats.

A

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ABSTRACT

Female Sprague-Dawley rats showed 2-fold higher total oral bioavailability (^{14}C genistein) than male rats. Due to experimental limitations of measurement of radioactivity count, the absolute oral bioavailability of genistein and its phase II metabolites is still unknown. In this study, gender-dependent differences in oral bioavailability of genistein (20mg/kg) in male and female SD rats were determined. Female rats showed significantly higher (2 fold) oral bioavailability of total genistein in female than in male rats. The gender difference observed was due to significantly higher (4 fold) plasma genistein glucuronide concentrations in female than in male SD rats. These results were consistent with the gender-dependent differences in genistein absorption and disposition in rats using rat intestinal perfusion model. In perfusion model, female rats showed significantly higher biliary excretion of genistein glucuronide than in male rats, suggesting higher enterohepatic recycling in female rats as primary mechanism of action for higher oral genistein bioavailability in female than male rats. We also identified that hormonal changes as the result of estrus cycle play an important role in genistein absorption and disposition in female rats. We further compared the oral bioavailability of genistein in control female and female ovariectomized SD rats to determine if the difference in gender was due to differences in female sex hormones produced by ovaries. To our surprise, female ovariectomized rats

showed higher (2.5 fold) oral bioavailability of total genistein than in control female rats, including higher plasma AUC of genistein (2 fold), genistein glucuronide (3 fold), and genistein sulfate (25 fold). Interestingly, exogenous dose of estrogen did not reverse any of bioavailability enhancement effects shown in female ovariectomized rats. The mechanisms responsible for the higher bioavailability of genistein in female ovariectomized rats remain unknown and require further investigation. In conclusion, genistein displayed substantial and significant gender-dependent differences in oral bioavailability of total genistein. Moreover, removal of sex hormones produced in ovary significantly impaired genistein metabolism in female rats, and the effects was not reversed by exogenous estrogen administration.

Keywords: male, female, ovariectomized, genistein, oral bioavailability, estrogen.

ACKNOWLEDGEMENT

I would like to dedicate my PhD thesis to my uncle Prof. Dr. Prasad Kulkarni, my parents Mr. Hridaynath S. Kulkarni and Snigdha H. Kulkarni, my brother Dnyanesh H. Kulkarni and my sister Akalpita H. Kulkarni for their unwavering love and support in my PhD career and life in whole. My uncle is my father-figure since my childhood when my father passed away.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION TO PROJECT

Polyhydroxylated phytochemicals such as flavonoids and isoflavones have received major attention for their health beneficial activities such as antioxidant [1-5], phytoestrogenic for postmenopausal symptoms [6], cancer chemo-prevention [7-10] and prevention of heart diseases [10-14].

Recent studies have shown that although absorption of these compounds (in the aglycone form) is very rapid, flavonoids show very low bioavailability [15]. This may be due to extensive phase II metabolism in gut and liver by various UGT and SULT isoforms even in the presence of significant enteric and enterohepatic recycling [16-19]. The flavonoid concentrations that showed the strong health beneficial activities in *in vitro* settings were in micromolar (μM) range whereas the *in vivo* aglycone concentrations obtained were in nanomolar (nM) range in rodents [20, 21]. The systemic flavonoids aglycone concentrations achieved in rats are less than 5% [21-23].

Only a few studies have been done to show gender-dependent difference in systemic bioavailability of flavonoids in rodents and humans. Coldham and coworkers have shown that oral bioavailability of total genistein to be 7 and 15% in male and female Sprague Dawley (SD) rats, respectively [24]. In that study,

radioactive genistein (4mg/kg) was orally administered as a single dose and radioactivity count was measured to determine the concentrations of total genistein in different organs of male and female SD rats. Since, the authors could not measure the concentrations of genistein and its metabolite concentrations separately they measured the total genistein radioactive count. The study, however, showed low and variable but overall two fold higher bioavailability of total genistein in female than in male SD rats [24]. There are no other publications which have shown gender-dependent systemic bioavailability of total or aglycone genistein and/or daidzein in SD or other strains of rats. In humans, significantly higher daidzein aglycone plasma serum levels were observed in pre-menopausal women than men after dietary intake of soy food [25, 26]. To fill the knowledge gaps in the genistein absorption and disposition in rats, we will determine the genistein/daidzein aglycone and its phase II conjugates concentrations in male and female rats for the first time. We will determine the gender-dependent differences in absorption, metabolism and disposition profiles of daidzein and genistein in male and female SD and Wistar rats. Lastly, we will also determine the effect of estrus cycle on the absorption and disposition profile of genistein and daidzein in female SD and Wistar rats respectively as well.

The thesis is divided into six chapters. In Chapter 1, detailed background with literature review as well as introduction for the proposed study is included. Chapter 2 discusses the in detail methods and models used for the proposed

study. In Chapter 3, research objective and long-term goals are discussed along with the hypothesis for the study. From Chapter 4 through Chapter 6, the proposed studies are discussed more in detail along with the research findings using one model at a time. In Chapter 4, we have discussed gender-dependent differences in total bioavailability of genistein in male, female and female ovariectomized SD rats. In Chapter 5, to determine mechanisms that explain a higher bioavailability of soy isoflavones in female, we studied gender-dependent differences in absorption and metabolism of genistein and daidzein in male and female rats of Sprague Dawley and Wistar rat strains using an intestinal perfusion model. To study the higher metabolite concentrations of genistein observed in PK studies in Chapter 4, we used rat intestinal and liver microsome model to determine the gender-dependent differences in microsomal metabolism of genistein and daidzein in Chapter 5. In Chapter 6, we again used rat intestinal perfusion model to study the possible role of estrus cycle as well as sex hormones on absorption and disposition of genistein and daidzein in female rats.

Chapter 1

1.1.A. Introduction to flavonoids

Flavonoids are a large group of polyphenolic compounds derived from plant origin. Along with phenolic acids, these two classes comprise the majority of polyhydroxylated phytochemicals which are found in fruits, vegetables and beverages like tea and wine[27]. In addition to this herbal remedies like Ginko biloba and Milk thistle also contain high amounts of these polyphenols [28].

1.1.B Sources of flavonoids and polyphenols

Flavonoids and other polyphenols are abundantly present in vegetables, fruits, tea and wine [29-33]. Vegetables such as spinach [34], onions [35], broccoli [36, 37], soy and fruits like apples [38], pear, grapes, strawberries, blueberries and cherries contain high amounts of flavonoids [34]. Tea leaves also contain large amounts of flavonoids [39, 40]. Interestingly coffee also has shown high levels of polyphenolic content [41, 42]. Therefore, we can say that green leafy salad, fruits, soy food, red wine and coffee can be a significant dietary source of polyphenols in U.S diet.

1.1.B.1. Introduction to isoflavonoids

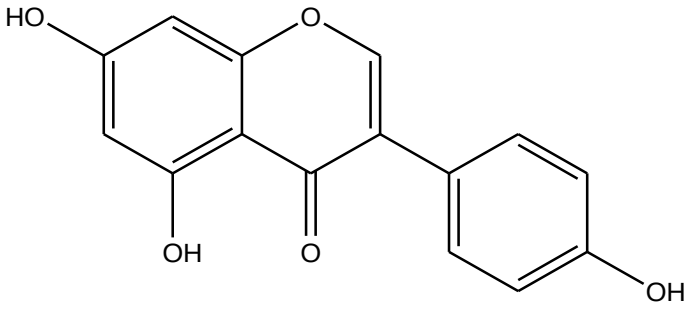
Isoflavonoids is a subclass of flavonoids, with the characteristic benzene ring attached to the 3 position of the benzopyran two ring system (Fig.1.1). The first recognition of isoflavones came in 1976 when legumes containing high amount of formononetin were effective in reducing fertility of quails [43].

1.1.B.2. Sources of isoflavonoids

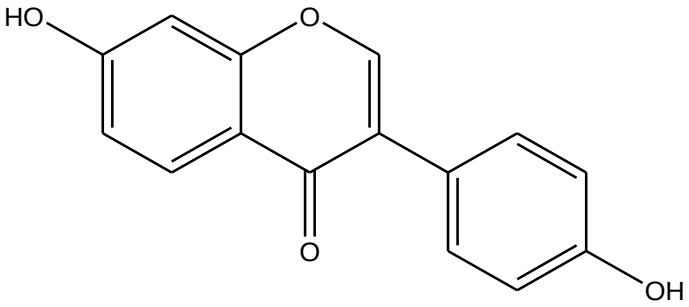
Isoflavonoids are selective with regard to their distribution and are found only in specific dietary plant products such as soybeans (high source of genistein and daidzein), chickpeas (biochanin A), legumes and alfa-alfa sprouts (formononetin) [44, 45].

1.1.C. Bioactivities of soy isoflavones

As mentioned earlier for the flavonoids, consumption of soy food was shown to correlate with increased antioxidant capabilities, prevention of heart disease, and relief of postmenopausal symptom relief [22, 46, 47]. Genistein, a major soy isoflavones, has also shown significant cancer chemo-preventive activity, and is under clinical trial for prostate cancer [7]. Recent studies have shown that genistein (Fig.1.1) in combination of daidzein (Fig.1.1) is significantly effective to potentiate radiotherapy in prostate cancer [48].



Genistein



Daidzein

Figure1.1: Chemical Structures of Soy isoflavones: Genistein and Daidzein

1.1.D. Mechanisms of action of soy isoflavones

Prevention of post menopausal symptoms by genistein and daidzein may be due to their phytoestrogenic activity [49-52]. Soy isoflavones derived from plant origin have estrogenic activity due to close chemical structure resemblance. Hence they are called phytoestrogens (estrogens derived from plant). Most of the chronic diseases prevented by flavonoid intake might be due to reduced levels of oxidative stress [53, 54]. Two theories exist for this mechanism of action. First one entails the antioxidant activity of soy isoflavones in tissue and blood whereas other one mentions the positive influence of flavonoids on the decreasing concentrations of uric acid (a metabolic antioxidant) in the body [55].

1.1.E. Challenges to develop soy isoflavones into “a pill”

The major obstacle for soy isoflavones to make into “pill” form is their low oral bioavailability. Upon single dose oral administration of radioactive genistein 4mg/kg, the highly variable total genistein bioavailability was 7 and 15% in male and female SD rats [24, 56]. In this total genistein bioavailability, very small amount can be attributed to aglycone concentration in plasma since it undergoes extensive first pass metabolism in gut and liver [16, 18, 19]. The concentrations required for soy isoflavone’s efficacy are typically in the range of micromolar (μM)

range whereas the concentrations observed for aglycone *in vivo* are in nanomolar (nM) range [20]. Therefore, the extensive metabolism in gut and liver and thereby higher metabolite concentrations in blood pose a major obstacle for the development of soy isoflavones into a viable “pill” form.

1.2. Xenobiotic metabolism pathways

Xenobiotic metabolism typically involves a variety of metabolizing enzymes that can convert a substrate into a more hydrophilic/water soluble compound thereby limiting its tissue deposition and facilitating its removal from the body. Two categories of xenobiotic metabolisms are well recognized and well documented as of today; phase I and phase II metabolism.

1.2.A. Gender-dependent phase I metabolism

Phase I metabolism reactions may precede the phase II metabolism reactions but this is not a necessarily a priority. Phase I metabolism comprises of three major reaction types: oxidation, reduction and hydroxylation. These reactions are mainly catalyzed by cytochrome P450 monooxygenase (CYP450) enzymes. Gender-dependent difference in male and female SD rats in CYP450 enzyme expression profile was significant, with males showing higher hepatic

expression of certain phase I metabolizing enzymes (CYP1A, CYP2E1) than in female SD rats [57, 58] However, hepatic CYP2D6 and CYP3A showed significantly higher expression in female than in male SD rats [57, 58]. In another study, male rats showed significantly higher expression levels of hepatic CYP450 2C1, 2A2, 2C13 and 3A2 enzymes than in female SD rats whereas hepatic CYP450 2C7, 2A1 enzyme expression levels were higher in female than in male SD rats [57, 59]. CYP2C11 is only expressed in male rats whereas CYP2C12 is only expressed in female rats. The authors also showed that the enzyme expression profiles changed significantly with the burst of sex hormone (estrogen, progesterone and testosterone) levels in male as well as female SD rats. Shapiro *et al* successfully showed that higher concentrations of estrogen and testosterone hormones significantly reduced the hepatic CYP2C11 and CYP2C12 expression levels in rats [57]. It has also been suggested that growth hormone plays a vital role in suppression and up-regulation of certain CYP450 metabolizing enzymes in male and female rat [57]. It has been suggested that rats is a better model to study the gender-dependent differences in test compound oral bioavailability due to large differences in metabolizing expression profiles in rats as compared to mice as well as humans [58]. However, as anticipated, the gender-related differences observed in rats cannot be translated into humans directly due to varying degree of gender-related enzyme expression differences in humans and rats.

In humans, higher CYP3A4, 2A6 and 2B6 activity has been observed in women than men whereas CYP2D6 and 1A2 levels are higher in men than women [60]. This fact can explain higher clearance of erythromycin, nifedipine and cyclosporine, all CYP3A4 substrates, in women than men. Caffeine, clozapine and olanzapine (CYP3A2) showed higher clearance rates in men than women who in turn showed higher sensitivity as well as adverse reactions to the antipsychotic drugs (e.g. clozapine and olanzapine) [60].

It has been shown that sex hormones also influenced gastrointestinal motility. For example estrogen inhibits gastric emptying and thereby can improve systemic bioavailability of certain compounds in female rats [58].

1.2.B. Gender-dependent phase II metabolism

The predominant family of phase II metabolizing enzymes responsible for metabolism of isoflavones is uridine diphospho (UDP)-glucuronosyltransferases (UGTs) that uses UDP-glucuronic acid as a co-substrate in the conjugation reaction. Another important family of phase II metabolizing enzymes is sulfotransferases (s). Sulfotransferases are cytosolic enzymes; whereas UGTs are endoplasmic reticulum membrane bound enzymes in the cell. The products

of these conjugation reactions are usually bulkier with a higher molecular weight due to an addition of glucuronide or sulfate group to the aglycone moiety.

In case of UGT metabolizing enzymes, only two enzyme isoforms have shown gender-related differences in rats. Female rats have shown higher hepatic expression levels of UGT1A6 and 2B12 enzymes than in male SD rats [61]. No study was done to show gender-dependant intestinal UGT enzyme expression levels in rats. The lack of data might be due to very few UGT isoform-specific antibodies available in the market.

In case of SULT isoforms, gender-related difference in rats was very obvious. Female rats showed significantly higher expression levels of hepatic SULT1A1, 1B1, 1C1 and 1E1 isoforms whereas SULT2A isoform showed higher expression levels in male SD rats [62]. No significant gender-related difference in hepatic SULT1C2 isoform expression was observed. No detectable amount of expression of above mentioned SULT isoforms was observed in male as well as female SD rats in small intestine. Only SULT1B1 and 1C2 enzyme isoforms were detected (significantly lower than liver) in colon and no gender-related difference was observed in their expression profiles in SD rats [62, 63].

Acetaminophen clearance is higher in men than women because of a higher rate of glucuronidation [63]. The higher bioavailability of aspirin in women

has been due to lower glucuronidation and slower rate of clearance as compared to men [64].

Gender-related differences can also be due to body weight (lower in women), body fat (higher in women), plasma volume (lower in women but changes according to the menstrual cycle), and organ blood flow (higher in women). In addition, body weight (lower in women) can influence the glomerular filtration rate which can influence the clearance of compounds that are eliminated through kidneys [65].

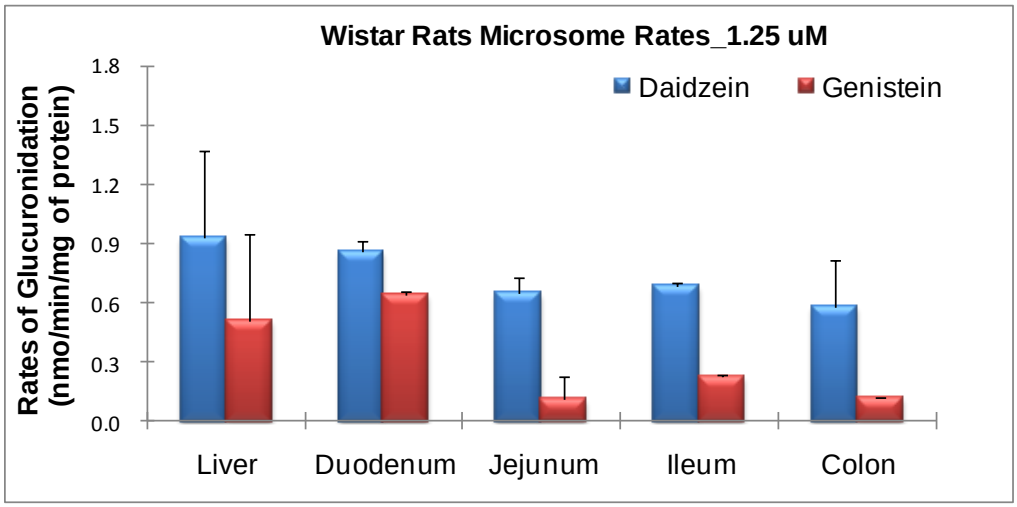
In mice, Klaassen's lab has shown gender-dependent difference in UGT enzyme expression profiles in liver and kidney tissues. Female showed significantly higher UGT1A1 and 2 as well as lower UGT2B1 expression levels in liver than in male C57BL6 of mice [66, 67]. Similarly higher UGT1A2 and lower UGT2B38 levels were observed in female than in male HX and GNX mice [66, 67].

1.2.C Strain-dependent differences in phase I and II metabolism

There are certain reports that showed strain-dependent differences in cytochrome P450 enzyme expression profiles in SD and Wistar rats [68]. Basal CYP1A1, 1A2 and 3A2 levels were significantly higher in male Wistar as

compared to male SD rats [68]. The phenobarbital treatment, a strong CYP inducer, showed significantly higher induced expression levels of CYP1A1, 1A2 and 3A2 in Wistar than in SD rats [68, 69]. However, no significant enzyme induction was observed in case of CYP2B1/2, 2C6 and 3A1 in Wistar and SD rats [68]. The authors also found out that expression levels of nuclear receptors such as PXR, CAR and FXR responsible for CYP1A and 3A hepatic enzyme inductions are significantly higher in Wistar than in SD rats [68].

Dr. Hu's lab has some unpublished data that suggests higher activity of certain UGT isoforms that are responsible for genistein and daidzein metabolism in Wistar than SD rats (Fig.1.2 and Fig.1.3). In figures, the rates of glucuronidation of genistein and daidzein were significantly higher (more than 2 folds) in male Wistar than in SD rats. No data has been published yet showing strain-dependent differences in phase II metabolizing enzyme expression profiles in rats. Similar to UGTs, no data has been published yet showing strain-dependent difference in SULT isoforms in rat as well.


Wistar Rats Microsome Rates_1.25 uM
Rates of Glucuronidation (nmo/min/mg of protein)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Daidzein
Genistein
Liver
Duodenum
Jejunum
Ileum
Colon

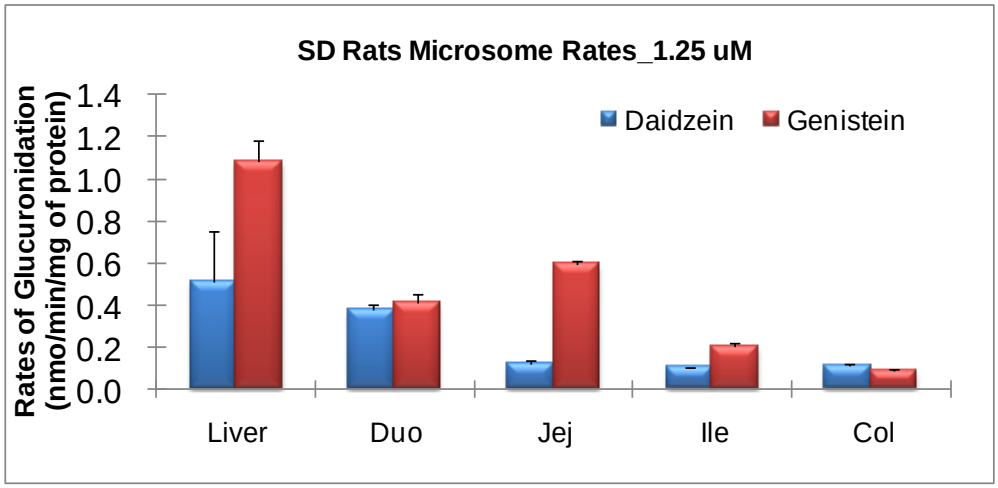
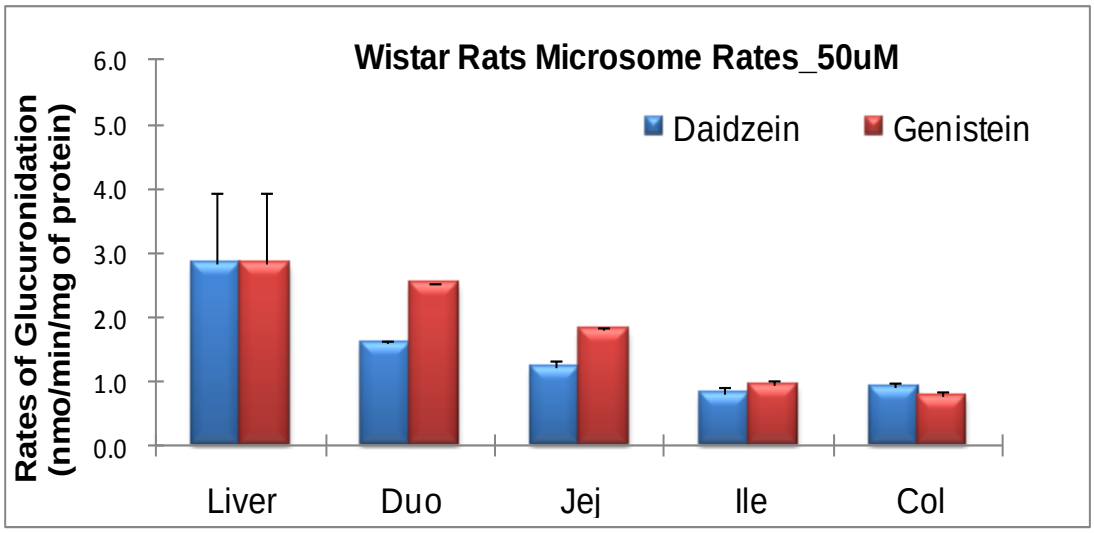

SD Rats Microsome Rates_1.25 uM
Rates of Glucuronidation (nmo/min/mg of protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Daidzein
Genistein
Liver
Duo
Jej
Ile
Col

Figure 1.2: Rate of metabolism of daidzein and genistein (1.25µM each) in male Wistar and SD rat intestinal and liver microsomes. The Metabolism rate is the average of three determinations and the error bar represents the standard deviation of the mean.


Wistar Rats Microsome Rates_50uM
Rates of Glucuronidation (nmo/min/mg of protein)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Daidzein
Genistein
Liver
Duo
Jej
Ile
Col

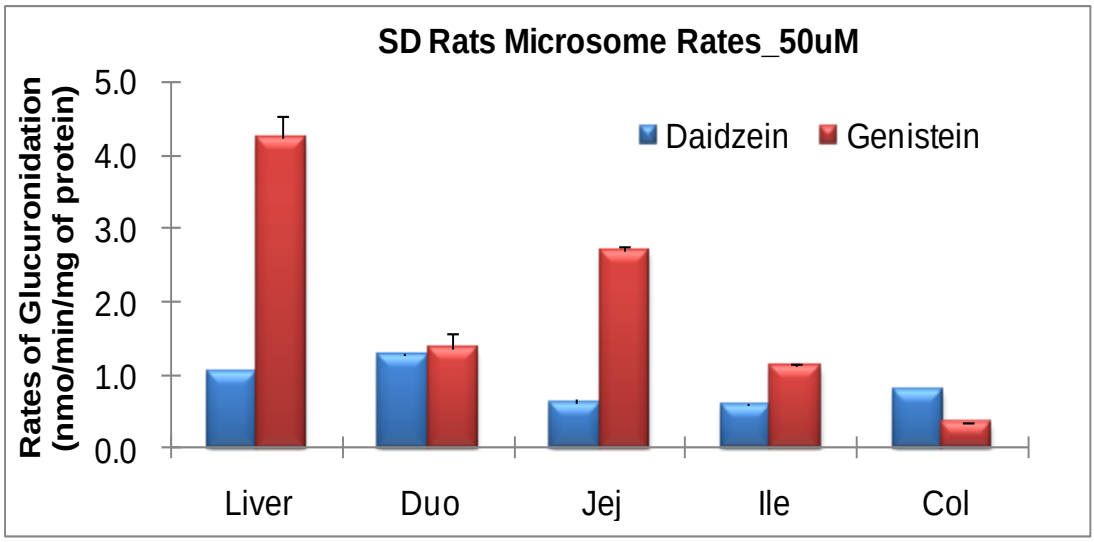

SD Rats Microsome Rates_50uM
Rates of Glucuronidation (nmo/min/mg of protein)
5.0
4.0
3.0
2.0
1.0
0.0
Daidzein
Genistein
Liver
Duo
Jej
Ile
Col

Figure 1.3: Rate of metabolism of daidzein and genistein (50µM each) in male Wistar and SD rat intestinal and liver microsomes. The Metabolism rate is the average of three determinations and the error bar represents the standard deviation of the mean.

1.3. Literature review of gender-dependent PK studies in rats

Gender-dependent PK studies are done to understand the possible differences in absorption and disposition of drugs in male and female rats. Understanding the gender-dependent differences in CYP450, UGTs and SULT enzyme isoforms in liver, intestine, kidneys and the rest of the organs and their implications on the overall bioavailability of compounds in male and female rats can only be accomplished by doing oral and intravenous pharmacokinetic studies in rats.

In the oral PK study done by Lash *et al* in Fisher F344 male and female rats for trichloroethylene at three known concentrations of 2, 5 and 15mM/kg body weight respectively showed concentration- and gender-dependent results [70]. Higher concentrations of the parent compound were observed in various tissues in male than in female rats. Similar to the parent compound, higher concentrations of CYP metabolites were observed in liver for males whereas in kidneys for female rats. However, significantly higher concentrations of glutathione metabolite concentrations of trichloroethylene were observed in liver and blood in female than in male rats. A biphasic pattern of trichloroethylene concentrations was also observed suggesting enterohepatic recycling in both male and female rats. The concentrations of the parent and the metabolite showed dose dependent increase in both male and female rats [70].

A study done by Wempe *et al* showed significantly higher bioavailability of letrozole in female (95%) than in male (38%) SD rats [71]. The possible reason for such a dramatic gender difference might have been due to difference in absorption. The change in drug delivery system to improve the solubility of letrozole, improved the rate as well as amount of absorption of letrozole in male rats whereas it decreased the rate but not the extent of absorption of letrozole in female SD rats. That shows the presence of gender-dependent difference in drug absorption and thereby bioavailability of certain polyphenolic compounds in SD rats [71].

The PK study of morphine in Wistar-Kyoto rats showed slower rate of glucuronidation of morphine in male than in female rats [72]. This caused significantly higher morphine aglycone levels whereas lower morphine -3-glucuronide levels in serum, urine and bile in male than in female Wistar-Kyoto rats. Phenobarbital, a nuclear receptor agonist, caused increase in morphine metabolism in male but not female rats, diminishing the gender-dependent difference in parent and metabolite concentration levels in Wistar-Kyoto levels [72].

In an interesting study done by Schlattjan *et al*, cellular sodium taurocholate clearance in proximal tubular region was slower in male (133.9 ± 29.8 µl/ml ×100 g b.w.) than in female (262 ± 45.4 µl/ml ×100 g b.w.) SD rats

[73]. Testosterone treatment did not show any significant difference on the levels of taurocholate clearance in female rats. However, ethylene-estradiol treatment showed significant lowering (61.6 ± 10.1% lower) of taurocholate clearance in male SD rats. Also ovariectomized female SD rats did not show any significant difference in cellular taurocholate clearance however, ethylene estradiol treatment again showed significant lowering (53.7 ± 15.0%) of proximal tubular cellular taurocholate clearance. This shows gender-dependent difference in sodium taurocholate proximal tubular clearance in male and female rats and also shows that sex hormones especially estrogen might be playing an important role in producing these effects [73].

Ifosfamide PK studies done in male and female SD rats also showed similar results with higher $AUC_{0-\infty}$ as well as plasma half life values in female than in male SD rats [74]. No gender-dependent difference was observed in volume of distribution, plasma protein binding and renal clearance values for ifosfamide in SD rats. The gender-related PK profile difference for ifosfamide was attributed to its stereoselective metabolism into 4-hydroxylation (R-ifosfamide) and N2/N3 dechlorethylation (S-ifosfamide) [74].

	2hr (ng) Genistein Eq		7hr (ng) Genistein Eq		24hr (ng) Genistein Eq	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Stomach	**8102 ± 4633**	**7394 ± 4332**	**4680 ± 1572**	**3205 ± 1129**	**498 ± 347**	**339 ± 67**
Small Intestine	**18458 ± 13060**	**25867 ± 14500**	**11894 ± 6501**	**18864 ± 6234**	**1088 ± 998**	**2714 ± 2261**
Coecum	**478 ± 344**	**288 ± 149**	**17397 ± 8344**	**5698 ± 3338**	**1920 ± 1359**	**2146 ± 1597**
Liver	**980 ± 261**	**2666 ± 1160**	**1062 ± 277**	**1975 ± 196**	**236 ± 85**	**314 ± 171**
Kidney	**686 ± 176**	**269 ± 118**	**515 ± 349**	**399 ± 100**	**57 ± 30**	**71 ± 21**
Testes/ Ovary	**117 ± 25**	**250 ± 119**	**96 ± 19**	**259 ± 18**	**23 ± 8**	**57 ± 32**
Prostate/ Uterus	**278 ± 220**	**253 ± 95**	**454 ± 192**	**293 ± 43**	**250 ± 294**	**77 ± 47**
Vagina		**322 ± 88**		**417 ± 144**		**289 ± 274**
Plasma	**785 ± 194**	**836 ± 445**	**779 ± 222**	**839 ± 67**	**116 ± 42**	**163 ± 84**

Table 1.1: Gender-dependent tissue disposition in male and female SD rats after 4mg/kg genistein oral dose

Until now, only one study has been published showing gender-related differences in soy isoflavones PK profiles in rats [24]. The oral genistein PK study has shown 7 and 15% systemic bioavailability of total genistein in male and female SD rats respectively (Table1.1). In the study, the authors used radioactive genistein which could not differentiate between parent and metabolite fractions. Therefore, the systemic bioavailability of genistein observed was the total genistein and not aglycone bioavailability alone. Gender-dependent differences in tissue disposition of radioactive genistein after 2, 7 and 24 hours were observed in male and female SD rats (Table 1.1). Significantly higher total genistein disposition was observed in liver in female than that in male rats up to 7hr time points. Other tissues that also showed higher total genistein disposition in male and female SD rats were small intestine, stomach, kidneys and sex organs. The observed higher systemic bioavailability of total genistein in female SD rats might be due to extensive phase II metabolism of genistein in gut and liver and highly efficient enterohepatic genistein glucuronide recycling [24].

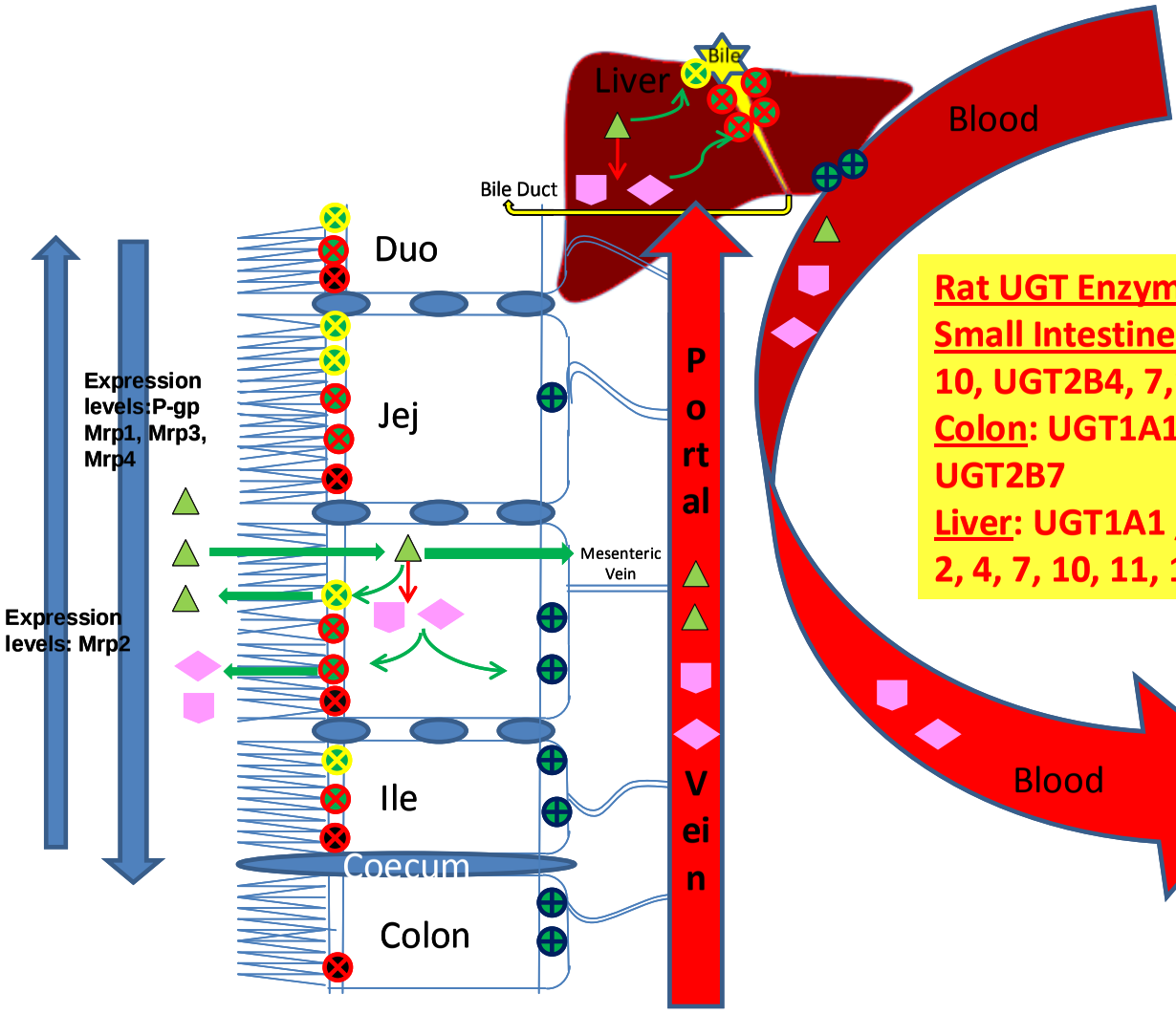
There is no literature available to show daidzein PK profile in male and/or female rats. Therefore, there is no data suggesting gender-related difference in plasma daidzein aglycone and/or daidzein metabolite (glucuronide and sulfate) concentrations in male and female rats. However, in a study done by Cassidy *et al* , men and premenopausal women showed no significant differences in plasma

concentration levels of daidzein after dietary intake of soy food containing daidzein, genistein and glycitein [25, 26].

In this project, we will perform single oral gavage dosing of daidzein and genistein 20mg/kg respectively and measure the plasma concentrations of the aglycone and its respective metabolites over the period of 24 hours in both male and female rats. This will give us insight into gender-dependent difference in overall and absolute systemic bioavailability of daidzein and genistein in rats.

1.4. Pathways of drug elimination

Soy isoflavones undergo extensive phase II metabolism in intestine and liver and their glucuronide and sulfate metabolites require efflux transporters for their excretion onto the luminal or serosal side. Metabolites being bulky and hydrophilic in nature cannot be passively diffused and therefore require efflux transporters for their elimination from the intestinal or hepatic cells. Once excreted on the luminal side of the enterocytes, the soy isoflavones metabolites as well as the aglycone are eliminated through feces. The aglycone and their phase II metabolites that are excreted on the serosal side of the enterocytes can be eliminated through kidneys or though bile into the intestinal lumen and thereby into feces (Fig 1.4).



Rat UGT Enzyme Expression Profile

Small Intestine: UGT1A1, 3, 4, 6, 8, 10, UGT2B4, 7,10, and 15

Colon: UGT1A1, 3, 4, 6, 8, 9, 10, and UGT2B7

Liver: UGT1A1 , 3, 4, 6, 9, UGT2B1, 2, 4, 7, 10, 11, 15, 17, and 28

Figure 1.4: Flow diagram showing the absorption and metabolism of flavonoids in intestine and liver. : BCRP transporter; : Mrp2 transporter; : Flavonoid aglycone; / : Flavonoid metabolites; : P-glycoprotein; :Mrp1, Mrp3, Mrp4 Transporter

The efflux transporters responsible for the elimination of soy isoflavones aglycone and their metabolites are P-gp, Mrp family (glucuronides) and BCRP (sulfate and glucuronide) transporter family proteins. These transporter proteins play an important and vital role in the soy isoflavone absorption and disposition in male and female rats. In rats, gender-dependent difference has been shown in expression levels of certain efflux transporters which may be linked to the observed differences in the soy isoflavones bioavailability in rats.

1.4.A Gender-dependent expression of P-glycoprotein

Many anticancer drugs are strong substrates of P-gp efflux transporter along with certain antibiotics, antivirals, calcium channel blockers, immunosuppressive agents and plant polyphenols found in regular diet. Substrates of P-gp are typically cationic and lipophilic in nature. Soy isoflavones such as genistein and daidzein have been shown to be weak substrates of P-glycoprotein efflux transporter protein [75] (Janneh, Anwar *et al* . 2009). The expression levels of P-glycoprotein go up from duodenum to ileum region of small intestine. Colon or large intestinal expression levels of P-glycoprotein transporter are similar to those in upper jejunum [76-78]. Significant expression levels of P-gp efflux transporter proteins are also observed in liver, especially in the bile duct canalicular membrane [79, 80].

Gender-dependent differences have been observed in the expression levels of P-gp in hepatocytes. In an *in-vitro* study, control female SD rats showed higher expression levels of P-gp in hepatocytes than in male SD rats. Upon treatment of high testosterone concentration of 50μM, significantly lower expression levels of P-gp were observed as compared to control in hepatocytes suggesting a possible role of sex hormones in regulation of expression levels of these transporter proteins in rats.

The major role of P-gp transporter protein is to limit the bioavailability of xenobiotics by excreting them out of the cell. The P-gp efflux transporter proteins are predominantly expressed on the apical side of the cells thereby driving the aglycone substrate back to the apical side. During simultaneous dosing regimen of pharmaceutical compounds along with xenobiotics may lead to unintentional increase in the bioavailability of active compounds leading to toxicity and health complications. This unintentional increase in the bioavailability may be due to the manipulation of the bioavailability barrier network by overwhelming of certain efflux transporters/metabolizing enzymes. However, the same concept can be used to intentionally improving the bioavailability of certain compounds of interest by carefully adjusting the dose and disrupting the P-gp barrier network. Certain flavonoids have been shown to improve the bioavailability of P-gp substrates when given in significantly high amounts [75].

1.4.B. Gender-dependent expression of multidrug resistance protein (Mrp)

Phase II metabolites of majority of dietary polyphenolics have been shown to be excellent substrates of multidrug resistance protein (Mrp) efflux transporter protein family. These ATP binding cassette efflux transporter proteins play a major role in tissue defense system by removing the hydrophilic metabolite out of the cells, reducing the chances of cell toxicity. The Mrp family has multiple efflux transporter proteins such as Mrp1, 2, 3, 4 5, 6, 7 and 8. Mrp1, 2, 3 and 4 are well characterized and their histological locations are well understood. Mrp1 and 2 are present on the apical side whereas Mrp3 and 4 are present on the basolateral side of the enterocytes. Mrp1 and 2 are also present on the canalicular membrane of the hepatocytes where as Mrp3 and 4 are present on the serosal side of the liver cells.

There is not much research being done to elucidate total gender-dependent differentiation of Mrp efflux transporter protein family tissue specific expression levels in rodents and/or humans.

In sham-operated nephroctomized SD rats, Mrp2 and Mrp3 transporter protein mRNA expression levels in hepatocytes were significantly higher in female than in male SD rats [81]. No significant gender-dependent differences

were observed in these rats for Mrp2 and Mrp3 mRNA expression levels in small intestinal and colon cells [82]. Significantly higher expression levels were also observed in kidneys for Mrp3 mRNA expression levels in female than in male sham-operated nephroctomized rats. Again no significant differences were observed in mRNA levels of Mrp1 efflux transporter protein in all regions of male and female SD rat intestine. Significantly higher Mrp4 and Mrp5 mRNA expression levels were observed in colon and in kidneys of female rats than in male sham-operated nephroctomized SD rats [81].

In another study, Rost *et al* found out that hepatic Mrp2 and Mrp3 efflux transporter protein expressions were higher in female than in male SD rats. Furthermore, 3 days treatment of DHEA increased the expression levels of Mrp3 but not Mrp2 protein expression levels only in female but not in male SD rats [83, 84].

In summary, hepatic Mrp2 (4 fold) and Mrp3 (2 fold) expression levels were significantly higher in female than in male SD rats. Similarly, hepatic P-gp efflux transporter expression levels were significantly higher in female than in male SD rats. No gender-dependent differences in hepatic BCRP, Mrp1, Mrp4, Mrp5 and Mrp6 transporters expressions were observed in male and female SD rats.

1.4.C. Gender-dependent expression of organic anion transporters (OATs)

Organic anion transporters (OATs) are important for the transport of sulfate metabolites of steroidal (and phytoestrogenic compounds) hormones in the body. OATs might also function primarily as transporters for endogenous substrates associated with sex hormones. Significant gender-dependent differences were observed in the expression levels of these transporters in rats and mice. These gender-dependent differences in OAT expression would directly influence pharmacokinetics and toxicokinetics of various pharmaceutical and dietary polyphenolic compounds.

Kobayashi *et al* demonstrated significantly higher expression levels of OAT2 in liver than in kidneys in male mice where as the expression levels were almost equivalent in female mice. The expression levels of OAT2 in kidneys were significantly lower in castrated mice. However, treatment with testosterone increased the expression levels of OAT2 back to normal [85].

Buist *et al* showed higher protein expressions of OAT1 and 3 in kidneys and liver respectively in male than infemale SD rats. Significantly higher protein expression of OAT2 was observed than in female liver than in male SD rats. Buist *et al a*lso showed that hypophysectomy decreased the expression levels of

OAT1, 2 and 3 in male rats. Similarly, hypophysectomy decreased the expression levels of OAT2 but increased the expression levels of OAT3 in female rats [86, 87]. Similar observations were done in mice studies as well. The expression levels changed with the treatment of testosterone and estrogen in rodents [88].

1.4.D. Gender-dependent plasma protein binding of soy isoflavones in rats

Age and sex have shown significant effect on plasma protein binding in rodents as well as humans. Plasma protein, especially albumin levels decrease as a function of age in both sexes. However alpha 1-acid glycoprotein levels increase with age, especially in men. Therefore the plasma protein binding of basic drugs was higher in male however no gender-dependent difference was observed in basic drugs in plasma protein binding of acidic drugs in rats.

Diazepam, an acidic drug showed significantly higher plasma protein binding in men (94%) than women (90%) [89]. Another acidic compound such as Zenarestat™, a statin, showed significant gender-dependent difference in its excretion profile after oral administration. Almost 45% of free drug was excreted in urine in female as compared to only 1% in male SD rats. However, no significant gender-dependent difference was observed in plasma protein binding

(99% each) in both male and female rats [90]. In case of single oral dose of 17β-estradiol glucuronide, female rats showed 250 folds lower urinary excretion than in male rats. Again, no significant differences were observed in plasma protein binding in male and female rats to explain the gender-dependent difference in glomerular filtration [91].

Plasma protein binding for propranolol showed no gender-dependent differences in humans. Furthermore, the binding did not change during the menstrual cycle and was maintained at 90% level. Testosterone cypionate also had no effect on protein binding in men. However, ethinyl oestradiol (50µg/day) alone or together with norethindrone in women produced an increase in the unbound percentage of propranolol due to decreased plasma concentration of alpha1-acid glycoprotein in women [92].

1.5. Role of sex hormones on metabolism and transport in female rats

As mentioned before, numerous studies have shown significant gender-dependent differences in plasma PK profiles for polyphenols in rats. It has also been established that estrogen and testosterone may influence the expression levels of metabolizing enzymes as well as uptake and efflux transporters in rodents. In female rats, the estrogen and progesterone levels change according

to the estrus cycle which on an average lasts for up to 3 to 4 days [93]. It has been shown that female rats show four different phases of estrus cycle which have same number of sex hormones and similar release pattern as compared to humans [94, 95]. In maternal female rats, the hepatic UGT1A, UGT1A1, UGT1A5, UGT1A6 and UGT2B1 enzyme expressions (western blots) were lower than in control female rats [96]. Furthermore, female rats in 10 to 12 days post partum showed significantly higher expression levels of hepatic UGT1A, UGT1A1, UGT1A5, UGT1A6 and UGT2B1 enzymes (western blots) than in control female rats. Prolactin, a lactating hormone secreted in female rats during and after pregnancy showed significant increase in the expression levels of only hepatic UGT1A6 enzyme expression (western blot) profile in control female rats [96]. There is no data suggesting effect of sex hormones on uptake and efflux transporters present on enterocytes and hepatocytes in female rats. Therefore, to delineate the possible role of estrus cycle on the metabolism and transport of soy isoflavones, we studied the estrus cycle in female rats and grouped the female rats according to high and low ratio of estrogen to progesterone in different phases of estrus cycles in female rats.

1.5.A. Identification of estrus cycle in female rats

To identify the phase of the estrus cycle using the method developed by Marcondes and coworkers, a vaginal smear was taken in the morning in between 8 and 9:00 am. The air dried smear was stained using methylene blue (Fig.1.5). The differentiation between the phases of the estrus cycle was based upon the presence as well as proportion of cornified cells, epithelial cells, leucocytes and mucus strands, made visible by methylene blue dye. As shown in the Figure 1.5, the four different phases in the estrus cycle in female rats are called Proestrus (Fig.1.5a/b), Oestrus (Fig.1.5c/d), Metoestrus (Fig.1.5e/f) and Diestrus (Fig.1.5g/h) respectively. Round nucleated cells are called epithelial cells. Irregular shaped cells are called cornified cells. Little round cells are called leucocytes [93]. Characteristics for pro-oestrus phase were the presence of more cornified and some epithelial cells and no leucocytes. Characterstic marks for oestrus phase were the presence of only cornified cells. Metoestrus phase showed large number of leucocytes, epithelial and cornified cells. Diestrus phase shows more mucus strands and least number of other cells (especially epithelial cells). The duration of each phase was on an average 12hrs, 12-18hrs, 12-18hrs and 24-48hrs respectively for each estrus cycle in female rats.

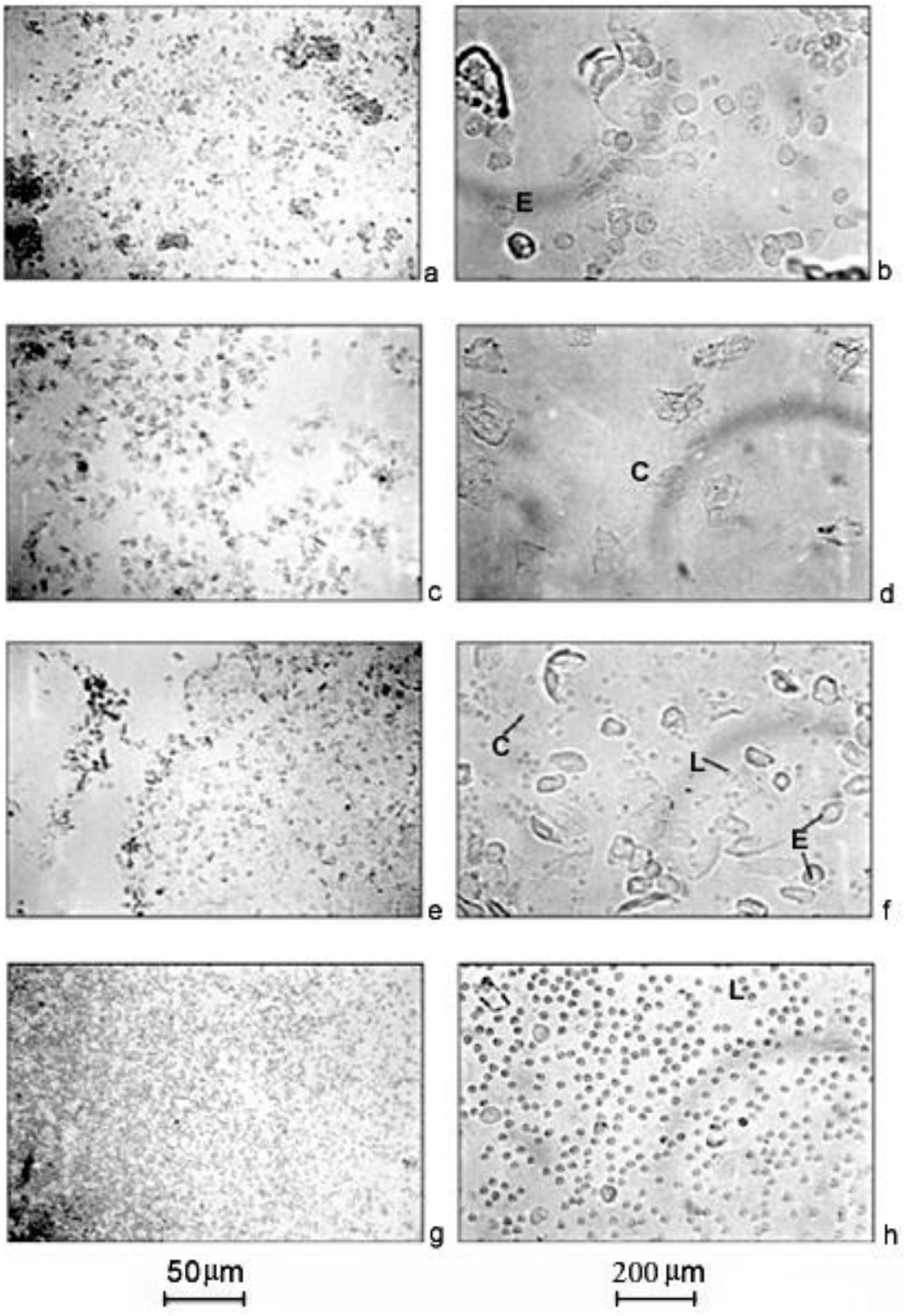

E
a
b
C
c
d
C
L
E
e
f
L
L
g
h
50 µm
200 µm

Figure 1.5: Unstained vaginal smears from female rats showing Proestrus (a, b) Oestrus (c, d) metoestrus (e, f) and diestrus (g, h) leukocytes (L) epithelial cells (E) and cornified cells (C). Proestrus phase is confirmed by presence of cornified cells. Oestrus phase consists of anucleated cornified cells. Metoestrus phase consists of cornified, epithelial cells and leukocytes. Diestrus phase consists of mucus strands and leukocytes only.

1.5.B. Physiological sex-hormonal changes in different phases of estrus cycle in female rats

In female rats, confirmed by the vaginal smears and the plasma concentrations of sex hormones, the estrogen levels start going up at the start of proestrus phase and peak at the end of the phase. By the end of the oestrus phase, the estrogen levels are back to normal. In the middle of the oestrus phase as well as during diestrus phase, the progesterone levels surge in female rats [97].

Therefore, to study the effect of estrus cycle on metabolism and transport of polyphenolics, we combined the female rats into two groups of proestrus + oestrus (high estrogen to progesterone ratio) and metoestrus + diestrus (low estrogen to progesterone ratio) phases of estrus cycles.

1.5.C. Estrus cycle-dependent differences in metabolizing enzyme and transporter expression levels in female rats

An interesting study done by Becedas and Ahlberg showed that female rats in luteal (metoestrus or diestrus) phase showed significantly higher hepatic phase II (UGT enzyme) expression profiles as compared to female rats in pre-ovulatory (proestrus and oestrus) phase of estrus cycle. Exactly opposite results

were obtained for hepatic phase I (CYP450) enzyme expression profiles. Significantly higher expression of phase I enzymes were observed in preovulatory phase than luteal phase of female rats. This phenomenon showed no correlation of phase I and phases II metabolizing activities on the basis of estrus cycle in female rats. In the same study, female rats treated with pregnant mare's serum gonadotropin (PMSG) showed significantly higher hepatic phase I and phase II metabolizing enzyme expression profiles as compared to female rats in all phases of estrus cycle. PMSG treatment was given to keep the female rats in the same phase of the estrus cycle [98].

Until today, no study has been done to show the estrus cycle dependent differences in P-gp, Mrp or BCRP transporters expression levels in enterocytes/hepatocytes in female rats.

1.5.D. Use of ovariectomized rats as a control to study the effect of sex hormones in female rats

Previously, female ovariectomized rats were used as a negative control to study the gender-dependent differences in rats. The most obvious possible mechanism for the gender-dependent differences in rats is assumed to be due to estrus cycle or sex hormones in female rats. Hence, female ovariectomized rats

were used as a negative control because of the absence of estrus cycle as well as reduced levels of sex hormones produced by ovaries in female rats. The hormonal levels of estrogen as well as testosterone in female ovariectomized rats would be considerably lower as compared to male and female rats which might give a very different perspective on the gender-dependent differences observed in rats.

1.5.E. Phase II metabolizing enzyme and transporter expressions in ovariectomized female rats

Luquita *et al* have shown that hepatic UGT1A (8 fold), UGT1A1 (6 fold), UGT1A5 (3.5 fold) and UGT1A6 (< 2 fold) are significantly higher in female ovariectomized rats than in control female SD rats [96]. Furthermore, Ntcp and Bsep transporter protein levels have been shown to be higher in female ovariectomized rats than control female rats [99]. There is no study for other uptake and efflux transporter expression levels in enterocytes and hepatocytes in female ovariectomized rats to compare with control female SD rats.

1.6. Summary

Soy isoflavones, daidzein and genistein have shown strong anti-cancer, anti-oxidant and post-menopausal symptom preventive phytoestrogenic activities. However, no significant amount of research has been done to conclude gender-dependent differences in genistein and daidzein's oral bioavailability in male and female rats. Furthermore, no study has been done to study the gender-differences in absorption and disposition of these two compounds in rats. This information has remained as a black box in the bioavailability barrier network and we have proposed to shed some light on these topics in this thesis project.

2. **Objective and Hypothesis**

The oral bioavailability of genistein aglycone in humans and rodents is extremely low due to extensive first pass metabolism in intestine and liver [20, 24, 56]. Furthermore, only one study has shown gender-dependent difference in oral bioavailability of ^{14}C genistein in male (7%) and female (15%) SD rats [24]. Due to experimental limitations of usage of ^{14}C genistein, the absolute oral bioavailability of genistein aglycone and its phase II metabolites in rats is still unknown. Also, mechanism for the observed 2 fold gender difference in total genistein (aglycone + metabolite) oral bioavailability remains unidentified.

Higher bioavailability of total genistein in female than in male rats may be accounted for differences in sex hormone levels in male and female rats. However, there are no studies done to delineate the differences in bioavailability of genistein in female rats based on the fluctuating levels of sex hormones in estrus cycle in female rats. Furthermore, there are no studies in ovariectomized rats delineating effect of loss of sex hormones produced by ovaries on the bioavailability of polyphenols in female rats.

2.1 **Objective:**

The overall goal of the study is to determine the gender differences in oral bioavailability of genistein and its phase II metabolites in male and female rats. The objectives of the project were to determine: 1. oral bioavailability of genistein and its phase II metabolites in male, female and female ovariectomized rats after single oral dose of genistein 20mg/kg using rat PK model. 2. Gender and sex hormone dependent differences in absorption and disposition of genistein in male, female and female ovariectomized rats using rat intestinal perfusion model. 3. Gender-dependent differences in metabolism of genistein in male and female rats using rat intestinal (duodenum, jejunum, ileum and colon) and liver microsomes model.

2.2 **Hypothesis:**

The hypotheses for this project are as follows:

H1(a). Genistein will show gender- and sex hormone-dependent difference in oral bioavailability of genistein and its phase II metabolites in male and female rats after single oral dose of genistein 20mg/kg.

H1(o). Genistein will not show gender-dependent difference in oral bioavailability of genistein and its phase II metabolites in male and female rats after single oral dose of genistein 20mg/kg.

H2(a). Genistein absorption and disposition will show gender-dependent difference in male and female rats.

H2(o). Genistein absorption and disposition will not show gender-and sex hormone-dependent difference in male and female rats.

H3(a). Genistein phase II metabolism (glucuronidation) will show gender-dependent difference in male and female rat microsomes.

H3(o). Genistein phase II metabolism (glucuronidation) will not show gender-dependent difference in male and female rat microsomes.

Chapter 3

3. General methodology

3.1 Pharmacokinetic Study:

3.1.A. Objective:

Perform Pharmacokinetic studies in rats using tail vein blood sampling procedure.

3.1.B. Advantages:

It is simple and easy to execute with minimal pain to the animal.

It is useful for multiple routes of administration including IV, IP and oral

3.1.C. Disadvantage:

Small sample volume can be taken. Therefore, a potential loss in sensitivity is observed.

3.1.D. Procedure:

1. Day before PK study experiment, fast n=4 rats at 6pm by removing only food but keeping water to drink ad libitum.

2. Make sure the fasted rats are placed on a stainless steel cage bottom so the rats will not have access to eat their own feces or the bedding.

3. On the day of the experiment, weigh the rat and record the weight along with date of birth, sex and strain.

4. Single oral dose of 20mg/kg of soy isoflavone is administered by simple gavage method.

5. Fifteen minutes after the oral gavage dose, the rat is anesthetized using isoflurane gas.

6. Alcohol patch is used to clean the rat tail and rat is placed on a diaper with warm electric blanket underneath. Warm light is used from top making sure it will not burn the skin of the rat to maintain the rat body temperature while it is under complete anesthetic plane.

7. Less than 2mm of rat tail is cut to collect blood samples (100µl volume) for 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12 and 24 hour sample points.

8. After collecting the blood for each time point, pressure is applied using piece of gauge for about 30 seconds on the tail to stop the blood flow before the rat is placed back in its cage.

9. Care must be taken to keep the animal in right posture so that its eyes will not get hurt by the metal cage or bedding underneath.

10. Rat is carefully observed until it is completely conscious and moving freely in the cage.

11. After the PK study, rat is sacrificed using CO_2 gas chamber.

3.2. Microsome Study

3.2.A. Objective:

To determine the K_m and intrinsic clearance (CL_{int}) values of daidzein and genistein in male and female rats of SD and wistar strains.

3.2.B. Procedure:

1. Day before the experiment, micro-centrifuge tubes (n=4 for each concentration) are labeled with known serial dilution concentrations of 0.78 to 50µM and 0.87 to 60µM and stored in freezer.

2. The known serial dilution concentrations of 0.78 to 50mM and 0.87 to 60mM for daidzein and genistein are prepared in 100% methanol a day before the experiment and a standard curve is made from these dilutions to confirm the concentrations.

3. Prepare 50mM KPI solution, solution A, solution B and 250mM sucrose as explained earlier and store it in freezer until the start of the experiment.

Potassium phosphate (KPI) buffer (50mM): pH 7.4

a. 0.5M KH_2PO_4 = 68.5g KH_2PO_4 + 1000ml dd water
b. 0.5M K_2HPO_4 = 87.09g K_2HPO_4 + 1000ml dd water
c. 150ml (a) + 700ml (b) = pH 7.4
d. 50ml (C) + 450ml dd water = 500ml 50mM KPI buffer.

Solution A:

a. 25mM UDPGA (Uridine 5'-Diphosphoglucuronic Acid Triammonium Salt FW=765.4) in H2O is what we need.
b. Therefore, simply add 191.4mg UDPGA to 10ml of water

c. Or we use (Uridine 5'-Diphosphoglucuronic Acid Trisodium Salt FW=646.2) in H2O and thus add 161.55mg UDPGA to 10ml of water
d. Store this at –20C
e. Separate this into centrifuge tubes for 1ml per tube.

Notes:

#1. FW for UDGPA varies depending on form of compound

Solution B:

a. 25mM Saccharolactone (for the purpose of pushing the reaction forward to completion) (FW=210.13) is needed
b. Therefore, simply add 105.065mg of the Saccharolactone in 20ml of already prepared 5mM MgCl2 (made with H2O)
c. Now we need a concentration of 0.125mg/ml of Alamethicin (for surfactant purposes)
d. Take the stock solution of Alamethicin (at 50mg/ml diluted in 100% methanol) and put 50ul of this in the 20ml of our solution
e. Store this at –20°C
f. Separate this into centrifuge tubes for 1ml per tube.

4. On the day of the experiment, water bath is warmed up to 37°C temperature.

5. Three buckets are filled with crushed ice.

6. Bring out 50mM KPI solution, solution A, solution B, test microsomes and 250mM sucrose along with the different serial dilution concentrations of daidzein/genistein from the freezer and place them in one of the ice bucket.

7. Bring the ice cold micro-centrifuge tubes and place them in second ice bucket.

8. Defreeze the 250mM sucrose and microsomes and prepare known concentration of microsomes by diluting them with sucrose solution.

9. Now defreeze solution A and solution B as well and place all the solutions back in ice as soon as possible.

10. Add desired (422µl) 50mM KPI volume to single row of previously labeled micro-centrifuge tubes using same pipette and pipette tip.

11. Add solution B (120µl) to the tubes again using single pipette and pipette tip. Please make sure to touch the pipette tip to the side of the micro-centrifuge tube wall to deliver all the contents.

12. Add microsomes (36µl) to the tubes making sure the pipette tip does not touch the wall.

13. Add test compound of known concentrations (6.8µl) to respective micro-centrifuge tubes by touching the pipette tip to the wall and delivering all the contents in the tube.

14. In same way, add solution A (96µl) to the tubes using single pipette and pipette tip and close the tops of the micro-centrifuge tubes.

15. Shake the tubes physically by turning them upside down at least three times.

16. The contents of these tubes are split (200µl each) into three other tubes.

17. Place these tubes in 37°C water bath for known incubation time.

18. After the known time of incubation, the tubes are taken out of the water bath and right away placed in ice bucket.

19. Open the tops of the tubes and 50µl of internal standard (100µM of testosterone in 94% acetonitrile and 6% acetic acid) is added to stop the microsome reaction by increasing the organic solvent content and denaturing the protein in microsomes.

20. Centrifuge the tubes at 15500 RPM for 15 minutes to precipitate the protein.

21. Carefully collect the supernatant and run it on UPLC along with the standard curve for the test compound.

3.3. Four site rat intestinal perfusion study:

Following method for four site rat intestinal perfusion studies has been written by I and Dr. Hu to submit to the animal care facility, University of Houston.

3.3.A. Objective:

Four sites of rat intestinal perfusion surgery (in-situ experiment) is done to study comparative absorption and/or metabolism of test compound in four different regions of rat intestine such as duodenum (upper 10 cm), jejunum (middle 10 cm), ileum (10 cm before cecum) and colon (entire large intestine). Smaller number of segments of the intestine may be used to compare the absorption and/or metabolism of test compound at fewer sites (e.g., jejunum vs colon or jejunum vs ileum and colon) (Fig.3.1 Page 55).

3.3.B. Remark:

Rat intestinal perfusion study is done via perfusion of compound through 10 to 15 cm of various above-mentioned intestinal regions. The perfusion experiment is carried out over the period of approximately 2.5 hours, although longer perfusion time may be used if the test compound is absorbed slowly. The

latter is because we need to achieve a reasonable % of absorption (>5%) to ensure we can measure the difference in absorption. During the entire surgery and experiment process, the rat is under the same desired anesthetic plane. Rat intestinal perfusion experiment is a non-survival surgery experiment and the rats are sacrificed at the end of the study. Patient and careful monitoring of the rats for achieving appropriate anesthetic plane is essential before the start of the surgery and afterwards to obtain clean and consistent absorption and metabolism data for each study design (n=4).

3.3.C. Advantages:

The rat stays completely unconscious for the total duration of time from the start of the surgery to the end of the experiment, once the desired anesthetic plane is reached. No additional urethane dose is required which reduces the numbers of animals that may be killed due to drug overdose. This significantly lessens the numbers of animals needed for each set of experiments (4-5 successful rats), when comparing to the use of other anesthetic agents such as ketamine. Most important advantage of the rat intestinal perfusion study is the comparative knowledge of intestinal absorption and metabolism profiles obtained for the test compound that are otherwise impossible to get even with oral and intravenous pharmacokinetic or other *in vivo* studies. The skill learnt to do four-

site rat intestinal perfusion study is highly important and useful to understand intestinal drug absorption and metabolism.

3.3.D. Disadvantages:

The anesthetic used for the study is urethane, which is a known carcinogen. The experiment needs skilled personnel to get consistent and reproducible data. Only way to achieve this skill is careful observation of the study and lots of hands on experience.

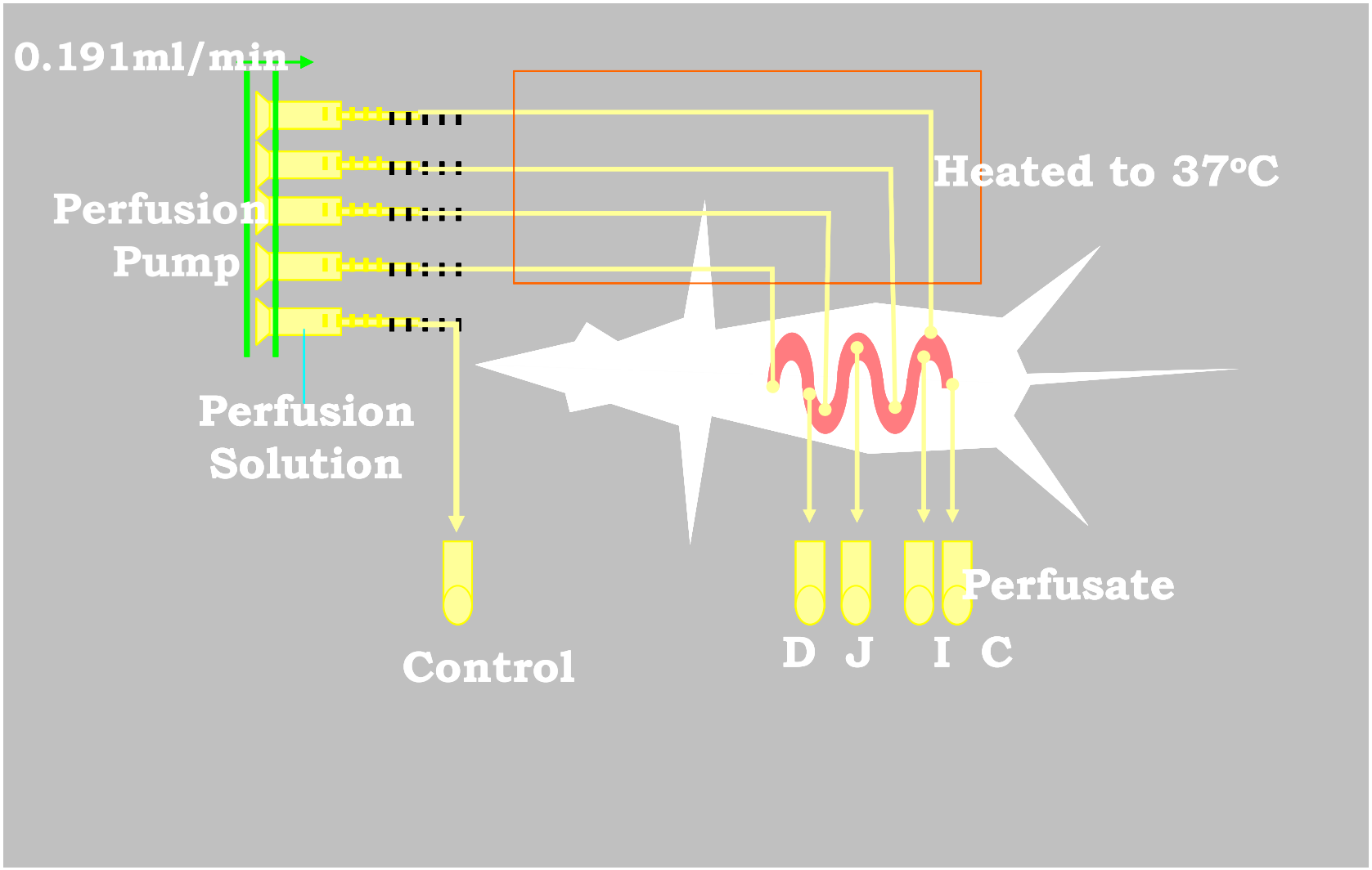

0.191ml/min
Perfusion
Pump
Perfusion
Solution
Heated to 37°C
Control
D J I C
Perfusate

Figure 3.1: Schematics of four-site rat intestinal perfusion model with bile duct cannulation.

3.3.E. Procedure:

1. 15 hours (usually at 5pm) before the start of the experiment, rats are kept in the metabolic cage (regular cage with wire mesh over the bedding). This is done to fast the animal before the surgery to keep the rat intestine clean for the experiment. The wire mesh is used to prevent the animal from eating its own feces. Water is provided during the fasting.

2. On the day of the perfusion experiment, proper animal care robe is put on and rat (typically 250-350 gram) is weighed.

3. Urethane 50% is given to the rat in the doses mentioned in the urethane anesthesia standard operating procedure.

4. The hot water baths are turned on to keep the perfusion tubing at 37 ºC for the entire duration of the experiment. Saline is kept in the water bath to be warmed up to 37 ºC before the start of the rat intestinal surgery.

5. While the rat is going to through the anesthetic process (usually takes 1 and half hours), known concentration (usually 10uM) of the compound is prepared in HBSS buffer (e.g., pH 7.4).

6. Five 60 cc plastic syringes are filled up to 50 ml of perfusate solution and placed on the perfusion pump (Harvard Apparatus).

7. The four perfusion tubing are observed to be completely dry from the experiment the day before and then are washed with double distilled water (60 ml each tube).

8. After washing the tubes, four syringes are connected to the four perfusion tubing and one syringe is kept as a control which is connected to small tubing and kept at one end of the perfusion pump.

9. Once connected, the Harvard Apparatus pump is turned on with the speed of 3ml/min to allow the liquid to pass through the five tubing and all the air bubbles are taken out of the system.

10. Once the bubbles are removed and the system is ready up to this step, the flow rate of the pump is reduced to a targeted speed (e.g., 0.191ml/min).

11. During and after this set up process, rat is carefully monitored for its vitals such as breathing pattern to ensure that it stays in the proper anesthetic plane. Once the proper plane is achieved, additional dose of urethane anesthetic will not be required.

12. The anesthetic plane is re-conformed once more by pinching the hind toes of rat. Once the rat shows no sign of twitch while pinched, the surgery is started.

13. All the required apparatus is gathered at this time such as two pairs of small scissors, 8 small 6 cm length chops of silk sutures, 10 cm long bile duct tubing, 8

small custom made perfusion tubing and precision micro-scissors for bile duct surgery.

14. Couple of gauge and a saline bottle is kept on the side for cleaning the blood oozing out while performing the surgery.

15. Once the animal is completely under anesthetic plane, it is placed on a warm electric blanket covered with animal diaper maintained at around 28 ºC. A low wattage lamp (25 watts) above the animal's body ensures that it does not feel hypothermic while under anesthetic effect. The skin temperature is checked every 15 minutes by placing a hand on the animal.

16. The animal is put on its back and the abdominal portion is shaved with rodent electric trimmer 2 inches from the tail up to the start of the rib cage of the animal.

17. The abdominal skin is held upwards approximately 3 cm and a deep incision (1 cm) is made on the very bottom part of the rat abdominal skin until you see the inside of the rat abdominal muscle. Cut the abdominal skin further for an opening that is 4-5 cm lengthwise. The pink muscle and center white line should be visible at this time.

18. Use sharp scissors to make a small (3-4 mm) opening on the white line and cut along the white line (to avoid excessive bleeding) to open the abdominal

cavity. The total opening is 3-4 cm long. Care must be taken not to harm the internal organs such as intestine and not to cause excessive bleeding.

19. Once opened, warm saline is sprayed on the entire intestine that is visible.

CAUTION: During the entire operation described below, special care must be taken to minimize the bleeding as little as possible. Handling of the intestine must be extra gentle or it will bruise the intestinal segment. Avoid heavy and large contact area using fingers or multiple fingers. Use pointed or narrow tweezers when needed when handle the intestine. This is because intestine tissue is very fragile and even slight rough handling will cause permanent bruise, which can results experiment failure. By using surgical apparatus with small contact area, the bleeding can be minimized or restricted to non-perfused segment, which will have minimal impact on the study.

20. Duodenum region of the small intestine is found on the right side of the rat right under the stomach.

21. Once found, the bile duct is carefully separated and after a tiny incision on the bile duct, small bile duct tubing is inserted inside to collect the bile from the rat during the entire experiment. The tubing is secured by small surgical suture. Bile duct cannulation may be optimal and not all rats undergoing perfusion experiments will have bile duct cannulation.

22. After bile duct surgery, an incision is made on duodenum and inlet perfusion tubing is placed inside the duodenum (approx 1 cm inside) and secured tight with surgical suture. This is the inlet cannulae for the duodenum.

23. At approximately 10 cm down (away from the stomach) from the inlet cannula, another incision is made to secure outlet perfusion tubing for duodenum.

24. Approximately 5 cm down from the duodenum outlet cannula, insert cannula is inserted into the jejunum, which is again secured using surgical suture.

25. At approximately 10 cm down from the jejunum inlet, another incision is made to secure outlet perfusion tubing for jejunum. Outlet cannula will be inserted after a brief wash of the jejunal segment with 5 ml saline to remove any remaining food content. Saline wash can be skipped if the operator is assured that no food content is present.

26. To cannulate terminal ileum, the end of the small intestine is located by finding the cecum. 27. At approximately 1 cm from the ileo-colonic junction, one incision is made for outlet tubing for ileum, which is secured by silk suture.

28. At approximately 10 cm up from the outlet cannula, another incision is made to secure inlet perfusion tubing for terminal ileum. Inlet cannula will be inserted after a brief wash of the terminal ileal segment with 5-10 ml saline to remove any

remaining food content. Saline wash can be skipped if the operator is assured that no food content is present (this does not happen very often).

29. One more incision is made on the other side of the cecum for colon inlet tubing, which is secured using suture.

30. Anal opening is taken as an outlet for the colon surgery. Outlet cannula is inserted after a thorough wash of the colonic segment with 20-40 ml saline to remove any remaining food content. Saline wash can be skipped if the operator is assured that no food content is present (this seldom happens).

31. The rat is carefully transferred to the perfusion table where the rat is again placed on a warm electric blanket covered with animal diaper and maintained at around 28°C. A low wattage lamp (25 watts) above the animal's body ensures that it does not feel hypothermic while under anesthesia. All four rat intestinal inlet perfusion tubings are connected to the perfusion pump tubings already maintained at 37°C.

32. The Harvard Apparatus pump is started with the targeted flow rate (e.g., 0.191 ml/min).

33. A micro-centrifuge is placed to collect bile from the bile duct tube.

34. A clean gauge is placed to cover the intestine and warm saline is sprinkled frequently (e.g., every 15 min) on the gauge to keep it moist at all times.

35. First 30 minutes perfusate from all four regions is discarded and then perfusate from each region is collected from each 30 minutes time period for next 2 hours (this is flow rate around 0.2 ml/min). Different sampling interval may be used. In general, shorter interval for faster flow rate and vice versa.

36. During the first 30 minutes and afterwards, rat was continuously and carefully watched for its vitals such as heart rate and breathing patterns. This is done at approximately once every 5 min.

37. Slow heart rate with no jerking motion and consistent breathing is a good sign of surgery and deep anesthesia effect, which is a proper and desired anesthetic plane.

38. After the 2 and half hour perfusion mentioned above, blood (200µl) is collected from the kidney vein. Animal dies from the blood sampling procedures.

39. The length of each region of the intestine is measured by careful cutting of each segment and putting it on the ruler inside a glass tray containing warm saline.

40. After this, the remaining urethane is injected into the rat carcass and the rat is put in small black colored plastic bag along with all the intestine segments. The black bag is taped shut and thrown in -80°C freezer.

41. The surgery tools are washed thoroughly with hot water for blood drops and rat feces.

42. The perfusion tubings are washed first with 100% double distilled water and then with 70% isopropyl alcohol. To expel the alcohol, air is pushed through the tubings, which are allowed to dry completely overnight.

43. Safety Addendum is the same as those described under Urethane Administration SOP.

3.4. Female Rat estrus cycle identification study

3.4.A. Objective:

To identify the phase of estrus cycle of female rats of both Wistar and SD strain using vaginal smear.

3.4.B. Procedure:

1. Female rat is restrained using a rat restrainer device.

2. A glass Pasteur pipette is dipped in double distilled water and by capillary motion, the water is taken up into the Pasteur pipette.

3. The Pasteur pipette is inserted in female rat's vagina and after 2 seconds, it is pulled out.

4. The contents of the Pasteur pipette are dropped on a glass slide and this smear is allowed to dry.

5. Methylene blue (0.1%) aqueous solution dye is used to stain the smear and once dried, it is observed under microscope and pictures are taken of the smears to identify the phases of estrus cycle of the rat (Fig.3.2).

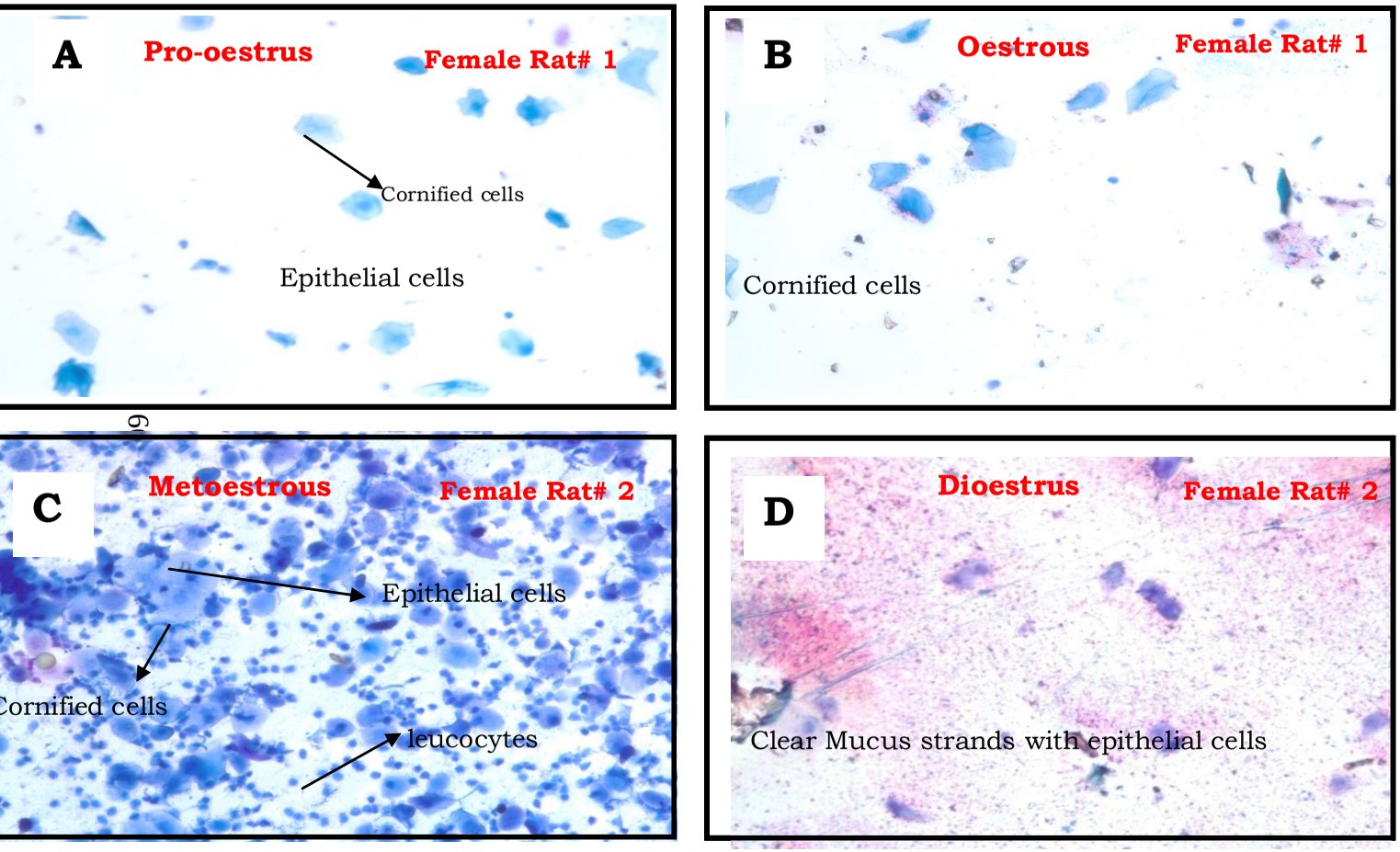

A
Pro-oestrus
Female Rat# 1
Cornified cells
Epithelial cells
B
Oestrous
Female Rat# 1
Cornified cells
C
Metoestrous
Female Rat# 2
Epithelial cells
Cornified cells
leucocytes
D
Dioestrus
Female Rat# 2
Clear Mucus strands with epithelial cells

Figure 3.2: Vaginal smears showing four different phases of estrus cycle: (A) proestrus phase with cornified and epithelial cells visible; (B) oestrus phase with anucleated cornified cells (C) metoestrus phase with large number of cell count of cornified cells. Epithelial cells and leukocytes (D) diestrus phase with lots of mucus strands and no cells except small number of leukocytes.

3.5. Plasma protein binding study

3.5.A. Objective:

To determine plasma protein binding of genistein in male and female rats using dialysis model.

3.5.B. Procedure:

1. A day before the experiment, dialysis bags with molecular weight cut off values of 6000-30000 Daltons are dipped in pH 7.4 HBSS buffer.

2. 3ml plasma is collected from male and female Wistar rats each.

3. On the day of the experiment, 0.5ml of plasma is incubated with 30µM of genistein in dialysis bag for n=3 male and female rats each.

4. The dialysis bag is dipped in 10ml HBSS buffer, which is maintained at 37°C and 0.5ml HBSS buffer samples are collected at 0, 1, 2, 4 and 6 hour sample time points to measure the free genistein that has crossed the dialysis bag.

5. At the end of the experiment, the bound genistein in the dialysis bag is also measured.

3.6. HPLC/UPLC-MS/MS analysis

3.6.A. Objective:

Analysis of daidzein, genistein and their respective metabolites using HPLC, UPLC and UPLC-MS/MS instruments.

3.6.B. Procedure:

HPLC analysis:

High pressure liquid chromatography conditions for analyzing daidzein/genistein and its conjugates are: system Agilent 1080 with UV detector; column Aqua (phenomenex, Gilroy, CA 5µm, 150 × 4.6mm). Mobile phase used for detection of these compounds consists of mobile phase A 0.04% (v/v) phosphoric acid + 0.06% (v/v) triethylamine in water (pH 2.9), mobile phase B, 100% acetonitrile. Gradient used for this mobile phases is: 0-3min, 15% B; 3-15min, 15 to 19%B, 15-35min, 19 to 41%B, wavelength 254nm and injection volume of 200µl. A 15min delay was placed between two injections to allow the column to be re-equilibrated with 15% mobile phase B.

3.6.C. UPLC analysis:

Ultra performance liquid chromatography conditions for analyzing daidzein/genistein and its conjugates are: system Waters UPLC with UV detector; column Acquity UPLC BEH C_{18} (Waters, CA 1.7µm, 2.1 × 50mm). Mobile phase used for detection of these compounds consists of mobile phase A 2.5mM ammonium acetate in water (pH 7.6), mobile phase B, 100% acetonitrile. Gradient used for this mobile phases is: 0-2min, 0 to 25% B; 2-3min, 25 to 70%B, 3-3.5min, 70 to 0%B, flow rate of 0.5ml/min and injection volume of 10µl. A representative chromatogram for genistein and its metabolites is shown in Fig. 3.3.

3.6.D. UPLC-MS/MS analysis:

An API 3200 QTRAP® triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSpray™ source was operated in negative ion mode to perform the analysis. The flow dependent parameters for introduction of the samples to the mass spectrometers ionization source were set as follows: ionspray voltage, -4.0 kV; ion source temperature, 700 °C; the nebulizer gas (gas1), nitrogen, 40 psi; turbo gas (gas2), nitrogen, 40 psi; curtain gas, nitrogen, 10 psi. The quantification

was performed using multiple reaction monitoring mode (MRM) with ion pair transitions to monitor each analyte and the internal standard are listed in Table 3.2 (genistein) and 3.3 (daidzein). Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. The representative MS full scan and MS/MS full scan spectra of genistein, genistein conjugates and daidzein (IS) are show in Figure 3.4.

Analyte	Q1 Mass	Q3 Mass	Dwell time	DP	CEP	CE	CXP
	u	u	ms	V	V	V	V
Genistein glucuronide	445	269	150	-80	-22	-40	-2
Genistein sulfate	349	269	150	-75	-22	-40	-2
Genistein	269	133	150	-75	-22	-40	-2
Daidzein	253	132	150	-75	-30	-52	-2

Table 3.2: Compound dependent parameters for genistein, its phase II conjugates and daidzein (IS) in MRM mode UPLC-MS/MS analysis.

Analyte	Q1 Mass	Q3 Mass	Dwell time	DP	CEP	CE	CXP
	u	u	ms	V	V	V	V
Daidzein glucuronide	429	253	150	-75	-30	-52	-2
Daidzein sulfate	349	269	150	-75	-30	-52	-2
Daidzein	253	132	150	-75	-30	-52	-2
Genistein	269	133	150	-75	-22	-40	-2

Table 3.3: Compound dependent parameters for daidzein, its phase II conjugates and genistein (IS) in MRM mode UPLC-MS/MS analysis.

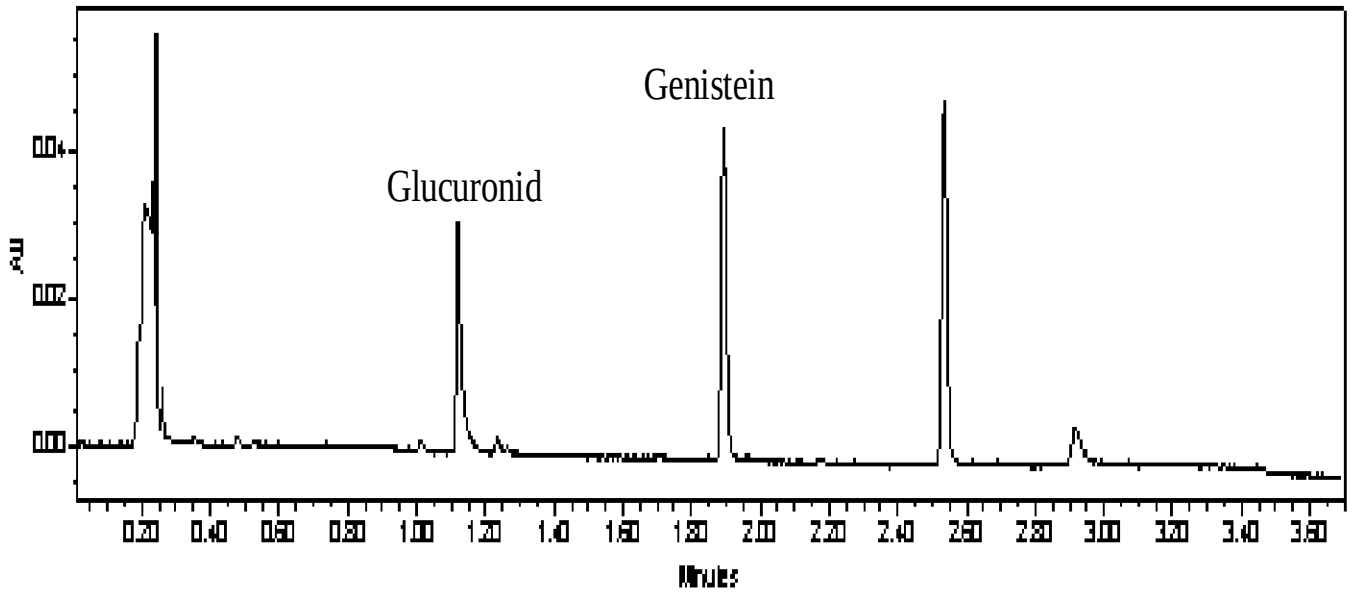

Genistein
Glucuronid
AU
Minutes

Figure 3.3: Representative UPLC chromatogram of 120min male SD rat intestinal genistein (10µM) perfusion model duodenum sample showing genistein glucuronide (1.1min), genistein (1.9min) and testosterone (IS) (2.5min).

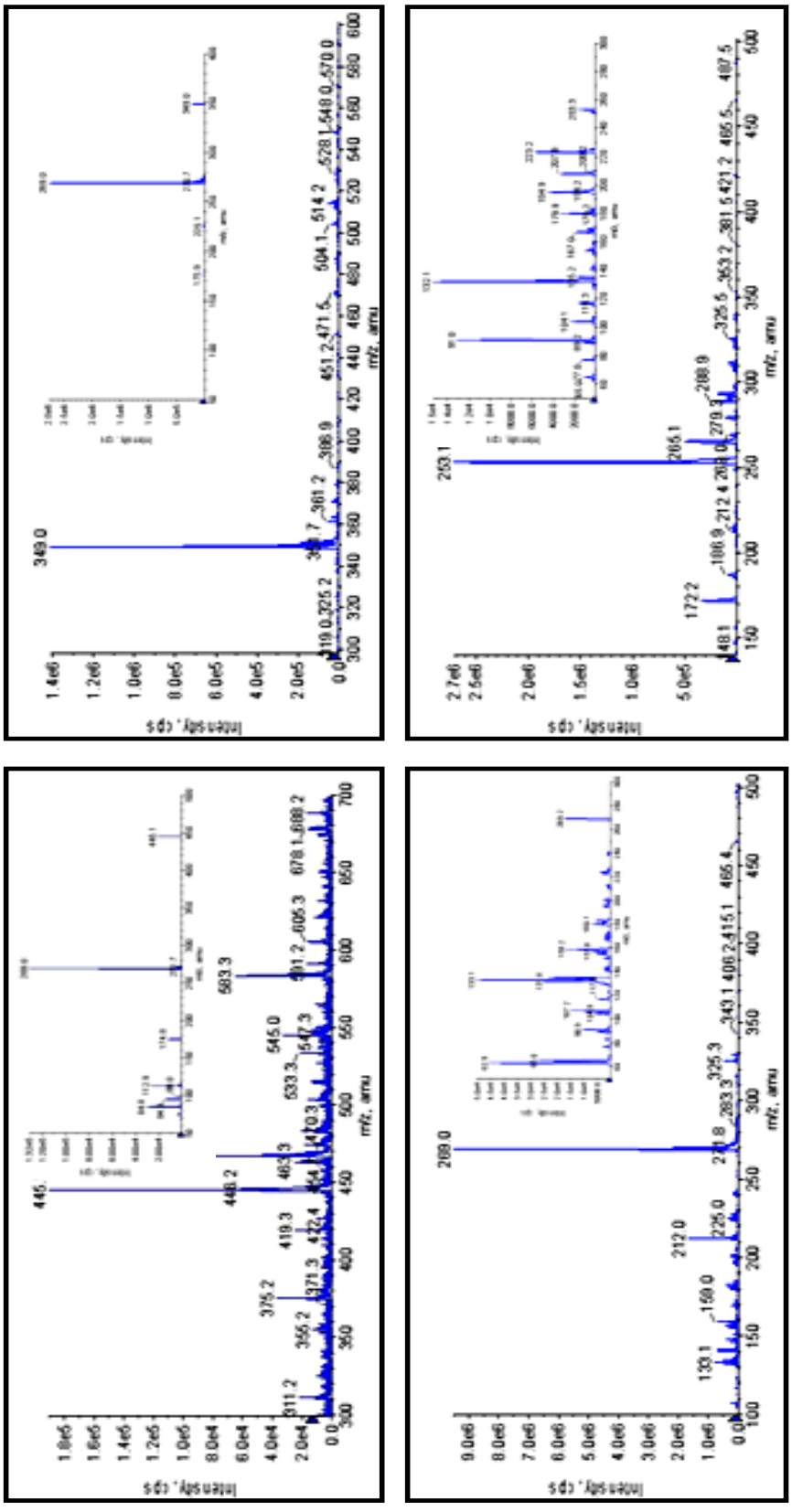


Figure 3.4 Representative MS spectra for genistein, its phase II metabolites and daidzein (IS). Panel (A), (B), (C) and (D) show the MS full scan spectra of genistein glucuronide, genistein sulfate, genistein and daidzein (IS). The small window in each panel shows the MS2 full scan for representative analyte.

Chapter 4.

Higher gender-dependent total genistein bioavailability in female SD rats is estrogen independent.

4.1. Abstract

Female Sprague-Dawley rats showed 2-fold higher total oral bioavailability (^{14}C genistein) than male rats. Due to experimental limitations of measurement of radioactivity count, the absolute oral bioavailability of genistein and its phase II metabolites is still unknown. In this study, gender-dependent differences in oral bioavailability of genistein (20mg/kg) in male and female SD rats were determined. Female rats showed significantly higher (2 fold) oral bioavailability of total genistein in female than in male rats. The gender difference observed was due to significantly higher (4 fold) plasma genistein glucuronide concentrations in female than in male SD rats. To determine the effect of sex hormones, we further compared the oral bioavailability of genistein in control female and female ovariectomized SD rats to determine if the difference in gender was due to differences in female sex hormones. To our surprise, female ovariectomized rats showed higher (2.5 fold) oral bioavailability of total genistein than in control female rats, including higher plasma AUC of genistein (2 fold), genistein glucuronide (3 fold), and genistein sulfate (25 fold). Interestingly, exogenous dose of estrogen did not reverse any of bioavailability enhancement effects

shown in female ovariectomized rats. In conclusion, genistein displayed substantial and significant gender-dependent differences in oral bioavailability of total genistein. Moreover, removal of sex hormones produced in ovary significantly impaired genistein metabolism in female rats, and the effects was not reversed by exogenous estrogen administration.

Keywords: male, female, ovariectomized, genistein, oral bioavailability, estrogen.

4.2. Introduction

Genistein, a major soy isoflavones, has shown potential anti-neoplastic and antioxidant activity in clinical trials [4, 37]. Furthermore, genistein showed additional potentially beneficial effects *in vitro*, such as protection from coronary heart disease, decreased risk or stroke and prevention of menopausal symptoms [100]. However, the efficacy was achieved at significantly higher (micromolar range) concentrations *in vitro*, whereas concentrations of genistein achieved *in vivo* were quite low (nanomolar range) with normal diet [20].

The absolute bioavailability of genistein in rats has been shown to be significantly lower (in nanomolar range)[24, 101, 102] as compared to genistein's effective therapeutic concentrations (in micromolar range) [20]. In oral bioavailability study, the total bioavailability of radioactive genistein (4mg/kg) was shown to be 7 and 15% in male and female Sprague Dawley (SD) rats respectively [24]. Significantly higher first pass metabolism of genistein might be a major factor influencing the poor bioavailability of genistein [24]. The two fold gender-dependent difference (15 and 7%) in total genistein bioavailability in SD rats observed was primarily accounted for the possible higher enterohepatic recycling in female than in male SD rats [24]. However, in the study, no biliary accumulation of genistein and/or its metabolites was measured to confirm the proposed theory.

Coldham *et al* used ^{14}C radioactive genistein to compare the gender difference in oral PK of genistein in rats [24]. Therefore, the authors lacked the ability to measure the aglycone concentrations which could have been significantly low even with high radioactivity count in hepatocytes and enterocytes due to high first pass metabolism. This observed 2 fold gender-dependent differences in total genistein oral PK profiles might be due to the difference in the level of sex hormones in male and female SD rats. There are no other gender-dependent genistein bioavailability studies done in rats. Furthermore no study has been done to determine the role of sex hormones on genistein PK using control female and female ovariectomized rats.

Genistein PK studies in humans showed no gender difference in genistein plasma concentrations in pre, postmenopausal women and men after taking dietary relevant levels of genistein formulations [25, 26, 103].

Until now, to our surprise, we did not see many references studying pharmaceutical or dietary compound's bioavailability difference in pre- and post-menopausal women or control female and female ovariectomized rats for that matter. The only available study showed significantly higher concentrations of tamoxifen, a classical anti-cancer drug used in breast cancer treatment, and tamoxifen metabolites in postmenopausal women than in premenopausal women.

Female ovariectomized rat is the model of choice used to test compounds for breast cancer and bone loss. Raloxifene, tamoxifen and genistein are three extensively studied compounds for breast cancer treatment in female ovariectomized rats. Breast cancer is not just limited to postmenopausal women and can occur in premenopausal women as well. Considering these facts, no comparative oral bioavailability (aglycone and phase II metabolites) data for these compounds in control female and female ovariectomized rats are rather surprising but required.

In this proposed study, we have determined the plasma concentrations of male and female SD rats after single oral gavage dose of genistein 20mg/kg to study gender-dependent differences in genistein aglycone and metabolite (glucuronide and sulfate) concentrations separately. We have also determined plasma protein binding of genistein and BMI (Body mass index) to identify gender-dependent differences in rate of elimination of genistein in male and female SD rats.

In order to determine the role of sex hormones in single dose oral genistein PK, we measured plasma concentrations of genistein and its metabolites in female ovariectomized SD rats. To study the effect of estrogen on single dose oral genistein PK, exogenous dose of subcutaneous estrogen 10ug/kg was administered 5 hours before the start of the PK study to achieve the peak concentrations of estrogen in female ovariectomized SD rats.

4.3. Materials and Methods:

4.3.A. Materials:

Genistein and daidzein (Internal Standard) were purchased from Indofine chemicals (Somerville, NJ). Hanks balanced salt solution (HBSS, powder form) was purchased from Sigma-Aldrich (St. Louis, MO). All other materials (analytical grade or better) were used as received.

4.3.B. Animals:

Male, female and female ovariectomized Sprague Dawley rats (6 to 8 weeks old) were ordered from Harlan Laboratories (Madison, WI). The rats were fed AIN 876 non soy diet (W) purchased from Harlan Laboratories (Madison, WI). The rats were fasted for up to 15 hours before the day of the experiment to ensure no food interaction with oral bioavailability of genistein.

4.3.C. Methods:

The methods used in the study for single dose oral genistein PK with tail vein sampling were similar to the ones described in section 3.1 of this thesis.

The UPLC-MS/MS analysis methods used to measure genistein, daidzein and their respective metabolites in perfusion and bile samples were similar to the ones described in section 3.8.C.

4.3.D. Data analysis:

Amounts of genistein absorbed (M_{ab}), amounts of genistein conjugate excreted into the intestinal lumen (M_{gut}), amounts of genistein conjugate excreted in bile (M_{bile}) and the %absorbed and %metabolized values were calculated as described previously.

4.3.E. Statistical analysis:

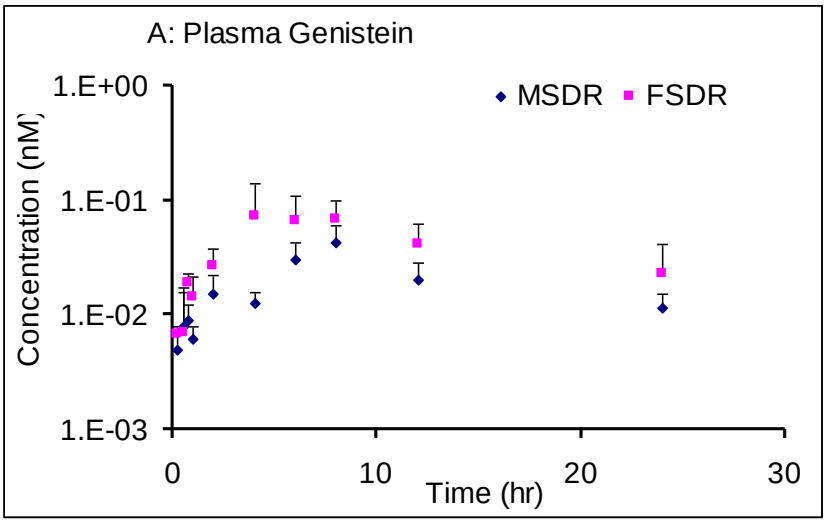
One way anova with posthoc test was used to analyze the gender-dependent differences in absorption and metabolite excretion of genistein in male, female and female ovariectomized SD rats. The prior level of significance was set at 5% or $p< 0.05$.

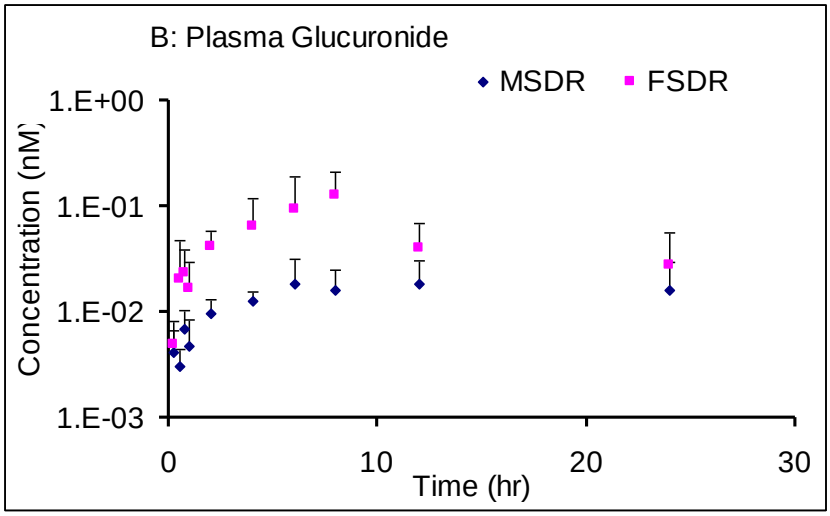
4.4. Results:

4.4.A.. Gender-dependent differences in oral bioavailability of genistein in male and female SD rats.

Single dose oral gavage PK for genistein 20mg/kg was done in male, control female and female ovariectomized SD rats to identify the gender-dependent as well as sex hormone-dependent differences in plasma profiles of genistein aglycone and its metabolites (glucuronide and sulfate) respectively using UPLC-MS/MS analysis.

Significant gender-dependent difference was observed in C_{max} and AUC values for genistein glucuronide and genistein sulfate plasma concentration profiles in male and female rats (Fig.4.1, Table 4.1). Female rats showed almost 4 fold higher plasma C_{max} (2134nM > 409nM) and AUC (20064nM/hr > 5786nM/hr) values for genistein glucuronide than in male rats respectively. However, female genistein sulfate plasma C_{max} (0.025nM > 0.003nM) and AUC (0.266nM/hr > 0.034nM/hr) values were almost 8 fold lower than in male rats. No significant difference was observed in $C_{max,}$ and AUC values of genistein aglycone in male and female rats (Table 4.1). Significant gender-dependent difference was observed in total genistein (aglycone+metabolite) plasma AUC values with females showing higher AUC values than male rats (Table 4.1).


A: Plasma Genistein
MSDR
FSDR
Concentration (nM)
1.E+00
1.E-01
1.E-02
1.E-03
0
10
20
30
Time (hr)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FSDR
Concentration (nM)
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1.E-01
1.E-02
1.E-03
0
10
20
30
Time (hr)

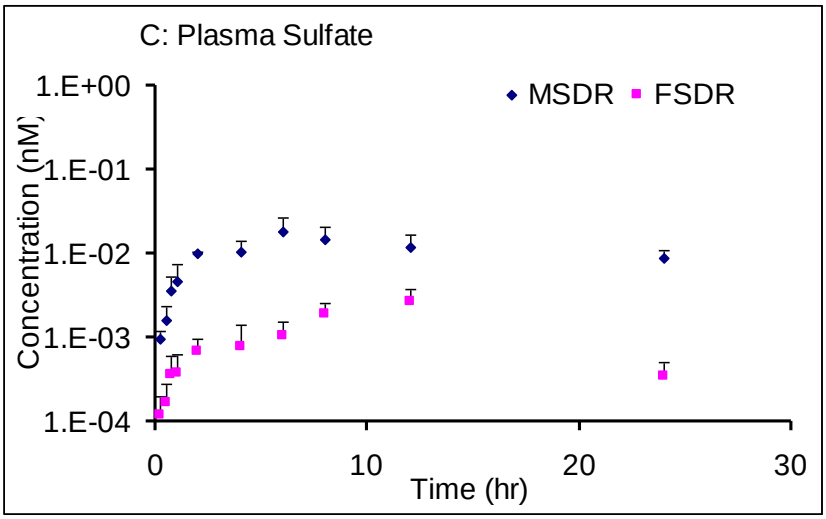

C: Plasma Sulfate
MSDR
FSDR
Concentration (nM)
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
0
10
20
30
Time (hr)

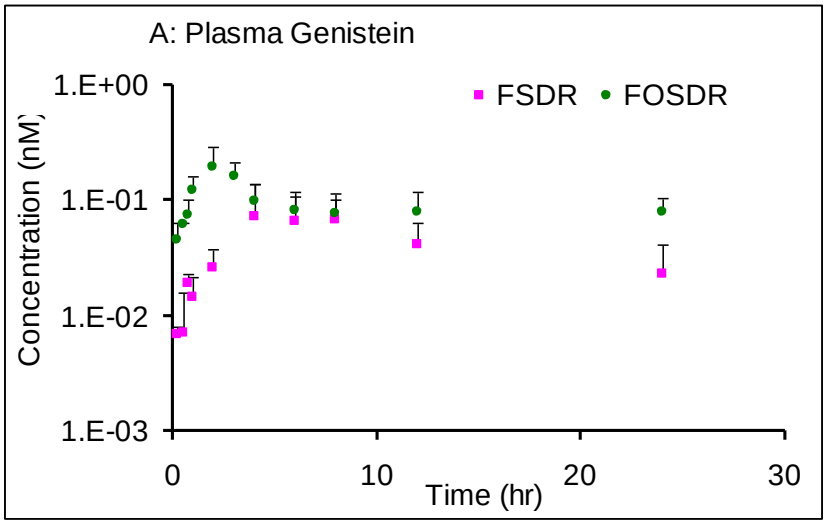
Figure 4.1: Pharmacokinetic profile of (A) Genistein, (B) Genistein glucuronide and (C) Genistein sulfate in male and female SD rats after single oral dose of genistein 20mg/kg.

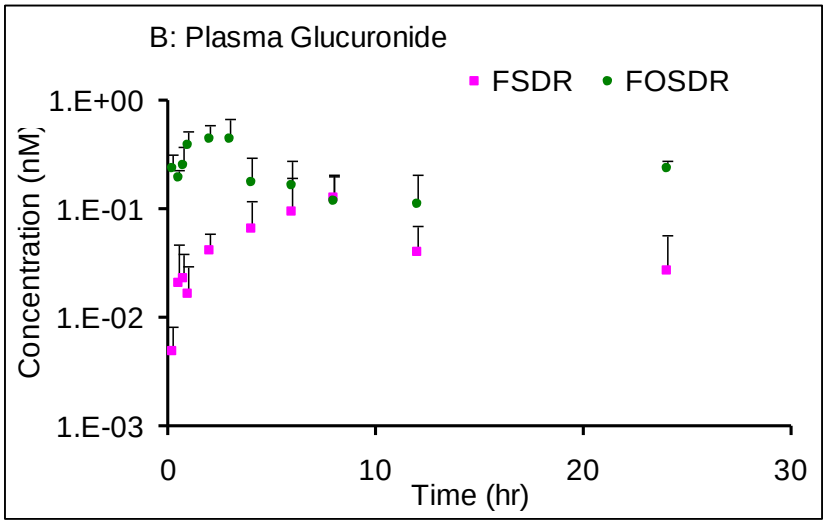
Table 4.1: Pharmacokinetic parameters for Genistein, Genistein glucuronide and Genistein sulfate in male and female SD rats after single oral dose of genistein 20mg/kg.

	Genistein		Glucuronide		Sulfate		Total	
	MSDR	FSDR	MSDR	FSDR	MSDR	FSDR	MSDR	FSDR
C_{max} (μM)	0.048 ± 0.012	0.092 ± 0.055	0.031 ± 0.004	0.120 ± 0.065	0.025 ± 0.004	0.003 ± 0.001	0.101 ± 0.013	0.214 ± 0.118
T_{max} (Hr)	8.5 ± 2.2	10.5 ± 7.9	12.5 ± 7.0	4.5 ± 2.6	8.5 ± 2.2	10.0 ± 2.0	9.83 ± 2.95	8.33 ± 2.60
AUC (μM/hr)	0.459 ± 0.044	1.003 ± 0.521	0.376 ± 0.088	1.457 ± 0.758	0.266 ± 0.027	0.034 ± 0.009	1.101 ± 0.116	2.630 ± 1.135

4.4.B. Role of sex hormones on oral bioavailability of genistein in female rats.

In another set of studies, to determine the effect of sex hormones by removal of ovaries in oral PK profile of genistein and its metabolites, we compared the pharmacokinetic parameters in control female and female ovariectomized rats (Fig4, table 2). To our surprise, female ovariectomized rats showed more than 2, 4 and 16 fold higher C_{max} values for genistein (0.25>0.09uM), genistein glucuronide (0.52>0.12uM) and genistein sulfate (0.05>0.003uM) than control female rats. Similarly, female ovariectomized rats also showed more than 2, 3 and 15 fold higher AUC values for genistein (2.38>1.03), genistein glucuronide (4.38>1.45) and genistein sulfate (0.53>0.03) than control female rats. Significant difference due to loss of ovaries was observed in total genistein (aglycone+metabolite) plasma AUC values with female ovariectomized rats showing higher AUC values than control female rats (Table 4.2).


A: Plasma Genistein
FSDR
FOSDR
Concentration (nM)
1.E+00
1.E-01
1.E-02
1.E-03
0
10
20
30
Time (hr)


B: Plasma Glucuronide
FSDR
FOSDR
Concentration (nM)
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1.E-01
1.E-02
1.E-03
0
10
20
30
Time (hr)

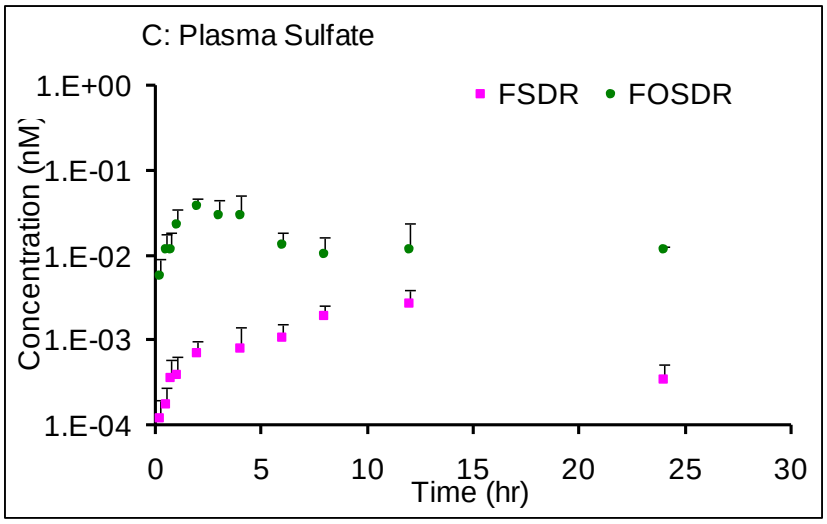

C: Plasma Sulfate
FSDR
FOSDR
Concentration (nM)
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
0
5
10
15
20
25
30
Time (hr)

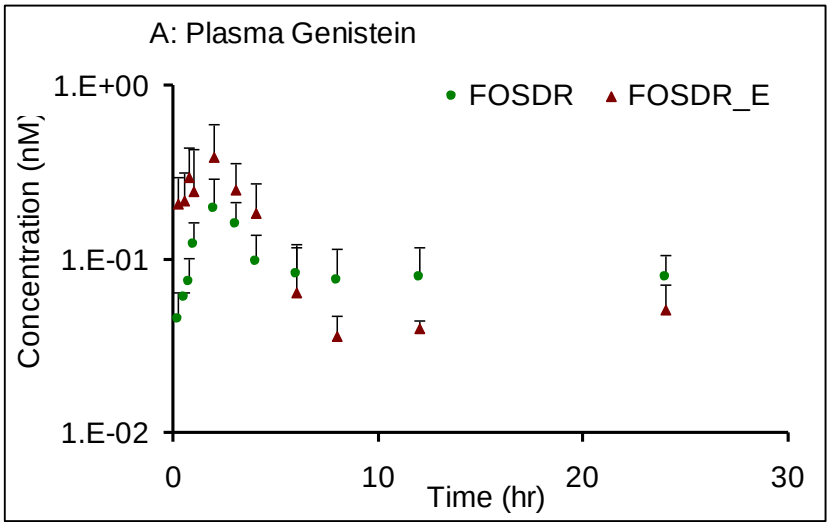
Figure 4.2: Pharmacokinetics profile of (A) Genistein, (B) Genistein glucuronide and (C) Genistein sulfate in control female and female ovariectomized SD rats after single oral dose of genistein 20mg/kg.

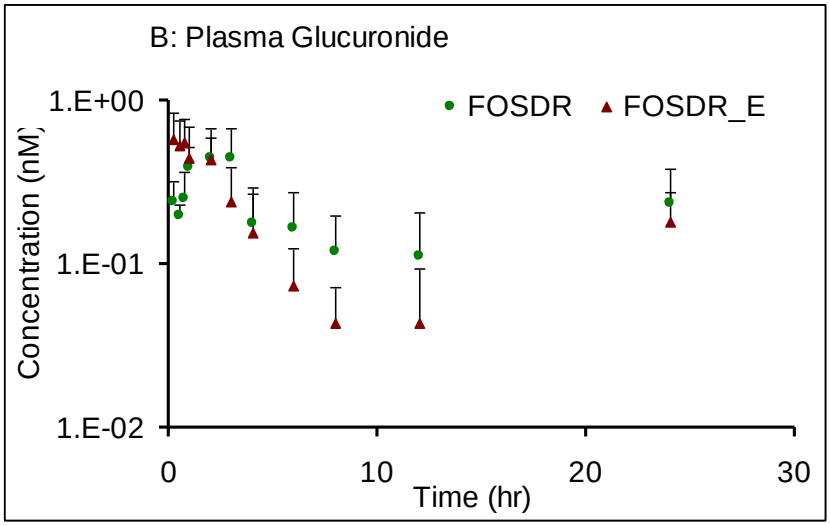
Table 4.2 Pharmacokinetic parameters for Genistein, Genistein glucuronide and Genistein sulfate in control female and female ovariectomized SD rats after single oral dose of genistein 20mg/kg.

	Genistein		Glucuronide		Sulfate		Total	
	FSDR	FOSDR	FSDR	FOSDR	FSDR	FOSDR	FSDR	FOSDR
C_{max} (μM)	0.092 ± 0.055	0.25 ± 0.09	0.120 ± 0.065	0.52 ± 0.12	0.003 ± 0.001	0.05 ± 0.01	0.214 ± 0.118	0.830 ± 0.205
T_{max} (Hr)	10.5 ± 7.9	6.0 ± 4.24	4.5 ± 2.6	2.5 ± 0.50	10.0 ± 2.0	2.5 ± 0.87	8.33 ± 2.60	3.66 ± 1.50
AUC (μM/hr)	1.003 ± 0.521	2.38 ± 1.12	1.457 ± 0.758	4.38 ± 1.97	0.034 ± 0.009	0.53 ± 0.47	2.630 ± 1.135	7.297 ± 3.480

4.4.C. Effect of Exogenous dose of estrogen on oral bioavailability of genistein in female ovariectomized rats.

In female ovariectomized rats, removal of ovaries caused significant loss of estrogen with plasma concentrations reaching more than 25 to 30 fold less than control female rats [104]. To determine the role of single exogenous dose of estrogen in female ovariectomized rats on oral PK profile of genistein and its metabolites, we gave subcutaneous dose of estrogen 10µg/kg 5 hours before the start of the PK study. Comparing the plasma profiles of genistein and its metabolites in these two groups of rats, no significant differences in C_{max} and AUC values were observed (Fig.4.3, Table 4.3). However, the T_{max} values for genistein glucuronide were significantly lower in female ovariectomized rats after the single exogenous dose of estrogen (Table 4.3). Overall, no significant difference due to exogenous subcutaneous dose of estrogen was observed in total genistein (aglycone+metabolite) plasma AUC values in female ovariectomized rats (Table 4.3).


A: Plasma Genistein
FOSDR
FOSDR_E
1.E+00
1.E-01
1.E-02
Concentration (nM)
0
10
20
30
Time (hr)


B: Plasma Glucuronide
FOSDR
FOSDR_E
1.E+00
1.E-01
1.E-02
Concentration (nM)
0
10
20
30
Time (hr)

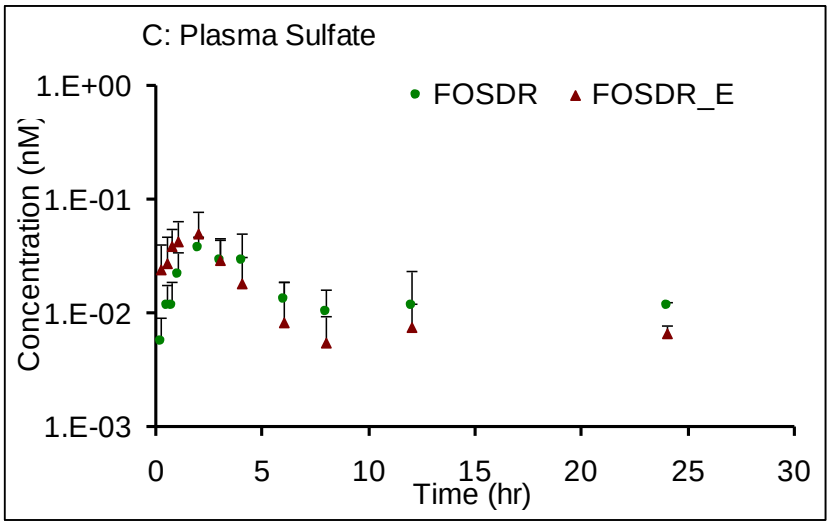

C: Plasma Sulfate
FOSDR
FOSDR_E
1.E+00
1.E-01
1.E-02
1.E-03
Concentration (nM)
0
5
10
15
20
25
30
Time (hr)

Figure 4.3: Pharmacokinetic profile of (A) Genistein, (B) Genistein glucuronide and (C) Genistein sulfate in female ovariectomized SD rats with and without exogenous subcutaneous dose of estrogen 10µg/kg after single oral dose of genistein 20mg/kg.

Table 4.3 Pharmacokinetics parameters for Genistein, Genistein glucuronide and Genistein sulfate in female ovariectomized SD rats with and without exogenous subcutaneous dose of estrogen 10µg/kg after single oral dose of genistein 20mg/kg.

	Genistein		Glucuronide		Sulfate		Total	
	FOSDR	FOSDR-E	FOSDR	FOSDR-E	FOSDR	FOSDR-E	FOSDR	FOSDR-E
C_{max} (µM)	0.25 ± 0.09	0.41 ± 0.14	0.52 ± 0.12	0.62 ± 0.17	0.05 ± 0.01	0.06 ± 0.01	0.830 ± 0.205	1.091 ± 0.316
T_{max} (Hr)	6.0 ± 4.24	2.0 ± 0.70	2.5 ± 0.50	0.5 ± 0.25	2.5 ± 0.87	1.5 ± 0.5	3.66 ± 1.50	1.33 ± 0.34
AUC (µM/hr)	2.38 ± 1.12	2.10 ± 0.50	4.38 ± 1.97	2.78 ± 0.81	0.53 ± 0.47	0.28 ± 0.05	7.297 ± 3.480	5.164 ± 1.303

4.5. Discussion

We concluded that a gender-dependent difference in oral genistein bioavailability was evident in rats with females displaying higher oral bioavailability of total (aglycone+metabolite) genistein than males. Furthermore, female ovariectomized rats showed significantly higher total as well as individual genistein aglycone, genistein glucuronide and genistein sulfate plasma AUC than control female SD rats, and the effect was not reversed by an exogenous single dose of estradiol (10μg/kg).

The conclusion that total genistein bioavailability was higher in female rats is supported by significantly higher plasma total genistein and genistein glucuronide C_{max} and AUC values in female than in male rats. This conclusion is also consistent with Coldham and coworkers' study showing significantly higher total radioactive genistein bioavailability in female (15%) than in male (7%) SD rats [24]. In the study, significantly higher genistein radioactivity count was recovered in small intestine and liver of the female rats than in male rats. Furthermore, significantly higher biliary excretion of genistein and genistein glucuronide was observed in female than in male SD rats, which is discussed more in detail later in Chapter 5.

Interestingly and very unexpectedly, significantly higher oral bioavailability of genistein aglycone and total genistein was observed in female ovariectomized rats than in control female rats. Furthermore, an exogenous single dose of

estradiol (10µg/kg) did not reverse this effect, suggesting that estradiol concentration difference alone was not the reason for the observed higher bioavailability in female ovariectomized rats than in control female rats. Curiously, subcutaneous administration of estradiol reduced T_{max} value of genistein glucuronide considerably in these rats, suggesting it may have limited impact on the rate of metabolism in the upper GI. Luquita *et al* , have shown significantly higher expression levels of tested UGT1A (8 fold), UGT1A1 (6 fold), UGT1A5 (3.5 fold) and UGT1A6 (< 2 fold) enzyme isoforms suggesting higher glucuronidation of genistein in female ovariectomized rats than in control female rats [96]. This observed increase in the UGT enzyme expression profiles might be due to higher levels of sex hormone prolactin (3 fold), [105] LH (2 fold) and FSH (2 fold) in female ovariectomized rats than in control female rats [104]. No detectable concentration of estradiol was found in serum for ovariectomized rats whereas (0.3ng/ml) estradiol basal levels were observed in control female rats [104].

Most human clinical trials will not stratify pharmacokinetic responses with regard to the hormonal states in women such as pre- or post-menopause [106-108] with certain exceptions. Only one study has shown difference in bioavailability of tamoxifen aglycone and its metabolite in pre- and postmenopausal women. Significantly higher plasma concentration of tamoxifen, desmethyltamoxifen, didesmethyltamoxifen and 4 hydroxytamoxifen in

postmenopausal than in premenopausal women after tamoxifen dose normalization [109].

Tamoxifen [110], raloxifene [111] and genistein [112] have shown good anti-cancer activity in animal models of breast cancer . These compounds have shown significantly higher phase II metabolism in rats in *in situ* and *in vivo* experiments because they are good substrates of intestinal and hepatic UGT1A enzyme isoforms in rats [19, 113]. Pharmacokinetic studies for raloxifene have shown less than 2% bioavailability in postmenopausal women[114]. The experiments were done using ^{14}C raloxifene. Therefore, no data suggesting difference in bioavailability, absorption and most importantly active metabolite formation is available for pre- and postmenopausal women[114]. The packing slip insert for Evista^{TM} (active ingredient raloxifene) has clearly stated no efficacy study being done in pre-menopausal women [http://www.rxlist.com/evista-drug.htm] and therefore Evista^{TM} is not recommended for premenopausal women by FDA. To our surprise there are no studies showing bioavailability studies in pre and postmenopausal women or in control female and female ovariectomized rats for paclitaxel, another classical breast cancer compound used in clinical practice.

In PubMed search conducted on 04/22/2010, there were more than 15,000 papers that suggested use of “ovariectomized” animal species with about 10,000 papers using “ovariectomized rats” as breast cancer and other disease

study models. However, there is no study showing mechanistic effect of loss of ovaries on bioavailability of any polyphenolic compound in female rats. As mentioned before, Luquita *et al* have shown increased hepatic UGT1A enzyme expression in female ovariectomized rats possibly due to compensatory increased levels of sex hormones other than estradiol in ovariectomized female rats [96]. There is no data available on the expression levels of uptake and efflux transporters (except Ntcp and Bsep) in female ovariectomized rats[99]. The unexpected effects of removal of ovary on absorption and disposition of pharmaceutical and dietary polyphenols may have a significant impact on study of drugs in female species. Traditionally, effects of hormones and phytoestrogens on breast cancer[115-117], bone loss[118-120], cognitive activities [121-123] and cardiac functions [124-126] were all completed using ovariectomized female animal models without regard to the fact that compound absorption and disposition may change significantly.

In a PubMed search conducted on 04/22/2010, we got about 400 hits for the search terms “breast cancer and ovariectomized”. Furthermore, we got 195, 128 and 59 searches for “genistein+ovariectomized” “genistein+ovariectomized rats” and “genistein+ ovariectomized mouse” terms respectively. In all these studies, the effect of removal of ovaries on the bioavailability of test compounds has been overlooked. This unintentional oversight might be of significant

importance due to our observed 4 fold increase in genistein and its phase II conjugates in female ovariectomized rats than in control female rats.

In addition to use in cancer model, genistein has shown a lowering of breast cancer incidences after dietary intake of tofu in females not separated in groups of pre and postmenopausal women [127, 128]. Genistein administered at high (>10µM) or low (<1µM) concentrations to ovariectomized mice showed significant inhibition or promotion of breast cancer growth [129]. These two results based on many fold differences in bioavailability of genistein aglycone in control female and female ovariectomized species may explain the controversy over health beneficial effects of soy food intake in females with breast cancer.

There is a direct evidence for low levels of endogenous estrogen promoting proliferation and growth of breast cancer [130]. However, there are no studies displaying any effect of estrogen (estradiol) on bioavailability of phytoestrogens (genistein) in female rats. Therefore in the study to identify the effect of estrogen (estradiol) on oral genistein PK, subcutaneous exogenous dose of estradiol 10ug/kg was administered 5 hours prior to the start (time to show peak plasma concentration) of the oral PK study in female ovariectomized SD rats [131]. The results showed no significant difference in C_{max}, T_{max} and AUC values of genistein and its metabolites except for genistein glucuronide where the T_{max} value was significantly shortened after exogenous administration of estradiol

10ug/kg. At this time, we do not have a concrete explanation available for this observed phenomenon.

Female ovariectomized rats are also used as a model for osteoporosis [132, 133], cognitive functions [134-137] and hypertension [135-137]. Again, similar to breast cancer model, these studies did not take into account the possible higher bioavailability of parent compound as well as its phase II metabolites in ovariectomized rats giving overestimated health beneficial effects of the test compounds.

In conclusion, female rats showed gender-dependent higher total genistein oral bioavailability than in male SD rats. Furthermore, female ovariectomized rats showed estrogen independent higher total genistein oral bioavailability than in control female SD rats.

Chapter 5

Role of gender in absorption and disposition of soy isoflavones in rats

5.1. Abstract

Female rats showed higher total genistein bioavailability than male SD rats in Chapter 4. To study the mechanism of action for this observed effect, we studied the gender-dependent differences in intestinal absorption and disposition of genistein in male and female SD rats. At 10 and 60µM concentration, genistein showed no significant gender-dependent difference in intestinal genistein absorption in rats. However, at 10µM concentration, intestinal (4-5 fold) and biliary (7 fold) excretion of genistein glucuronide was significantly higher in female than in male SD rats. At 60µM genistein concentration, intestinal excretion was not different whereas biliary excretion of genistein glucuronide was still significantly higher (< 2 fold) in female than in male rats. These results are consistent with higher oral bioavailability of total genistein in female than in male rats, as shown in Chapter 4. The latter may be through higher and more efficient enterohepatic recycling in female than in male SD rats. Similar to observed higher plasma genistein glucuronide and lower genistein sulfate concentrations in oral PK studies in Chapter 4, we observed higher biliary excretion of glucuronide and lower biliary excretion of genistein sulfate in female than in male rats. Higher glucuronide excretion in female than in male rats is consistent with higher

expression levels of UGT1A [61], where as lower sulfate excretion in female rats may be the result of lower SULT2B expression [62] . Lastly, intestinal and liver microsome studies also showed higher intrinsic clearance (CL_{int}) values for genistein glucuronidation in female rats, which are consistent with the results obtained in oral bioavailability and rat intestinal perfusion studies.

Similar to genistein, daidzein, another soy isoflavone, showed higher biliary excretion of glucuronide and higher glucuronidation CL_{int} values in female than in male rats.

In conclusion, female rats showed higher biliary excretion of genistein glucuronide suggesting higher enterohepatic recycling as a major pathway for higher oral bioavailability of total genistein in female than in male rats.

Keywords: Genistein, gender, isoflavone, intestine, metabolism, disposition, enterohepatic recycling.

5.2. Introduction

Genistein, an anti-cancer and antioxidant compound [36, 138] has shown significant gender-dependent differences in its total bioavailability in male and female SD rats (chapter 4). The possible mechanism of action for the higher bioavailability of genistein in female rats is suggested to be due to efficient enterohepatic recycling. However, it has not been confirmed by measuring the absorption and disposition differences in male and female rats. There are no other studies available suggesting gender-dependent differences in genistein bioavailability in male and female rats.

Significantly higher genistein glucuronide concentrations in female than male rats suggested higher genistein first pass metabolism and efficient phase II metabolite turnover through enterohepatic recycling in female rats. Until now, there is no data showing predominant rat UGT enzyme isoforms responsible for the metabolism of genistein in rats. Our lab has shown human UGT1A1, 1AIn literature, there are no “Fingerprinting” studies done to show gender-dependent differences in the expression/activity of entire set of phase I (CYP450) and II (UGT and SULT) metabolizing enzymes in male and female SD rats. UGT1A6 and UGT2B12 enzyme isoforms have shown higher hepatic expression levels in female than in male SD rats [61]. The lack of “Fingerprinting” data for entire set of various intestinal and hepatic UGT and SULT enzyme isoforms might be due to unavailability of rat specific UGT/SULT enzyme isoforms antibodies in the

market. Furthermore, there is also no data confirming the rat intestinal and/or hepatic UGT and SULT isoforms predominantly responsible for the metabolism of soy isoflavones genistein and daidzein in rats.

Therefore, to study the gender-dependent differences in absorption and disposition of genistein and daidzein, we used four-site rat intestinal perfusion model with bile duct cannulation. We used two soy isoflavones genistein and daidzein to determine if gender-dependent differences in absorption and disposition are also compound dependent. We used Sprague Dawley rats to study genistein whereas SD and Wistar rats to study daidzein absorption and disposition in rats to also observe gender-differences across different strains of rats. We further used high (60µM) concentrations of genistein to overcome gender-dependent metabolism driven absorption difference for genistein (10µM) (achievable through asian soy diet) and to study the concentration dependent differences in absorption and disposition of genistein in male and female SD rats.

5.3. Materials and Methods:

5.3.A. Material:

Genistein and daidzein were purchased from Indofine chemicals (Somerville, NJ). Hanks balanced salt solution (HBSS, powder form) was

purchased from Sigma-Aldrich (St. Louis, MO). All other materials (analytical grade or better) were used as received.

5.3.B. Animals:

Male and female Sprague Dawley and Wistar rats (6 to 8 weeks old) were ordered from Harlan Laboratories (Madison, WI). The rats were fed Tekland F6 rodent diet (W) purchased from Harlan Laboratories (Madison, WI). The rats were fasted for up to 15 hours before the day of the experiment to ensure clean intestinal and colon regions for genistein perfusion.

5.3.C. Methods:

The methods used in the study for four and two site male and female rat intestinal perfusion were similar to the ones described in section 3.4 of this thesis.

The UPLC-MS/MS analysis methods used to measure genistein, daidzein and their respective metabolites in perfusion and bile samples were similar to the ones described in section 3.8.C.

5.3.D. Data analysis:

Amounts of genistein absorbed (Mab), amounts of genistein conjugate excreted into the intestinal lumen (Mgut), amounts of genistein conjugate excreted in bile (M_{bile}) and the precent absorbed and percent metabolized values were calculated as described previously.

5.3.E. Statistical analysis:

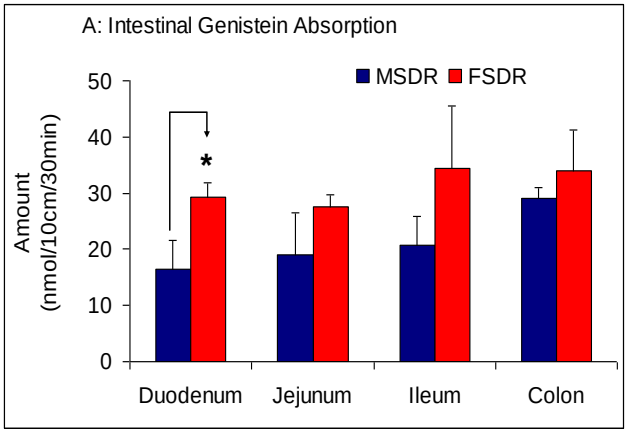
One way anova with posthoc test was used to analyze the gender-dependent differences in absorption and metabolite excretion of genistein in male, female and female ovariectomized SD rats. The prior level of significance was set at 5% or $p< 0.05$.

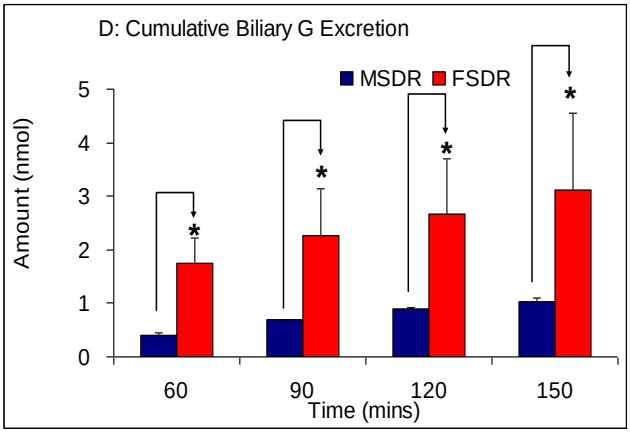
5.4. Results

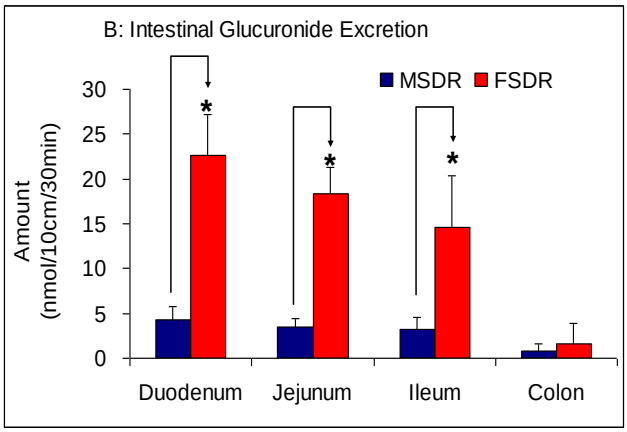
5.4.A. Effects of Gender on Intestinal Absorption and Disposition of Genistein.

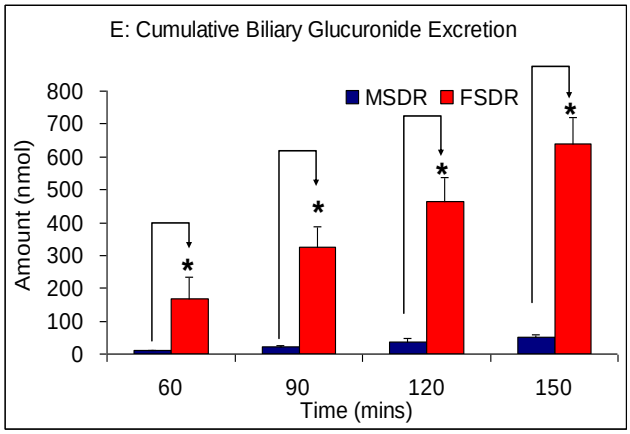
In rat intestinal perfusion experiments, no gender-dependent difference in genistein (10µM) absorption profiles in all four regions of the intestine was observed (Fig.5.1A) except duodenum where genistein absorption was higher in female than in male SD rats. In case of intestinal genistein metabolite efflux, genistein glucuronide excretion profiles were significantly higher in all parts of the intestine except colon (no difference) in female than in male SD rats (Fig.5.1B). However, intestinal genistein sulfate excretion profiles did not show any significant gender-related difference in any intestinal region of male and female SD rats (Fig.5.1C). Overall genistein intestinal absorption in male and female SD rats at 10µM was in the range of 40-60%.

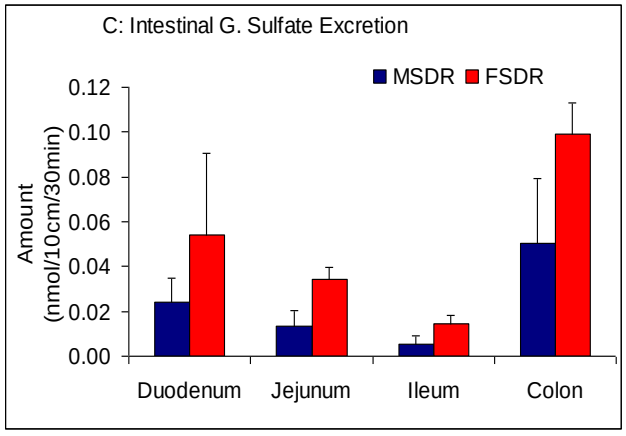
In four-site rat intestinal perfusion experiment, cumulative biliary excretion profiles for genistein and its conjugates showed significant gender-dependent differences in SD rats. Biliary genistein aglycone concentrations (0.5-4nmol range) were significantly higher in female rats (6 folds) than in male SD rats (Figure 1D). Similarly, genistein glucuronide concentrations (100-600nmol range) were significantly higher in female rats (more than 7 folds) than in male SD rats


A: Intestinal Genistein Absorption
MSDR
FSDR
Amount (nmol/10cm/30min)
50
40
30
20
10
0
Duodenum
Jejunum
Ileum
Colon
*


D: Cumulative Biliary G Excretion
MSDR
FSDR
Amount (nmol)
5
4
3
2
1
0
60
90
120
150
Time (mins)
*


B: Intestinal Glucuronide Excretion
MSDR
FSDR
Amount (nmol/10cm/30min)
30
25
20
15
10
5
0
Duodenum
Jejunum
Ileum
Colon
*


E: Cumulative Biliary Glucuronide Excretion
MSDR
FSDR
Amount (nmol)
800
700
600
500
400
300
200
100
0
60
90
120
150
Time (mins)
*


C: Intestinal G. Sulfate Excretion
MSDR
FSDR
Amount (nmol/10cm/30min)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Duodenum
Jejunum
Ileum
Colon

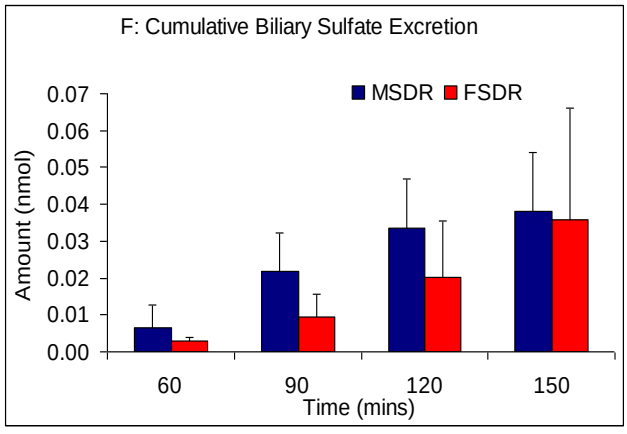

F: Cumulative Biliary Sulfate Excretion
MSDR
FSDR
Amount (nmol)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
60
90
120
150
Time (mins)

Figure 5.1. For genistein 10μM four-site male and female rat intestinal perfusion model: Intestinal (A) absorption of genistein aglycone (B) excretion of genistein glucuronide (C) excretion of genistein sulfate. Cumulative biliary excretion of (D) genistein (E) genistein glucuronide and (F) genistein sulfate.

(Fig.1E). On the other hand, genistein sulfate concentrations (0.01-0.06nmol range) showed no difference in male and female SD rats (Fig.5.1F).

5.4.B. Effect of Concentration on Gender-dependent Intestinal Absorption and Disposition of Genistein

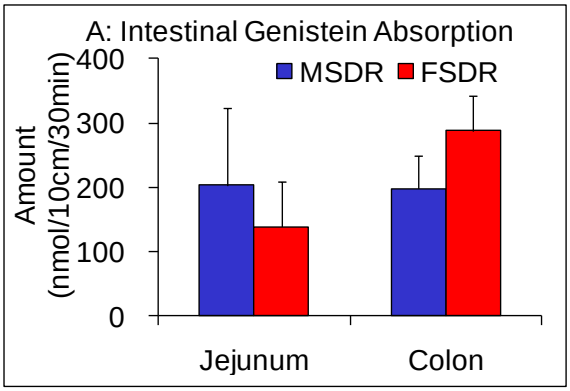
To determine gender-dependent differences in genistein absorption in male and female SD rats, we controlled the absorption in the range of 15 to 20% by increasing the perfusate concentration up to 60µM and doubling the flow rate of perfusate from 0.191ml/min to 0.382ml/min. In case of two-site (jejunum and colon) rat intestinal 60µM genistein perfusion experiments, no significant differences were observed in genistein absorption (Fig.5.2A) as well as intestinal genistein glucuronide (Fig.5.2B) and genistein sulfate (Fig.2C) metabolite excretion profiles in male and female SD rats.

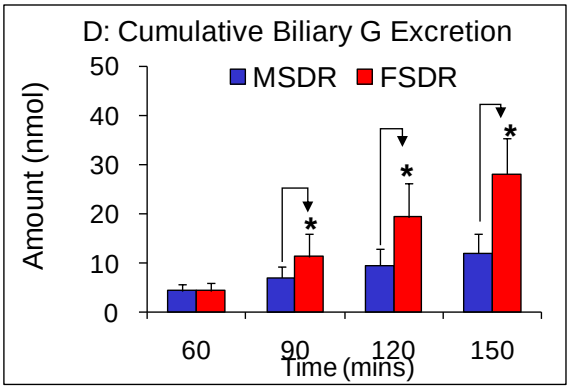
Compared to 10µM concentration, at 60µM, genistein showed more than 6 fold equivalent increased intestinal absorption in jejunum and colon regions in male and female rats. Similarly, more than 6 fold increase was observed in intestinal genistein metabolite efflux in male as well as female rats (Fig 5.2 and 5.3).

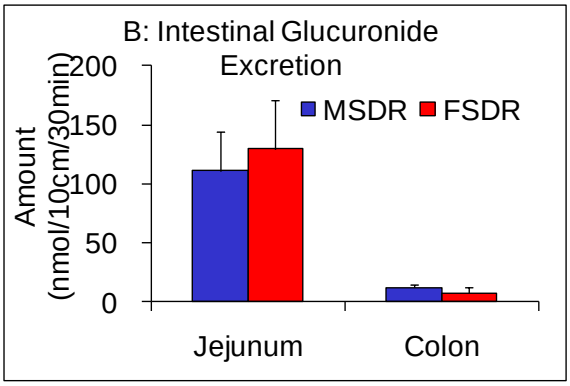
At 60µM concentration, biliary excretion profiles for genistein and its metabolites also showed gender-dependent differences in SD rats. Cumulative biliary genistein aglycone (Fig.2D) concentrations (5-30nmol range) were

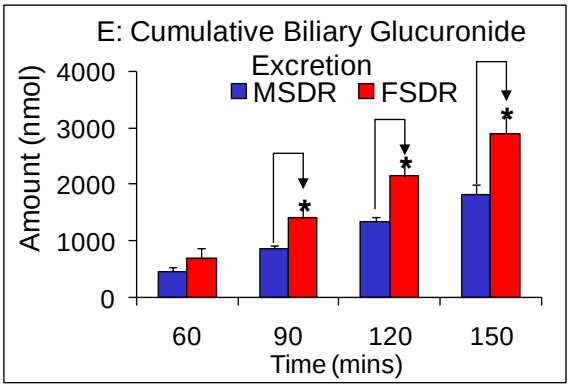
significantly higher in female rats (2 folds) than in male SD rats. Similarly, cumulative biliary genistein glucuronide (Fig.5.2E) concentrations (500-3000nmol range) were significantly higher in female (less than two folds) than in male SD rats. In contrast to biliary genistein glucuronide profiles, cumulative biliary genistein sulfate (Fig.5.2F) concentrations (0.5-7nmol range) were significantly lower in female (26 folds) than in male SD rats.

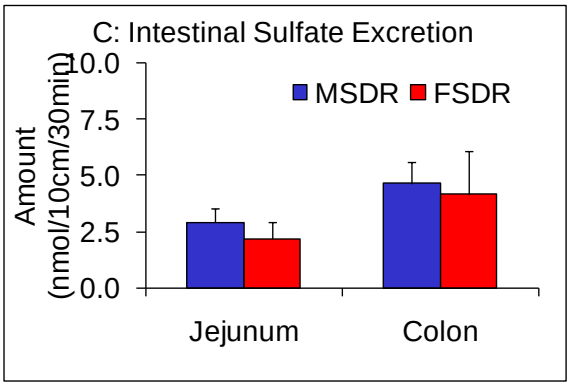
Compared to 10μM concentration, at 60μM, due to higher flow rate of the perfusate and genistein concentration, genistein showed more than 10 fold increase in biliary genistein efflux in both male and female SD rats(Fig.5.1 and 5.2). Interestingly only 5 fold increase was observed in genistein glucuronide efflux for 10 and 60μM genistein perfusion experiment in female rats. On the other hand, male rats showed almost 20 folds increase in biliary genistein glucuronide excretion for the same comparison (Fig.5.1 and 5.2). Almost 100 fold higher biliary genistein sulfate excretion was observed in male SD rats at 60μM than at 10μM concentration of genistein perfusion whereas only five folds increase was observed for female SD rats (Fig.5.1 and 5.2).


A: Intestinal Genistein Absorption
Amount (nmol/10cm/30min)
400
300
200
100
0
MSDR
FSDR
Jejunum
Colon


D: Cumulative Biliary G Excretion
Amount (nmol)
50
40
30
20
10
0
MSDR
FSDR
60
90
120
150
Time (mins)


B: Intestinal Glucuronide Excretion
Amount (nmol/10cm/30min)
200
150
100
50
0
MSDR
FSDR
Jejunum
Colon


E: Cumulative Biliary Glucuronide Excretion
Amount (nmol)
4000
3000
2000
1000
0
MSDR
FSDR
60
90
120
150
Time (mins)


C: Intestinal Sulfate Excretion
Amount (nmol/10cm/30min)
10.0
7.5
5.0
2.5
0.0
MSDR
FSDR
Jejunum
Colon

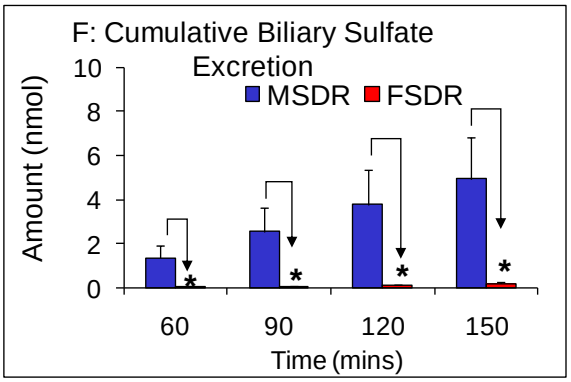
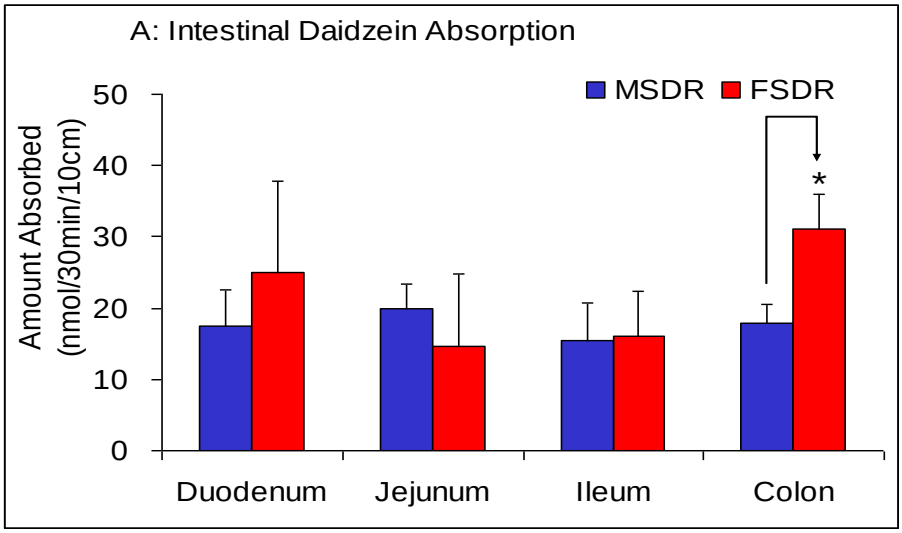

F: Cumulative Biliary Sulfate Excretion
Amount (nmol)
10
8
6
4
2
0
MSDR
FSDR
60
90
120
150
Time (mins)

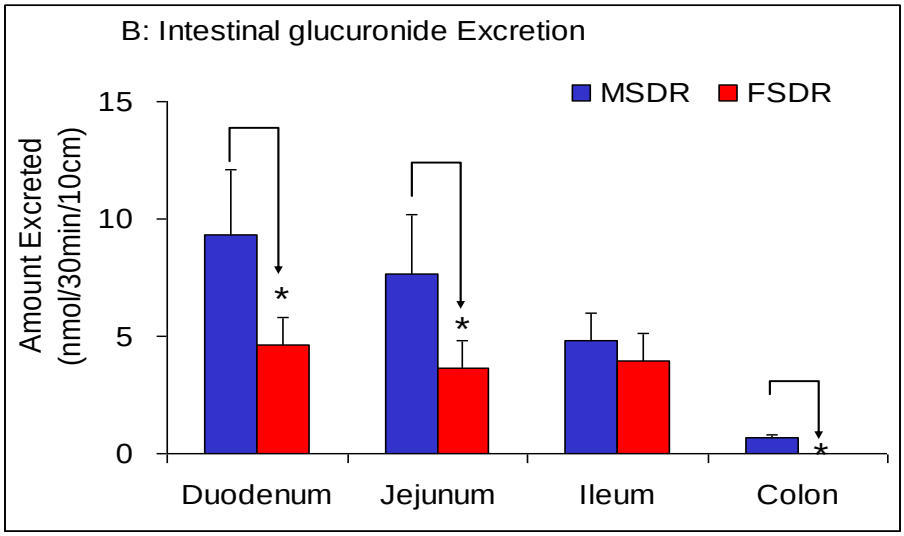
Figure 5.2. For genistein 60μM two-site male and female rat intestinal perfusion model: Intestinal (A) absorption of genistein aglycone (B) excretion of genistein glucuronide (C) excretion of genistein sulfate. Cumulative biliary excretion of (D) genistein (E) genistein glucuronide and (F) genistein sulfate.

5.4.C. Effects of Gender on Intestinal Absorption and Metabolism of Daidzein in SD Rats.

Similar to genistein, In rat intestinal perfusion experiments, no gender-dependent difference in daidzein (10μM) absorption profiles in all four regions of the intestine was observed (Fig.5.3A) except colon where daidzein absorption was higher in female than in male SD rats. In case of intestinal daidzein metabolite efflux, daidzein glucuronide excretion profiles were significantly lower in all parts of the intestine except ileum (no difference) in female than in male SD rats (Fig.5.3B). In four-site rat intestinal perfusion experiment, cumulative biliary excretion profiles for total daidzein showed significant gender-dependent differences in SD rats.

Similar to genistein biliary excretion profile for glucuronide metabolite, cumulative biliary total daidzein concentrations (50-600nmol range) were significantly higher in female rats (6 folds) than in male SD rats (Fig.5.3C).


A: Intestinal Daidzein Absorption
MSDR
FSDR
Amount Absorbed (nmol/30min/10cm)
50
40
30
20
10
0
*
Duodenum
Jejunum
Ileum
Colon


B: Intestinal glucuronide Excretion
MSDR
FSDR
Amount Excreted (nmol/30min/10cm)
15
10
5
0
*
*
*
Duodenum
Jejunum
Ileum
Colon

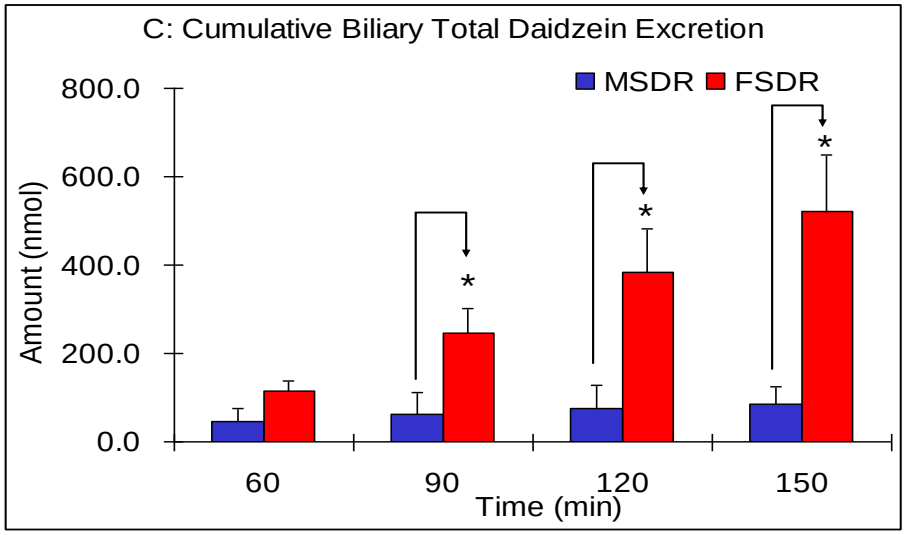
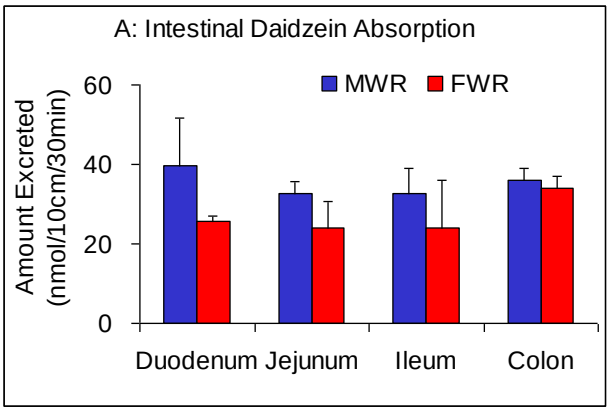
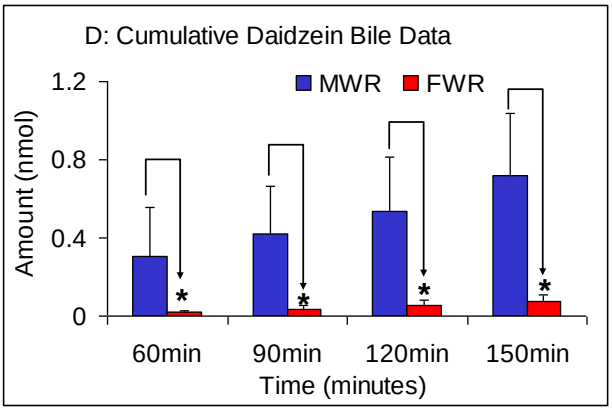
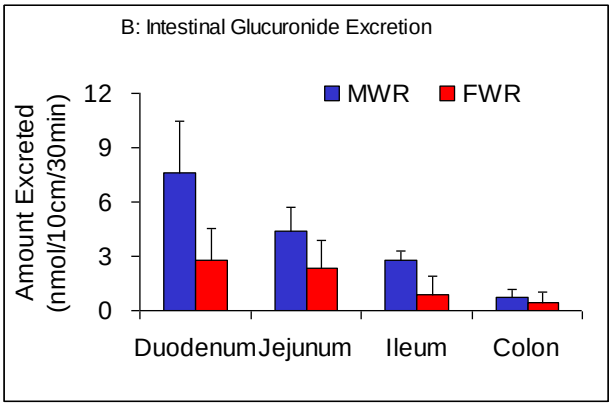
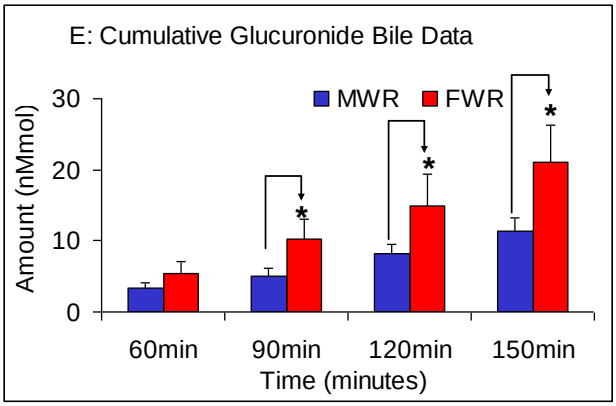

C: Cumulative Biliary Total Daidzein Excretion
MSDR
FSDR
Amount (nmol)
800.0
600.0
400.0
200.0
0.0
*
*
*
60
90
120
150
Time (min)

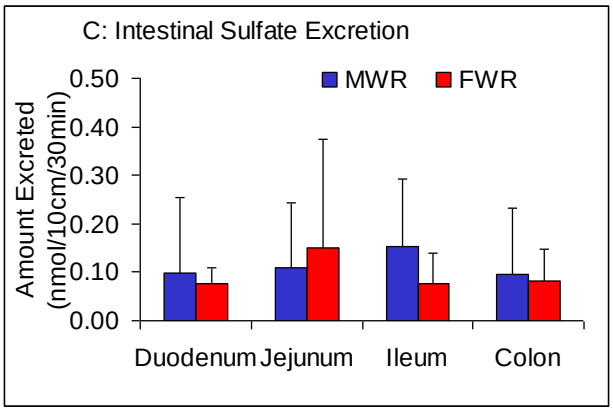
Figure 5.3. For daidzein 10μM four-site male and female rat intestinal perfusion model: Intestinal (A) absorption of daidzein aglycone (B) excretion of daidzein conjugates (C) Cumulative biliary excretion total daidzein (daidzein + daidzein glucuronide + daidzein sulfate).


A: Intestinal Daidzein Absorption
MWR
FWR
Amount Excreted (nmol/10cm/30min)
60
40
20
0
Duodenum
Jejunum
Ileum
Colon


D: Cumulative Daidzein Bile Data
MWR
FWR
Amount (nmol)
1.2
0.8
0.4
0
60min
90min
120min
150min
Time (minutes)


B: Intestinal Glucuronide Excretion
MWR
FWR
Amount Excreted (nmol/10cm/30min)
12
9
6
3
0
Duodenum
Jejunum
Ileum
Colon


E: Cumulative Glucuronide Bile Data
MWR
FWR
Amount (nMmol)
30
20
10
0
60min
90min
120min
150min
Time (minutes)


C: Intestinal Sulfate Excretion
MWR
FWR
Amount Excreted (nmol/10cm/30min)
0.50
0.40
0.30
0.20
0.10
0.00
Duodenum
Jejunum
Ileum
Colon

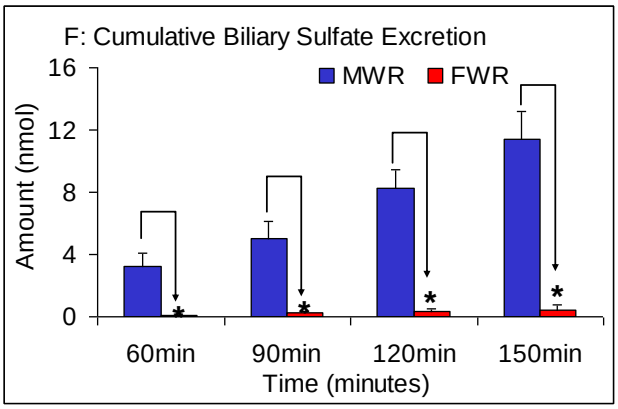

F: Cumulative Biliary Sulfate Excretion
MWR
FWR
Amount (nmol)
16
12
8
4
0
60min
90min
120min
150min
Time (minutes)

Figure 5.4. For daidzein 10μM four-site male and female rat intestinal perfusion model: Intestinal (A) absorption of daidzein aglycone (B) excretion of daidzein glucuronide (C) excretion of daidzein sulfate. Cumulative biliary excretion of (D) daidzein (E) daidzein glucuronide and (F) daidzein sulfate.

5.4.D. Effects of Gender on Intestinal Absorption and Disposition of Daidzein in Wistar Rats.

Similar to genistein 10µM perfusion studies, no gender-dependent difference in daidzein (10µM) absorption profiles in all four regions of the intestine was observed (Fig.5.4A) except duodenum where daidzein absorption was higher in female than in male Wistar rats. In case of intestinal daidzein metabolite efflux, daidzein glucuronide excretion profiles showed no differences in all parts of the intestine except duodenum (female > male) in male and female Wistar rats (Fig.5.4B). Similar to intestinal genistein sulfate efflux, intestinal daidzein sulfate excretion profiles also did not show any significant gender-related difference in any intestinal region of male and female Wistar rats (Fig.5.4C).

Again similar to biliary genistein excretion profiles, cumulative biliary excretion profiles for daidzein and its conjugates showed significant gender-dependent differences in SD rats. Biliary daidzein aglycone concentrations (0-1nmol range) were significantly lower in female (10 folds) than in male Wistar rats (Fig.5.4D). However, similar to genistein glucuronide, daidzein glucuronide concentrations (10-40nmol range) were significantly higher in female rats (less than 2 fold) than in male Wistar rats (Fig.5.4E). Similar to biliary genistein sulfate profile, cumulative biliary daidzein sulfate concentrations (0.1-7nmol range) showed significantly lower (17 fold) in female than in male Wistar rats (Fig.5.4F).

In rat intestinal and liver microsome studies, 13 different concentrations of genistein/daidzein in the range of 0.78 to 60µM were tested to determine the K_m and intrinsic clearance (CL_{int}) values for genistein/daidzein glucuronidation reaction. The representative Michaelis-menten kinetics profiles for jejunum and liver for male and female SD rats are shown in Fig.5.5 (genistein) and Fig.5.6 (daidzein). Summary of all the kinetic parameters for genistein and daidzein glucuronidation reaction using different intestinal and liver microsomes are given in table 5.1 and 5.2 respectively.

For genistein, the Michaelis-menten kinetics parameters suggested that female rats have significantly higher rate of glucuronidation (V_{max}) in all regions of intestine (except ileum with no gender-difference) and liver microsomes than in male SD rat microsomes. Similarly, the intrinsic clearance (CL_{int}) values for genistein were also significantly higher in female than in male SD rat upper intestinal (duodenum and jejunum) and liver microsomes. In case of Wistar strain, at this point, we can only show gender-differences in jejunal and liver microsomes for genistein because of lack of male rat intestinal microsome availability at this time. Again, similar to SD strain, Wistar rat female jejunal and liver microsomes showed significantly higher V_{max} and CL_{int} values than in male Wistar rat microsomes (table 5.1).

Again similar to genistein, the Michaelis-menten kinetics parameters for daidzein suggested significantly higher rate of glucuronidation (V_{max}) and intrinsic

clearance (CL_{int}) values in jejunum and liver microsomes than in male rat microsomes of both SD and Wistar strains (table 5.2).

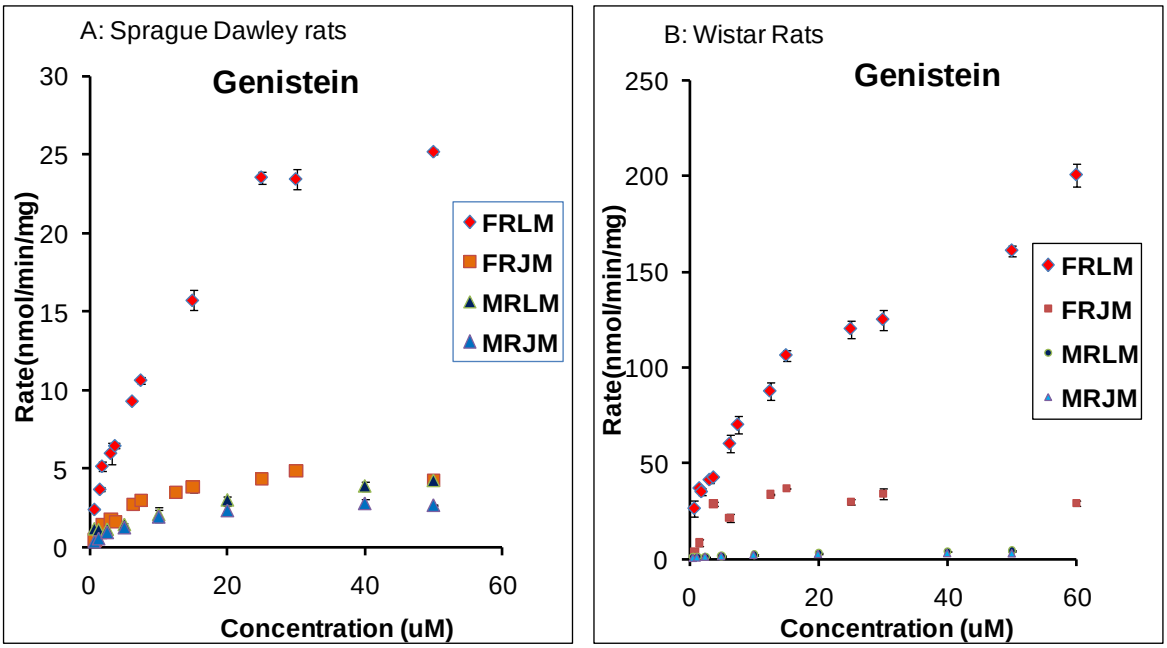


Figure 5.5. Representative Michaelis-Menten Kinetics profile for genistein in male and female SD rat jejunum and liver microsomes.

Table 5.1: Kinetic parameters of microsome metabolism of genistein in different regions of small and large intestine and liver of male and female Sprague-Dawley and Wistar rats (n=3)

Parameters		Duodenum		Jejunum		Ileum		Colon		Liver	
		SD	Wistar	SD	Wistar	SD	Wistar	SD	Wistar	SD	Wistar
V_{max} (nmol/min/mg)	Male	1.40		3.20	1.01	1.40		0.40		3.70	0.62
	Female	5.60	20.23	5.90	15.68	1.30	8.80	3.20	9.44	33.90	70.15
K_{m1} (µM)	Male	4.00		6.60	5.10	8.30		3.20		23.60	1.86
	Female	10.10	10.0	2.70	13.5	10.20	7.0	26.40	25.0	14.20	15.0
V_{max}/K_m (ml/min/mg)	Male	0.40		0.50	0.19	0.20		0.10		0.20	0.33
	Female	0.55	2.02	2.20	1.16	0.12	1.25	0.13	0.38	2.40	4.67
R^2	Male	1		1		1		1		1	
	Female	0.92	095	0.65	0.95	0.99	0.95	0.93	0.89	0.99	0.92

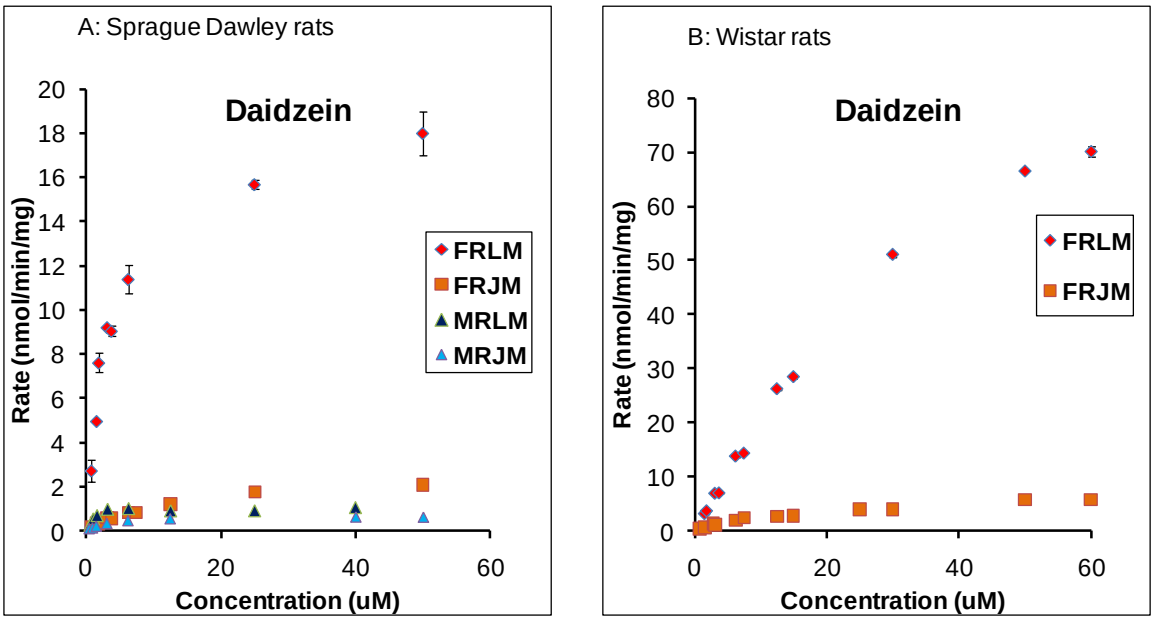


Figure 5.6 Representative Michaelis-Menten Kinetics profile for daidzein in male and female SD rat jejunum and liver microsomes.

Table 5.2 Kinetic parameters of microsome metabolism of daidzein in jejunum and liver of male and female Sprague-Dawley and Wistar rats (n=3)

Parameters		Jejunum		Liver	
		SD	Wistar	SD	Wistar
V_{max} (nmol/min/mg)	Male	0.60	1.01	1.20	0.62
	Female	2.65	15.68	18.5	70.15
K_{m1} (μM)	Male	2.40	5.10	1.10	1.86
	Female	3.67	13.5	3.73	15.0
$Cl_{int} = V_{max}/K_m$ (ml/min/mg)	Male	0.30	0.19	1.10	0.33
	Female	0.19	1.16	4.95	4.67
Ratio: Female/Male	V_{max}	4.17	15.68	15.42	113.15
	CL_{int}	0.63	2.65	3.39	8.06

5.5. Discussion

We concluded that gender plays an important role in absorption and disposition of soy isoflavones in rats. In four-site rat intestinal perfusion model, female rats showed significantly higher biliary excretion of genistein (6 folds) and daidzein (4 folds) than in male SD rats. Furthermore, daidzein also showed higher biliary excretion of daidzein glucuronide in female than in male Wistar rats.

In chapter 4, in single dose genistein (20mg/kg) oral PK studies, we observed 4 fold higher AUC values for plasma concentrations of genistein glucuronide in female than in male SD rats. While studying the mechanism of gender difference for higher total genistein bioavailability in female rats, genistein (10µM) rat intestinal perfusion showed more than 2 and 6 fold increase in cumulative biliary excretion of genistein and genistein glucuronide, respectively. This 6 fold higher biliary genistein glucuronide efflux can explain the higher total genistein oral bioavailability through efficient enterohepatic recycling in female rat theory suggested by Coldham *et al* [24]. High genistein (60µM) concentration perfusion studies showed similar results with higher cumulative genistein and genistein glucuronide efflux in female than in male SD rats. However, the 6 fold biliary excretion difference in genistein glucuronide efflux difference was diminished. This may be explained by following possible theories; 1. Male SD rats up-regulated hepatic genistein glucuronide metabolite formation and thereby biliary excretion at higher genistein concentration. 2. Female SD rats caused

saturation of Mrp2 efflux transporter protein at higher genistein concentrations causing more efflux of genistein glucuronide in to systemic circulation through Mrp3 and Mrp4 efflux transporters present on the on the serosal side of the hepatocytes.

Interestingly, similar to the gender dependent high male rat plasma concentrations of plasma genistein sulfate in genistein oral PK study, we also observed higher genistein sulfate biliary concentrations in male than in female SD rats. These higher plasma and biliary genistein sulfate concentrations in male rats might be due to saturation of UGT enzymes and thereby compensatory sulfate metabolism of genistein in male SD rats. From microsome studies, we saw that hepatic rate of glucuronidation (V_{max}) and intrinsic clearance (CL_{int}) values for genistein were 10 and 100 fold lower in male than in female rats. Furthermore, the hepatic genistein K_m values were also higher (less than 2 fold) in male than female SD rats suggesting possible saturation of UGT metabolism in male at genistein 60µM but not in female SD rats. Furthermore, male rats have shown higher hepatic expression profiles of SULT1A and SULT2A enzyme isoforms suggesting higher genistein sulfate formation and thereby excretion than in female SD rats.

Genistein 60µM concentration perfusion studies were done to clearly identify gender-dependent differences in intestinal genistein absorption in male and female rats. At 10µM concentration, average intestinal genistein absorption

differences (female > male) might have been due to high metabolism (40 to 60%) driven absorption in female than in male SD rats (Fig.5). Therefore, to minimize the impact of metabolism on absorption, we increased the concentration of genistein to 60µM and doubled the flow rate to decrease %absorption to the range of 15 to 20%, which further decreased percent metabolism. Interestingly, we did not see any gender-dependent difference in absorption of genistein at 60µM concentration as well.

Similar to genistein, daidzein (10µM) also showed significantly higher biliary excretion of daidzein glucuronide in female than in male rats of both SD and Wistar strains. Interestingly, we saw strain-specific gender differences in the biliary excretion profiles of total daidzein (daidzein + daidzein metabolites) in male and female rats. The biliary excretion of total daidzein was significantly higher in female SD (600-700nmol) than in female Wistar (20-30nmol) rats. Similarly, biliary excretion of total daidzein was also higher in male SD (100-150nmol) than in male Wistar (20-30nmol) rats. This data is little conflicting with the daidzein microsome studies with higher intrinsic clearance CL_{int} values for male and female Wistar than SD rats suggesting higher glucuronide formation and biliary excretion in male and female Wistar than in SD strain. At this point, we do not have concrete evidence to explain this phenomenon. However, the difference in biliary Mrp2 and hepatic Mrp3/Mrp4 efflux transporter expression

levels in male and female rats (female > male) might be accountable for the observed discrepancies.

In conclusion, female rats showed gender dependent higher intestinal and biliary efflux of genistein glucuronide suggesting higher enteric and enterohepatic recycling than in male SD rats. Furthermore, similar to genistein oral PK studies, genistein 60µM rat intestinal perfusions showed higher genistein sulfate metabolite formation in male than in female SD rats.

Chapter 6

Absorption and disposition of genistein in control female and female ovariectomized rats

6.1. Abstract

Estrus cycle played an important role in absorption and disposition of genistein in female rats. Female rats showed higher total genistein bioavailability than male SD rats in Chapter 4. In chapter 5, the gender-dependent mechanistic studies identified higher intestinal (4-5 fold) and biliary (7 fold) excretion of genistein glucuronide in female than in male SD rats. To further understand the role of sex hormones on genistein absorption and disposition in female rats, we separated control female rats into two groups (proestrus vs. metoestrus) based on ratio of levels of estrogen to progesterone. Female rats in proestrus phase group had higher ratio of estrogen to progesterone than metoestrus phase of estrus cycle group of female rats. Significantly higher biliary excretion of genistein and genistein glucuronide was observed in female rats in metoestrus phase than in proestrus phase of estrus cycle. The increased genistein glucuronide formation results are consistent with the increased expression levels of UGT1A in female rats in metoestrus phases which revert back to normal levels in proestrus phase of estrus cycle. Removal of ovaries in female rats did not result in any significant differences in intestinal absorption and disposition of

genistein in control female and female ovariectomized rats. This data is contradictory to the bioavailability data suggesting higher genistein, genistein glucuronide and genistein sulfate concentrations in female ovariectomized than in control female rats.

In conclusion, estrus cycle played a significant role in hepatic disposition of genistein in female rats.

Keywords: Genistein, gender, isoflavone, intestine, metabolism, disposition, enterohepatic recycling.

6.2. Introduction

Female rats showed significantly higher total genistein oral bioavailability than in male SD rats (Chapter 4). Furthermore, consistent to the bioavailability results, female rats also showed significantly higher metabolite (genistein glucuronide) formation (Fig.5.5) as well as biliary excretion (Fig.5.1 and 5.2) than in male SD rats. The observed gender-dependent differences might be due to involvement of effect of female sex hormones on phase II metabolizing enzymes as well as the efflux transporters involved in disposition of genistein in female rats.

Luquita *et al* showed sex hormone dependent differences in hepatic UGT enzyme expressions in female rats. Pregnant female rats showed significantly lower hepatic UGT1A, UGT1A1, UGT1A5, UGT1A6 and UGT1A8 enzyme expression levels than in control female SD rats[96]. The authors also showed decreased levels of UGT1A6 due to increased levels of prolactin, a sex hormone released during lactation period, in female rats. Furthermore, the authors also showed significantly higher levels of all the above tested hepatic UGT isoforms 10 to 12 days post partum than in control female rats [96]. There are no studies suggesting estrus cycle dependent differences in expression/activity levels of intestinal and hepatic metabolizing enzymes or uptake/efflux transporters in female rats.

In chapter 4, we also discovered significantly higher oral bioavailability of total genistein in female ovariectomized than in control female rats. Female ovariectomized rats showed higher AUC values of genistein, genistein glucuronide as well as genistein sulfate than in control female rats. Interestingly, Luquita et al have shown significantly higher hepatic UGT enzyme expression levels suggesting higher hepatic metabolite formation and excretion of genistein in female ovariectomized rats than in control female rats [96].

Therefore, to study the role of sex hormones on bioavailability of genistein, we proposed to divide female rats in two groups (proestrus and metoestrus) based on the levels of high and low estrogen to progesterone in different phases of estrus cycle in female rats. Proestrus phase showed higher ratio of estrogen to progesterone levels than metoestrus phase (basal levels) of estrus cycle in female rats. Absorption and disposition of genistein was compared between proestrus and metoestrus phase of female rats using four-site rat intestinal perfusion model. We also compared genistein absorption and disposition difference in ovariectomized female and control female rats to study the effect of lack of sex hormones produced by ovaries in female rats.

6.3. Materials and Methods:

6.3.A. Material:

Genistein was purchased from Indofine chemicals (Somerville, NJ). Hanks balanced salt solution (HBSS, powder form) was purchased from Sigma-Aldrich (St. Louis, MO). All other materials (analytical grade or better) were used as received.

6.3.B. Animals:

Control female and female ovariectomized Sprague Dawley rats (6 to 8 weeks old) were ordered from Harlan Laboratories (Madison, WI). The rats were fed non-soy food diet AIN 816 (W) purchased from Harlan Laboratories (Madison, WI). The rats were fasted for up to 15 hours before the day of the experiment to ensure clean intestinal and colon regions for genistein perfusion.

6.3.C. Methods:

The methods used in the study for four and two site rat intestinal perfusion were similar to the ones described in section 3.4 of this thesis.

The UPLC-MS/MS analysis methods used to measure genistein, daidzein and their respective metabolites in perfusion and bile samples were similar to the ones described in section 3.8.C.

6.3.D. Data analysis:

Amounts of genistein absorbed (Mab), amounts of genistein conjugate excreted into the intestinal lumen (Mgut), amounts of genistein conjugate excreted in bile (M_{bile}) and the percent absorbed and percent metabolized values were calculated as described previously.

6.3.E. Statistical analysis:

One way anova with posthoc tests were used to analyze the gender-dependent differences in absorption and metabolite excretion of genistein in male, female and female ovariectomized SD rats. The prior level of significance was set at 5% or $p< 0.05$.

6.4. Results

6.4.A. Identification of different phases of estrus cycle in female rats using vaginal smears.

In female rats, vaginal smears were taken in a day (9am) for ten female rats over a period of four days. After using methylene blue dye to stain the dried smears, the cells showed significant clarity and difference making it easier to differentiate the phases of the estrus cycle in female rats. In proestrus phase of the estrus cycle in female rats, the vaginal smears showed presence of nucleated epithelial cell and cornified (irregularly shaped anucleated) cells (Fig.6.1A). Oestrus phase of the estrus cycle showed only the presence of cornified cells (Fig.6.1B). In metoestrus phase of the estrus cycle, female rat vaginal smears showed large amount of cell count with presence of epithelial cells, cornified cells and leucocytes (Fig.6.1C). In diestrus phase of the estrus cycle, we observed clear smears with large amount of mucus strands and minimal number of cells (mostly leucocytes) (Fig.6.1D). For perfusion experiments, the female rats were grouped in “proestrus + oestrus” and “metoestrus + diestrus” groups. The groups were based on levels of estrogen to progesterone in female rats which were higher in proestrus group and were at the baseline in metoestrus phase of estrus cycle in female rats group.

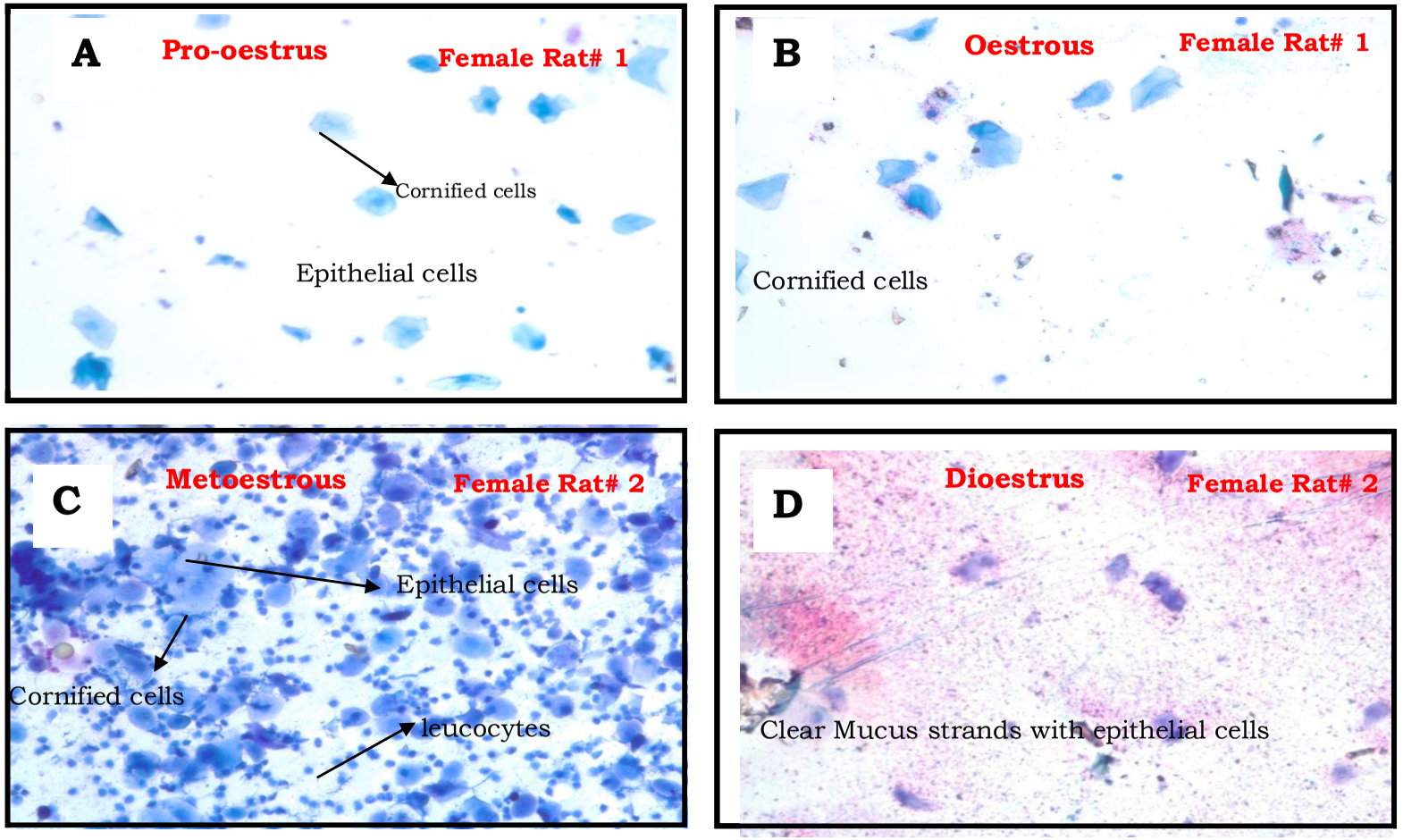

A
Pro-oestrus
Female Rat# 1
Cornified cells
Epithelial cells
B
Oestrous
Female Rat# 1
Cornified cells
C
Metoestrous
Female Rat# 2
Epithelial cells
Cornified cells
leucocytes
D
Dioestrus
Female Rat# 2
Clear Mucus strands with epithelial cells

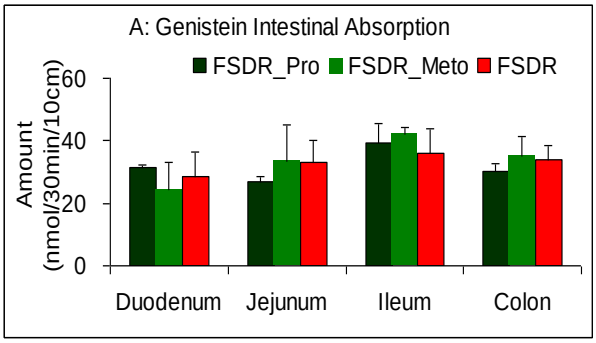
Figure 6.1: Vaginal smears showing four different phases of estrus cycle: (A) proestrus phase with cornified and epithelial cells visible; (B) oestrus phase with anucleated cornified cells (C) metoestrus phase with large number of cell count of cornified cells. Epithelial cells and leukocytes (D) diestrus phase with lots of mucus strands and no cells except small number of leukocytes.

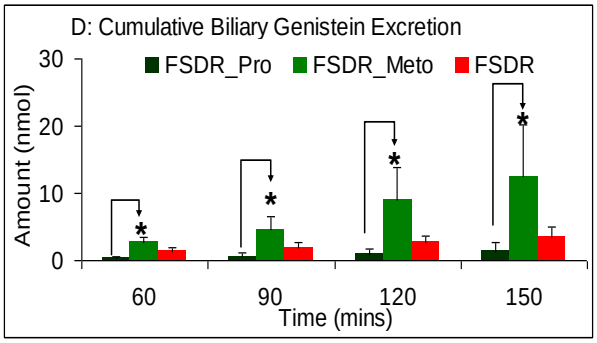
6.4.B. Genistein absorption and disposition in female rats in different phases of estrus cycle

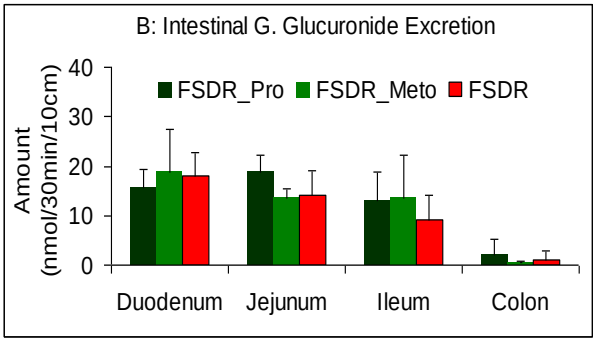
Genistein (10µM) showed significant estrus cycle dependent differences in absorption and disposition in female rats using four site rat intestinal perfusion model. No significant differences were observed in intestinal genistein absorption and metabolite (glucuronide and sulfate) excretion profiles in female rats in proestrus and metoestrus phase of estrus cycle (Fig.6.2A/B/C). However, significantly higher cumulative biliary genistein aglycone (Fig.6.2D) and genistein glucuronide (Fig.6.2E) excretion was observed in female rats in metoestrus phase than in proestrus phase of estrus cycle. No significant difference was observed in cumulative biliary excretion of genistein sulfate in female rats in different phases of estrus cycle (Fig.6.2F).

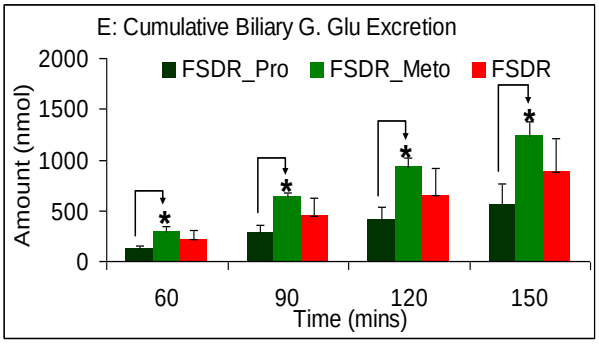
6.4.C. Genistein absorption and disposition in control female and female ovariectomized rats

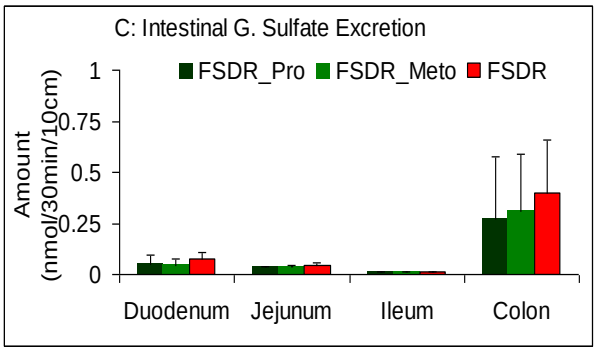
No significant difference was observed for genistein (10µM) intestinal absorption and disposition in control female and female ovariectomized rats using four-site rat intestinal perfusion model except one minor exception. No detectable amount of genistein aglycone was observed in biliary excretion in female ovariectomized rats as opposed to control female rats (Fig.6.3).


A: Genistein Intestinal Absorption
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol/30min/10cm)
0
20
40
60
Duodenum
Jejunum
Ileum
Colon


D: Cumulative Biliary Genistein Excretion
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol)
0
10
20
30
60
90
120
150
Time (mins)


B: Intestinal G. Glucuronide Excretion
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol/30min/10cm)
0
10
20
30
40
Duodenum
Jejunum
Ileum
Colon


E: Cumulative Biliary G. Glu Excretion
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol)
0
500
1000
1500
2000
60
90
120
150
Time (mins)


C: Intestinal G. Sulfate Excretion
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol/30min/10cm)
0
0.25
0.5
0.75
1
Duodenum
Jejunum
Ileum
Colon

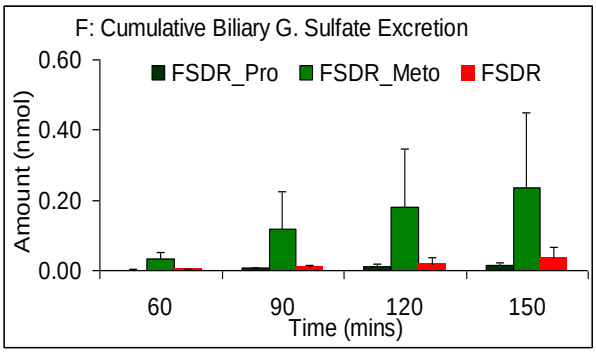

F: Cumulative Biliary G. Sulfate Excretion
FSDR_Pro
FSDR_Meto
FSDR
Amount (nmol)
0.00
0.20
0.40
0.60
60
90
120
150
Time (mins)

Figure 6.2: For genistein 10μM four-site female rat intestinal perfusion model: Intestinal (A) absorption of genistein aglycone (B) excretion of genistein glucuronide (C) excretion of genistein sulfate in female rats in proestrus and metoestrus phase of estrus cycle groups. Cumulative biliary excretion of (D) genistein (E) genistein glucuronide and (F) genistein sulfate in female rats in proestrus and metoestrus phase of estrus cycle groups.

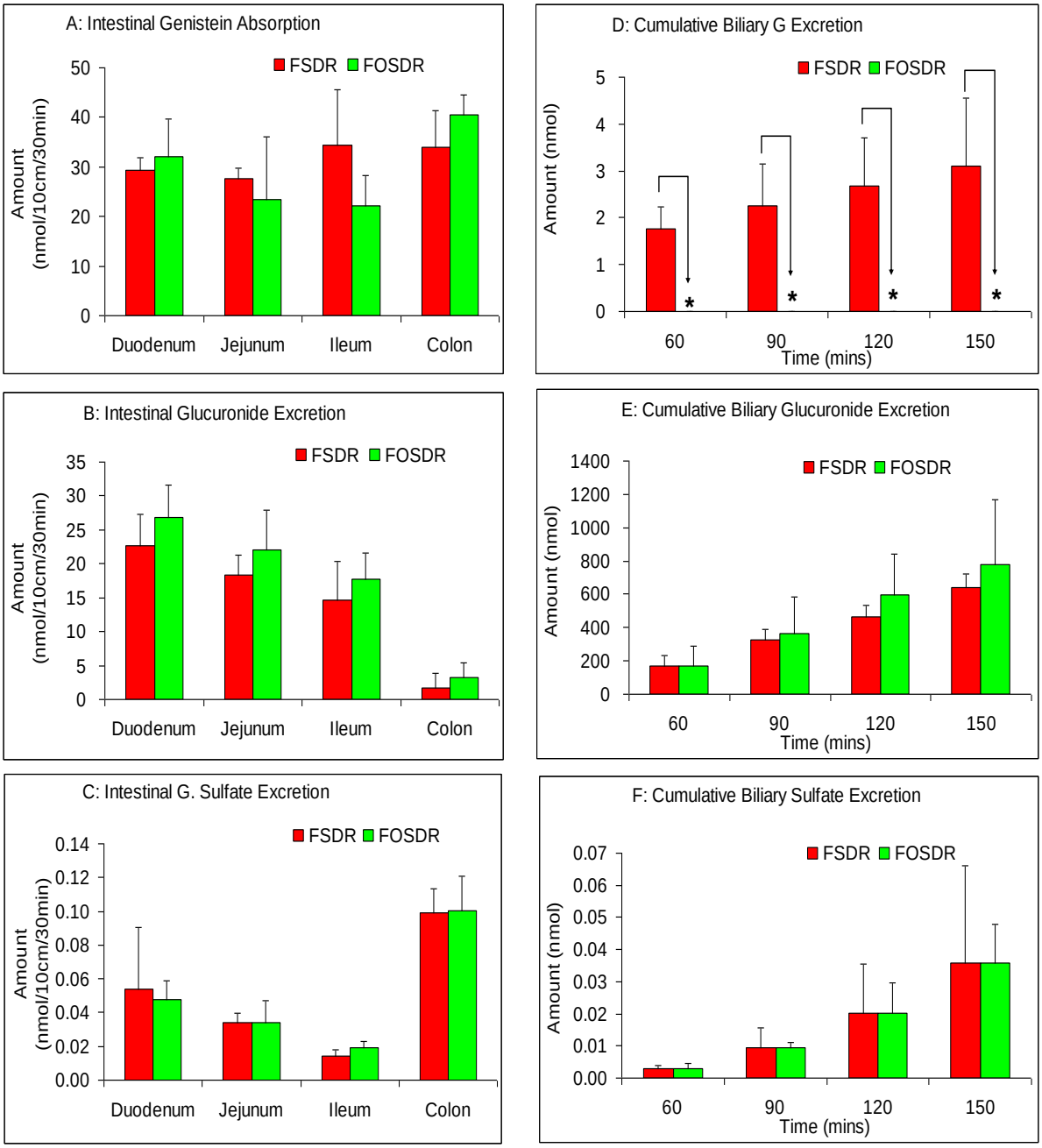

A: Intestinal Genistein Absorption
FSDR FOSDR
Amount (nmol/10cm/30min)
0 10 20 30 40 50
Duodenum Jejunum Ileum Colon
D: Cumulative Biliary G Excretion
FSDR FOSDR
Amount (nmol)
0 1 2 3 4 5
*
60 90 120 150
Time (mins)
B: Intestinal Glucuronide Excretion
FSDR FOSDR
Amount (nmol/10cm/30min)
0 5 10 15 20 25 30 35
Duodenum Jejunum Ileum Colon
E: Cumulative Biliary Glucuronide Excretion
FSDR FOSDR
Amount (nmol)
0 200 400 600 800 1000 1200 1400
60 90 120 150
Time (mins)
C: Intestinal G. Sulfate Excretion
FSDR FOSDR
Amount (nmol/10cm/30min)
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14
Duodenum Jejunum Ileum Colon
F: Cumulative Biliary Sulfate Excretion
FSDR FOSDR
Amount (nmol)
0.00 0.01 0.02 0.03 0.04 0.05 0.06 0.07
60 90 120 150
Time (mins)

Figure 6.3: For genistein 10μM four-site rat intestinal perfusion model: Intestinal (A) absorption of genistein aglycone (B) excretion of genistein glucuronide (C) excretion of genistein sulfate in control female and female ovariectomized rats. Cumulative biliary excretion of (D) genistein (E) genistein glucuronide and (F) genistein sulfate in female rats in control female and female ovariectomized rats.

6.5. Discussion

We concluded that estrus cycle plays an important role in absorption and disposition of genistein female rats. Female rats in metoestrus phase showed higher cumulative biliary excretion of genistein and genistein glucuronide than female rats in proestrus phase of estrus cycle.

Significantly higher plasma concentrations of genistein glucuronide in female than in male rats in single dose oral PK studies suggested role of sex hormones in absorption and disposition of genistein in female rats. Therefore, we grouped the rats in based on the levels of female hormones in different phases of estrus cycle. First group consisted of female rats in proestrus and oestrus phase where the estrogen levels surged by the start of proestrus phase and came back to the baseline by the end of the oestrus phase of the estrus cycle in female rats. Second group consisted of metoestrus and diestrus phase where the estrogen levels stayed at the basal levels. The metoestrus phase group showed higher levels of cumulative biliary genistein and genistein glucuronide than proestrus phase of estrus cycle female rats. This phenomenon may be explained by the following theory. In metoestrus phase of estrus cycle, the female rats have higher levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which is associated with higher levels of hepatic UGT1A metabolizing enzyme isoforms in female rats [96] thereby increasing hepatic glucuronidation of genistein. These enzyme isoforms expression levels came back to basal level by the start of the

next proestrus phase of the estrus cycle suggesting lower levels of glucuronidation in proestrus phase than in metoestrus phase of estrus cycle in female rats. These higher levels of genistein glucuronide formation may explain higher biliary excretion of genistein glucuronide in metoestrus than in proestrus phases of estrus cycle in female rats.

Cumulative biliary excretion of genistein sulfate did not show significant differences in proestrus and metoestrus phase of estrus cycles in female rats. This was due to below detection limit concentrations of genistein sulfate in bile for both proestrus and metoestrus phase of estrus cycle groups of female rats.

To our surprise, four site rat intestinal perfusion studies for control female and female ovariectomized rats did not show any significant differences in absorption and disposition of genistein except no detectable amount of biliary excretion of genistein in female ovariectomized rats (Fig.6.3). This data was rather unexpected and surprising for the following reasons. In chapter 4, more than 2 and 4 fold higher plasma genistein and genistein glucuronide AUC values were observed in female ovariectomized rats than in control female rats. At this point, we do not have any explanation for this phenomenon and further investigation is required to confirm the findings observed for female ovariectomized rats.

In conclusion, estrus cycle played an important role in genistein absorption and disposition in female rats. Female rats in metoestrus phase group showed

higher biliary excretion of genistein and genistein glucuronide than in proestrus phase of estrus cycle group of female rats.

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