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Effects of Estrogen and Estrus Cycle on Pharmacokinetics, Absorption, and Disposition of Genistein in Female Sprague–Dawley Rats

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Supporting Information

ABSTRACT: Genistein is an active soy isoflavone with anticancer activities, but it is unknown why it has a higher oral bioavailability in female than in male rats. Our study determined the effects of estrus cycle on genistein's oral bioavailability. Female rats with various levels of estrogen were orally administered with genistein or used in a four-site rat intestinal perfusion experiment. Rats in "proestrus" group (with elevated estrogen) had significantly reduced (57% decrease, $p < 0.05$) oral bioavailability of total genistein (aglycone + conjugates) than those in "metoestrus" group (with basal level of estrogen). Female ovariectomized rats, due to lack of estrogen, showed oral bioavailability of total genistein similar to the "metoestrus" group but higher (155% increase, $p < 0.05$) than the "proestrus" group. On the basis of intestinal perfusion studies, the increased bioavailability was partially attributed to the higher (>100% increase, $p < 0.05$) hepatic disposition via glucuronidation and possibly more efficient enterohepatic recycling of genistein in the "metoestrus" group. Furthermore, chronic exogenous supplementation of estradiol in ovariectomized rats significantly reduced (77%, $p < 0.05$) the oral bioavailability of total genistein, mostly via increased sulfation (>10-fold) in liver, to a level comparable to those in the "proestrus" group. In conclusion, the oral bioavailability of total genistein was inversely proportional to elevated estrogen levels in female rats, which is partially mediated through the regulation of hepatic enzymes responsible disposition of genistein.

KEYWORDS: *genistein, oral bioavailability, pharmacokinetics, estrus cycle, estrogen*

INTRODUCTION

Genistein, a soy isoflavone present in nature as genistin (genistein-7-*O*-glucoside) but absorbed as aglycone,[1] has been labeled as a "miracle" compound with lots of claimed beneficial effects such as its antioxidant and anticancer activities,[2,3] many of which have been demonstrated in small-scale clinical trials.[2−4] Genistein has also displayed potential favorable impact on prevention of coronary heart disease and postmenopausal symptoms.[5] To this date, the major difficulty in demonstrating the efficacy of genistein in humans is its poor, highly variable, and gender-dependent oral bioavailability.[6,7]

Gender-dependent oral bioavailability was demonstrated in an earlier study, where a significantly higher (2-fold) oral bioavailability of total ^{14}C-genistein (aglycone + metabolite) was observed in female (15%) than in male (7%) Sprague–Dawley or SD rats.[6] We observed similar results for oral PK study in male and female SD rats, in that significantly higher oral bioavailability of genistein (<2-fold) and genistein glucuronide (>4-fold) and thereby total genistein was observed in female than in male SD rats (see Supporting Information). Until now, no study has been done to determine if female sex hormone(s) impact oral bioavailablity of pharmaceutical or dietary polyphenol in rodents, primates, or humans. Furthermore, no study has been done to determine the role of estrus cycle in regulation of oral bioavailability of pharmacologically active polyphenols in female rats, which is important because expression levels of certain hepatic phase II metabolizing enzymes (e.g., UDP-glucuronosyltransferases, or UGTs) are female sex-hormone regulated.[8]

Female sex hormone appears to impact the expression levels of certain phase II enzymes in that the pregnant female rats showed significantly lower hepatic Ugt1A, Ugt1A1, Ugt1A5, Ugt1A6, and Ugt1A8 enzyme expression levels than in control female SD rats.[8] In the same study, significantly higher levels of these hepatic UGT isoforms were observed in female rats 10−12 days postpartum compared to those in the control female rats.[8] However, there is a lack of studies done to determine the regulation of uptake/efflux transporter proteins by female sex hormones in rodents.

Female ovariectomized rodents with a diminished level of estrogen (a major female sex hormone) have been the model of choice for testing the effectiveness of antiestrogens such as tamoxifen and phytoestrogens such as genistein against breast cancer and bone loss for more than two decades.[9,10] Raloxifene, tamoxifen, and genistein are three extensively studied compounds for breast cancer development, prevention, and treatment in female ovariectomized rats.[11−13] No studies have been done to clearly understand the effect of diminishing and resurrected levels of estrogen on oral bioavailability of these

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phenolic compounds in female ovariectomized rats, which could significantly impact their efficacy.

Therefore, the purpose of this study was to determine the role of sex hormones, estrogen, on oral bioavailability of genistein by using female ovariectomized rats and control female rats in different phases of the estrus cycle. We divided female rats into two groups (the "proestrus" and the "metoestrus") based on the levels of female sex hormones.[14] At proestrus and oestrus phases (called the "proestrus" group) of the estrus cycle, female rats showed elevated levels of estrogen, whereas at metoestrus and diestrus phases (called the "metoestrus" group) of the estrus cycle, female rats showed basal levels of estrogen. Oral bioavailability of genistein was compared between female ovariectomized and control female rats and between female rats in the "proestrus" and the "metoestrus" groups. We also determined the effect of a chronic exogenous dose of estradiol on oral bioavailability of genistein in female ovariectomized rats. Additional absorption and intestinal disposition studies were conducted using a four-site rat intestinal perfusion model with bile duct cannulation to determine the contribution of gut and liver metabolism to the observed differences in oral bioavailability.

MATERIALS AND METHODS

Chemicals. Genistein and daidzein (internal standard) were purchased from Indofine Chemicals (Somerville, NJ). Estradiol, mineral oil, and Hank's Balanced Salt Solution (HBSS powder form) were from Sigma-Aldrich (St. Louis, MO). All other materials used were analytical grade or better.

Animals. Female Sprague−Dawley rats (6−8 weeks old) were purchased from Harlan Laboratories (Madison, WI). The rats were fed with an AIN 76A nonsoy diet (W) purchased from Harlan Laboratories (Madison, WI). The rats were fasted for up to 15 h before the day of the pharmacokinetic (or PK) or perfusion experiment.

Estrus Cycle Status. Estrus cycle status of a female SD rat was monitored daily. Vaginal smears for all the female rats were taken twice (morning and evening) daily for four consecutive days at the same time, and only the female rats showing all four phases of estrus cycle were chosen for the study. The vaginal smear was taken using the same method described previously[15,16] with minor modifications. Briefly, the vaginal smear was taken using a plastic pipet on a clean glass slide, air-dried, and stained using methylene blue dye to identify the presence of different cells, confirming the phases of estrus cycle in female rats (see Supporting Information). For perfusion/PK experiments, the female rats were grouped as the "proestrus" group for those in "proestrus + oestrus" phases (at elevated plasma estrogen levels) or as the "metoestrus" group for those in "metoestrus" + diestrus" phases (at basal plasma estrogen levels).

Exogenous Estradiol Administration. Estradiol 100 μg/mL was prepared in mineral oil, and 10 μg/kg subcutaneous dose was administered to female ovariectomized rats every day at 9:00 a.m. for a period of 10 days. On the 11th day, a single dose of genistein (20 mg/kg) was administered orally. Another group of rats were dosed with vehicle (i.e., mineral oil) for seven days, and on the eighth day, they were dosed with a single dose of genistein (20 mg/kg).

Pharmacokinetic (PK) Experiment. The procedures for this and other animal experimentation were approved by the University of Houston's Institutional Animal Care and Use Committee. Genistein suspension (20 mg/mL) freshly prepared in oral suspending vehicle from PCCA Laboratories (Houston, TX) was administered by oral gavage at a dose of 20 mg/kg.[17] Blood samples were taken from the tail vein at 15, 30, 45, 60, 120, 180, 240, 360, 480, 720, and 1440 min after oral administration.

Sample Extraction. Blood samples (20 μL) from the pharmacokinetic studies were precipitated with 100% acetonitrile (80 μL) containing 3 μM of daidzein to collect >90% of genistein and its phase II metabolites. The mixture was centrifuged at 15500 rpm for 15 min and 80 μL of supernatant was collected and dried under air. The residue was reconstituted in 100 μL of 30% acetonitrile solution, centrifuged at 15500 rpm for 15 min, and injected in UPLC-MS/MS.

Animal Surgery. The intestinal surgical procedures were modified from our previous publications,[18,19] in that four segments of the intestines were cannulated simultaneously along with a bile duct cannulation.[1,20,21] The blood circulation to the liver and intestine was not disrupted in this model. To maintain the temperature of the perfusate constant at 37 °C, the inlet cannulae were insulated and kept warm by a 37 °C circulating water bath.

Transport and Metabolism Experiments in Perfused Rat Intestinal Model. A single-pass perfusion method described previously was used.[21,22] At least four rats were used for each set of the perfusion study. Briefly, four segments (duodenum, upper jejunum, terminal ileum, and colon) of the rat intestine approximately 10−15 cm each were perfused simultaneously with a Hanks' Balanced Salt Solution (HBSS, pH 7.4) containing 10 μM genistein as the perfusate. The flow of the perfusate was driven by an infusion pump (Harvard Apparatus, Cambridge, MA) at a flow rate of 0.191 mL/min. After a 30 min washout period, which is usually sufficient to achieve steady-state absorption, intestinal perfusate samples were collected from the outlet cannulae every 30 min. Bile samples (approximately 0.5 mL) were collected before perfusion started and every 30 min afterward. After perfusion, the length of the intestine was measured as described previously.[18,19] The perfusate sample concentrations of genistein and its phase II metabolites were determined using UPLC-MS/MS. Bile samples were diluted (1:40) with HBSS buffer, centrifuged, and supernatant removed for UPLC-MS/MS analysis.

UPLC-MS/MS Analysis of Genistein and Its Conjugates. We implemented a published method for analysis of genistein and its conjugates in rat plasma, perfusion, and microsome samples using UPLC-MS/MS.[17] 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 MRM method with the transitions of m/z 269 → m/z 133 for genistein, m/z 445 → m/z 269 for genistein glucuronides, m/z 349 → m/z 269 for genistein sulfate, and m/z 253 → m/z 132 for daidzein (IS). Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. The standard curves for genistein, genistein glucuronide, and genistein sulfate were prepared in blank rat blood. The standard curves were linear in the concentration range of 0.019−10 μM for genistein, 0.02−10 μM for genistein glucuronide, and 0.002−1.6 μM for genistein sulfate.

Data Analysis in Pharmacokinetic Studies. The plasma concentration−time profiles for each rat were analyzed by using WinNonlin3.3. C_{max} and the area under the concentration−time curve [$AUC_{(0-24\ h)}$] values were calculated according to a noncompartmental model using the Winnonlin software.

Data Analysis in Rat Intestinal Perfusion Experiments. Amounts of genistein absorbed (M_{ab}), amounts of conjugated genistein excreted into the intestinal lumen (M_{gut}), and amounts of conjugated genistein excreted via the bile (M_{bile}) values were calculated as described previously.[20,21,23] Briefly, M_{ab} was expressed as:

$$M_{ab} = Q \times \tau \times (CA_{in} - CA_{out}) \quad (1)$$

where Q is the flow rate (mL/min), τ is the sampling interval (30 min), and CA_{in} and CA_{out} are the inlet and outlet concentrations of genistein aglycone corrected for water flux, respectively. M_{gut} was expressed as

$$M_{gut} = Q \times \tau \times CM_{out} \quad (2)$$

where CM_{out} is the outlet concentration (nmol/mL) of metabolites corrected for water flux, and M_{bile} is expressed as

$$M_{bile} = V \times CM_{bile} \quad (3)$$

where CM_{bile} is the bile concentration (nmol/mL) of metabolites and V is the volume of bile collected over a 30 min time period.

Statistical Analysis. The data in this paper were presented as mean ± SD, if not specified otherwise. Significant differences were assessed by using unpaired Student's t test with "p" value of <0.05.

RESULTS

Estrus Cycle-Dependent Oral Bioavailability of Genistein in Female SD Rats. Female rats were divided into two separate groups: one for elevated (the "proestrus") and the other for basal (the "metoestrus") levels of estrogen using vaginal smears as described previously.[14] A single oral dose of genistein (20 mg/kg) was given to female SD rats from both groups to identify the effects of estrus cycle on plasma profiles of genistein and its metabolites (glucuronide and sulfate) using UPLC-MS/MS analysis.

Significant estrus cycle-dependent differences were observed in total genistein (aglycone + metabolite) plasma pharmacokinetic profiles (Figure 1, Table 1). Genistein plasma concentration profiles showed significantly higher C_{max} (<2-fold, $p < 0.05$) and $AUC_{(0-24\ h)}$ (>2-fold, $p < 0.05$) values in the "metoestrus" group than in the "proestrus" group (Figure 1A, Table 1). Furthermore, genistein glucuronide plasma concentration profiles also showed higher $AUC_{(0-24\ h)}$ (2.4-fold, $p < 0.01$) values in the "metoestrus" group than in the "proestrus" group (Figure 1B, Table 1). Genistein sulfate plasma concentrations, however, were too low to determine the effects of estrus cycle on its level in these rats.

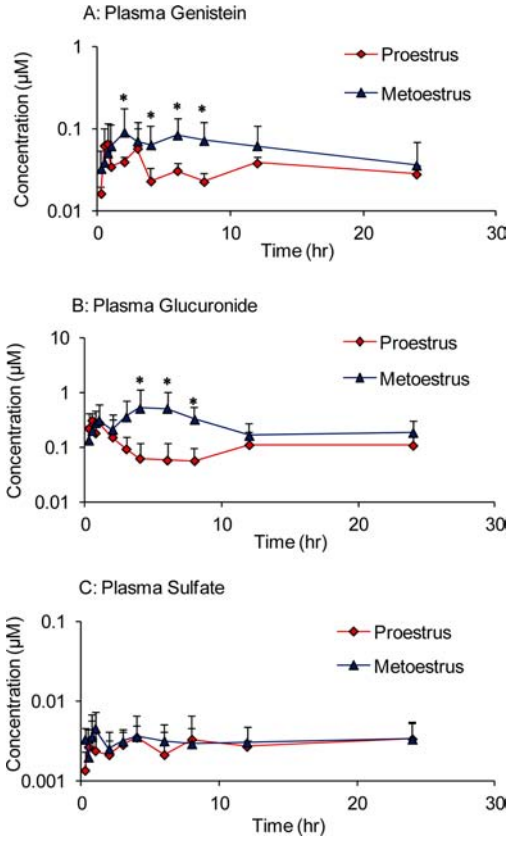

Figure 1. Plasma concentrations of genistein (A), genistein glucuronide (B), and genistein sulfate (C) in female rats in the "proestrus" (diamonds) or the "metoestrus" (triangles) phase of the estrus cycle after single oral dose of genistein 20 mg/kg. The symbol "*" indicates $p < 0.05$ compared with "proestrus" group.

Table 1. Comparison of Pharmacokinetic Parameters for Genistein (Aglycone), Genistein Glucuronide (Glucuronide), Genistein Sulfate (Sulfate), and Total Genistein (Aglycone + Conjugates or Total) in Female SD Rats at Different Estrus Cycle Phases and in Female Ovariectomized SD Rats with or without Estrogen Supplementation[a]

	aglycone				glucuronide				sulfate				total			
	Pro	Meto	Ova	Ova+E	Pro	Meto	Ova	Ova+E	Pro	Meto	Ova	Ova+E	Pro	Meto	Ova	Ova+E
C_{max} (μM)	0.10 ± 0.03	0.14 ± 0.07	0.25 ± 0.09	0.06 ± 0.02	0.47 ± 0.14	0.53 ± 0.37	0.52 ± 0.12	0.22 ± 0.04	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.53 ± 0.13	0.80 ± 0.30	0.83 ± 0.21	0.26 ± 0.04
AUC (0–24 h) (μM·h)	0.79 ± 0.10	1.70 ± 0.72	2.38 ± 1.12	0.36 ± 0.1	2.50 ± 1.11	5.97 ± 1.41	4.38 ± 1.97	2.00 ± 1.00	0.07 ± 0.02	0.07 ± 0.02	0.53 ± 0.47	0.04 ± 0.01	3.36 ± 1.18	7.74 ± 1.90	8.58 ± 3.09	2.38 ± 1.02

[a]A single dose of genistein at 20 mg/kg was given to each rat, and pharmacokinetic parameters were calculated using a non-compartmental model (Winnonlin 3.3). In this table, "Pro" represents ""proestrus" (elevated estrogen) phase," "Meto" represents ""metoestrus" (basal estrogen) phase, "Ova" represents ovariectomized female rats without estrogen, and "Ova+E" represents ovariectomized rats supplemented with exogenous estrogen. Comparisons of the results using various statistical means were shown in the Results section. Results of statistical analysis of data were shown in Supporting Information (Table S3).

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Overall, the 2-fold higher oral bioavailability of total genistein in the "metoestrus" group as compared to the "proestrus" group was contributed predominantly by 2.4-fold ($p < 0.01$) higher plasma concentrations of genistein glucuronide in "metoestrus" group than in "proestrus" group of female rats (Table 1).

Ovarian-Dependent Oral Bioavailability of Genistein in Female SD Rats. Single dose oral pharmacokinetics of genistein (20 mg/kg) was also determined in ovariectomized rats, and plasma concentrations of genistein and its metabolites (glucuronide and sulfate) were also analyzed. Significant ovarian (sex hormone)-dependent difference was observed in total genistein (aglycone + metabolite) plasma pharmacokinetic profiles (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information). Genistein plasma concentrations showed significantly higher C_{max} (>2.5-fold, $p < 0.05$) and $AUC_{(0-24\ h)}$ (3-fold, $p < 0.05$) values in ovariectomized rats than the "proestrus" group respectively (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information). Surprisingly, genistein sulfate plasma concentrations also showed significantly higher C_{max} and $AUC_{(0-24\ h)}$ (>5-fold each, $p < 0.01$) values in ovariectomized rats than not just the "proestrus" but also the "metoestrus" group (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information).

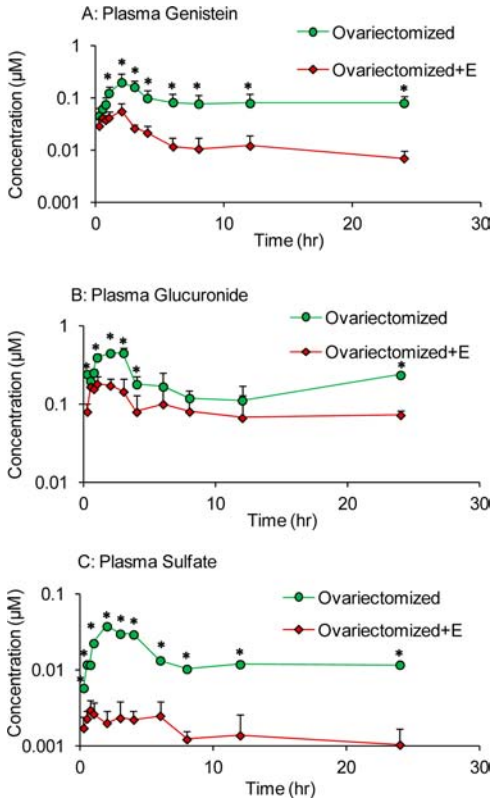

Figure 2. Plasma concentrations of genistein aglycone (A), genistein glucuronide (B), and genistein sulfate (C) in female ovariectomized (circles) and female ovariectomized SD rats with chronic exogenous estradiol (@10 μg/kg for 10 days) (diamonds) administration after single oral dose of genistein at 20 mg/kg. The symbol "*" indicates $p < 0.05$ compared with "ovariectomized + E" group.

Overall the 2.5-fold higher oral bioavailability of total genistein in ovariectomized rats as compared to the "proestrus" group was contributed predominantly by 3-fold ($p < 0.01$) higher plasma concentrations of genistein in ovariectomized rats than the "proestrus" group (Table 1). No significant difference in oral bioavailability of total genistein in ovariectomized rats was observed when compared to the female rats in the "metoestrus" group (Table 1).

Exogenous Estradiol-Dependent Oral Bioavailability of Genistein in Female SD. The pharmacokinetics of genistein after a single oral dose (20 mg/kg) was further determined in ovariectomized rats after chronic (10 days) subcutaneous estradiol (10 μg/kg/day) administration, and plasma concentrations of genistein and its metabolites (glucuronide and sulfate) were again analyzed using UPLC-MS/MS analysis. Our hypothesis was that the exogenous estrogen supplement might reverse the higher oral bioavailability plasma profiles of genistein and its metabolites (glucuronide and sulfate) in female ovariectomized rats. The results indicated that a significant exogenous estradiol-dependent difference was observed in total genistein (aglycone + metabolites) plasma pharmacokinetic profiles in ovariectomized rats after chronic estradiol administration. Genistein, genistein glucuronide, and genistein sulfate plasma concentrations showed significantly lower C_{max} (4-, 2-, and 5-fold, $p < 0.05$) and $AUC_{(0-24\ h)}$ (6.5-, 2-, and 13-fold, $p < 0.05$) values after exogenous estradiol supplement in ovariectomized rats (Figure 2A–C, Table 1).

Overall, the 3-fold lower oral bioavailability of total genistein after chronic (10 days) estradiol (10 μg/kg/day) administration in ovariectomized rats as compared to the control ovariectomized rats was contributed predominantly by 6.5-fold ($p < 0.01$) and 2-fold ($p < 0.05$) lower plasma concentrations of genistein and genistein glucuronide respectively (Table 1).

Comparison of Oral Bioavailability of Total Genistein between Chronic (10 days) Estradiol (10 μg/kg) Administered Ovariectomized and Control Rats in the "Proestrus" Group. Oral bioavailability of total genistein (aglycone + metabolite) plasma pharmacokinetic profile comparison showed no significant difference (except one) between exogenous estradiol supplemented ovariectomized rats and the "proestrus" group. Genistein plasma concentrations showed significantly lower $AUC_{(0-24\ h)}$ (<2-fold, $p < 0.05$) with no difference in C_{max} values in estradiol supplemented ovariectomized rats than the "proestrus" group (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information). Genistein glucuronide and total genistein (aglycone + metabolite) plasma concentrations showed no significant difference in C_{max} and $AUC_{(0-24\ h)}$ values between estradiol supplemented ovariectomized rats and the "proestrus" group (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information). Furthermore, genistein sulfate plasma concentrations in both groups of rats were too low to determine the effects of exogenous estradiol administration on its level (Table 1, Figure 1 and 2, and Figure S3 in Supporting Information).

Overall, no significant difference in oral bioavailability of total genistein in ovariectomized rats after chronic estradiol administration and control rats in "proestrus" group was observed (Table 1).

Comparison of Oral Bioavailability of Total Genistein between Ovariectomized Rats after Chronic (10 days) Estrogen (10 μg/kg) Dose Administration and Control Rats in the "Metoestrus" Group. Oral bioavailability of total genistein (aglycone + metabolite) plasma pharmacokinetic profile comparison showed significant difference between exogenous estradiol supplemented ovariectomized rats and the "metoestrus" group. Genistein and genistein glucuronide plasma

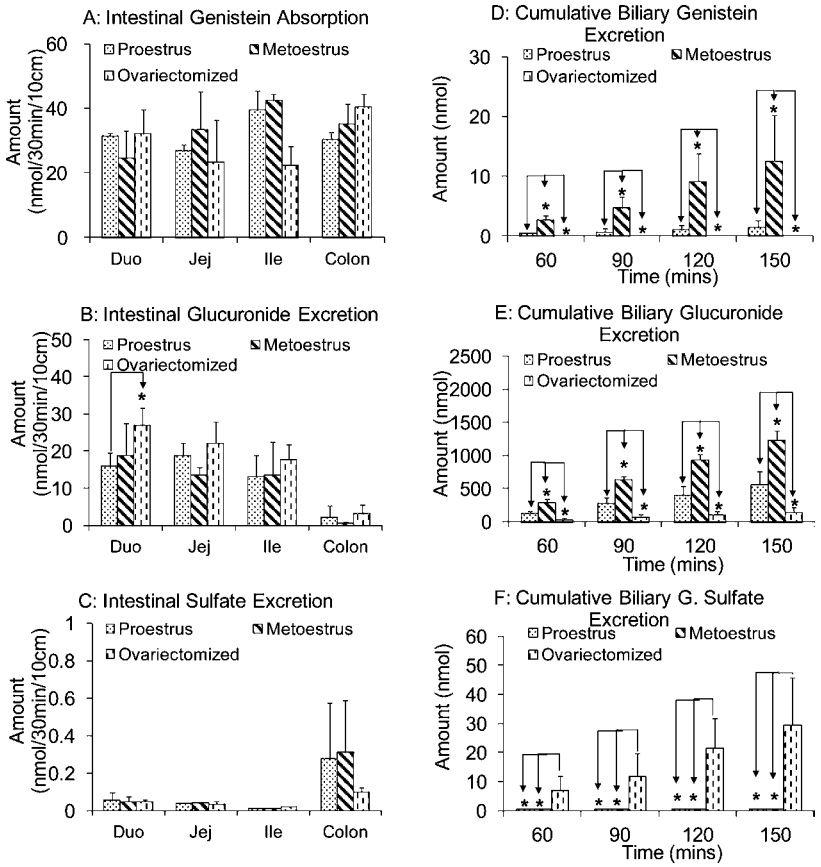

Figure 3. Absorption and disposition of genistein in a four-site rat intestinal perfusion model ($n \geq 4$). Four segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously at a flow rate of 0.191 mL/min using perfusate containing 10 μM genistein in the "proestrus" group (columns with dots), the "metoestrus" group (columns with stripes), and ovariectomized rats (columns with broken vertical lines). Amount absorbed (A), intestinal glucuronide excreted (B), and intestinal sulfate excreted (C) were normalized to 10 cm intestinal length using eqs 1−3 in data analysis. Biliary excretion samples ($n \geq 4$) were collected at every 30 min time interval during the entire perfusion experiment. Cumulative amount of biliary excretion of genistein aglycone (D), glucuronide (E), and sulfate (F) was depicted. The arrow indicates a statistically significant difference between two groups.

concentrations showed significantly lower C_{max} (>2-fold each, $p < 0.05$) and $AUC_{(0-24\ h)}$ (4- and >2.5-fold, $p < 0.01$) values in estradiol supplemented ovariectomized rats than the "metoestrus" group respectively (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information). Genistein sulfate plasma concentrations, however, in both groups of rats were too low to determine the effects of exogenous estradiol administration on its level (Table 1, Figures 1 and 2, and Figure S3 in Supporting Information).

Overall, the 3-fold lower oral bioavailability of total genistein after chronic estradiol (10 μg/kg) administration in ovariectomized rats as compared to the control rats in the "metoestrus" group was contributed predominantly by 2.5-fold ($p < 0.01$) lower plasma concentrations of genistein glucuronide (Table 1).

Estrus Cycle-Dependent Genistein Absorption and Disposition in Female SD Rats. Female rats were divided into two separate groups: elevated (the "proestrus") and basal (the "metoestrus") levels of female sex hormone, estrogen, using vaginal smears as described previously.[14] Four-site rat intestinal perfusion with bile duct cannulation was used to identify the estrus cycle-dependent differences in intestinal perfusate and biliary excretion profiles of genistein and its metabolites (glucuronide and sulfate). Significant estrus cycle-dependent difference was observed in hepatic disposition but not in intestinal absorption and disposition of genistein (Figure 3). Cumulative biliary excretion profiles showed significantly higher (<2-fold each, $p < 0.05$) amount of genistein (1−12 nmol) and genistein glucuronide (500−1500 nmol) in the "metoestrus" than the "proestrus" group (Figure 3D,E). Intestinal absorption and metabolite (glucuronide and sulfate) excretion of genistein showed no difference between the "metoestrus" and the "proestrus" groups (Figure 3A−C). Cumulative biliary excretion of genistein sulfate in both groups was too low to determine the effects of changes in the estrus cycle (Figure 3F).

Overall, significantly higher biliary excretion of genistein and conjugates in the "metoestrus" as compared to the "proestrus" group was mainly due to the higher (~2 fold, $p < 0.05$) cumulative biliary efflux of genistein glucuronide (micromolar range) in the "metoestrus" than in the "proestrus" group of rats.

Ovarian-Dependent Genistein Absorption and Disposition in Female SD Rat. Four-site rat intestinal perfusion with bile duct cannulation was used to determine the impact of ovary on intestinal and biliary excretion of genistein conjugates. Significant ovarian-dependent difference in biliary excretion was observed in ovariectomized rats when compared to rats in either the "proestrus" or the "metoestrus" or the control (containing both proestrus and metoestrus) group, but intestinal absorption and disposition of genistein was not observed, with one minor exception (Figure 3): duodenal genistein glucuronide excretion was significantly higher (<2-fold, $p < 0.05$) in female

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ovariectomized rats as compared to the "proestrus" group (Figure 3B). In case of biliary excretion of genistein conjugates, no detectable amount of genistein aglycone and significantly lower genistein glucuronide (150−1250 nmol range) (4- and 8-fold, respectively, $p < 0.01$) was observed in cumulative biliary excretion of genistein conjugates in ovariectomized rats (Figure 3E). In contrast, significantly higher amount of genistein sulfate (10−30 nmol range) was observed in biliary excretion in ovariectomized rats as compared to undetectable levels in the other groups (Figure 3F).

Overall, significantly lower biliary excretion of genistein and conjugates in the ovariectomized rats as compared to control group of rats (containing rats from both the "proestrus" and the "metoestrus" groups) was mainly due to 4- and 8-fold lower ($p < 0.01$) cumulative biliary efflux of genistein glucuronide in the ovariectomized rats.

DISCUSSION

Our results showed for the first time that the estrus cycle differences and associated changes in sex hormones, especially estrogen, significantly affected oral bioavailability of total genistein in female rats. The observed higher oral bioavailability (AUC value >2-fold, $p < 0.05$) of total genistein in the "metoestrus (low estrogen)" group as opposed to the "proestrus (high estrogen)" group (Table 1) supports the conclusion that higher level of female sex hormone estrogen down regulates phase II enzyme activities. The conclusion is further corroborated by the fact that ovariectomized rats (without ovarian sex hormone estrogen) also showed equally higher oral bioavailability (AUC value >2-fold, $p < 0.05$) of total genistein than that in the "proestrus" group (Table 1). Interestingly, chronic (10 days) estradiol supplementation in ovariectomized rats significantly reduced the oral bioavailability (AUC value >2-fold, $p < 0.05$) of total genistein in ovariectomized rats (Table 1) to a level that is comparable to that in "proestrus" group (Table 1), suggesting that estradiol alone may contribute to the bulk of the female hormonal regulation of phase II enzymes.

Until now, to our surprise, we did not see any publications studying the differences in oral bioavailability of pharmaceutical or dietary compounds in female rats in relation to differences in female sex hormone (especially estrogen) levels. Control female and female ovariectomized rats have been used as a model of choice for pharmacological testing of these compounds in a variety of pharmacological models including breast cancer.[12,24,25] The major assumption for using ovariectomized rats as an ideal model for testing the effectiveness of these agents against breast cancer was that sex hormones did not alter their oral bioavailabilities. Considering that breast cancer is not just limited to postmenopausal women (completely diminished plasma levels of major sex hormone, estrogen), one should consider how to extrapolate the results of various translational research that test the pharmacological activities of these compounds' efficacy using ovariectomized rats. For antiestrogenic agents such as raloxifene and genistein, which are extensively studied in female ovariectomized rats,[12,26] it is now imperative to determine the effect of female sex hormones on their oral bioavailability and efficacy.

While studying the effect of fluctuating levels of female sex hormone, estrogen, during the estrus cycle phases on single oral dose pharmacokinetics of genistein (20 mg/kg), we observed that plasma estrogen levels played an important role in determining oral bioavailability of total genistein (>2-fold, $p < 0.05$) in female rats. Significantly higher oral bioavailability of total genistein in the "metoestrus" (basal levels of estrogen) than the "proestrus" (elevated levels of estrogen) group (Figure 1, Table 1) suggested that oral bioavailability of total genistein was inversely proportional to the elevated plasma sex hormone levels in female rats.

To further confirm our theory, we used female ovariectomized rats to identify the effect of hormone ablation on oral bioavailability of total genistein in female rats. As expected, we observed significantly higher oral bioavailability of total genistein (2.5-fold, $p < 0.05$) with predominant contribution from genistein (3-fold, $p < 0.01$) and genistein glucuronide (<2-fold, $p < 0.05$) in ovariectomized than the "proestrus" (elevated estrogen) group. Furthermore, there was no significant difference in oral bioavailability of total genistein in ovariectomized rats and the "metoestrus" (basal estrogen) group. This further confirmed our theory that oral bioavailability of total genistein is inversely proportional to elevated estrogen levels in female rats.

In the next set of studies, after chronic (10 days) exogenous estradiol (10 μg/kg/day) supplementation to ovariectomized rats, oral bioavailability of total genistein significantly dropped (Figure 2, Table 1), making it comparable to the "proestrus" group. This observed significant drop in oral bioavailability further bolstered our theory that oral bioavailability of total genistein is inversely proportional to elevated plasma estrogen levels in female rats.

Because oral bioavailability is affected by both absorption and first-pass metabolism, we determined if intestinal absorption and metabolism of genistein contributed to the differences in oral bioavailability observed above. To study the differences in absorption and disposition of genistein, we used a four-site rat intestinal perfusion model with bile duct cannulation. The results suggested that female sex hormone, estrogen, predominantly regulated the hepatic disposition (phase II metabolizing enzymes and/or efflux transporters) of genistein and its conjugates in female rats because the bile excretion patterns were drastically altered (Figure 3). The basal levels of estrogen in the "metoestrus" group of rats were associated with a significantly higher biliary efflux of genistein glucuronide (Figure 3), which in turn meant more efficient enterohepatic recycling than in the "proestrus" group (elevated levels of estrogen). Efficient enterohepatic recycling of genistein metabolites contributed to a longer apparent half-life, which in turn meant higher oral bioavailability of total genistein in the "metoestrus" than in the "proestrus" group of rats, as observed in oral pharmacokinetic studies.

In the case of ovariectomized rats, the rat intestinal perfusion experiments showed significantly lower biliary efflux of genistein glucuronide than in the "proestrus" as well as the "metoestrus" group of rats (Figure 3). The lowered biliary excretion was inconsistent with the oral pharmacokinetics data observed in ovariectomized rats. Currently, we do not have any explanation for these observed inconsistencies. A possible explanation is that intestinal phase II metabolism was significantly increased but the majority of metabolites were transported to the plasma via the basolateral efflux transporters that were up-regulated in absence of the ovary hormones. More work is still required to explain this phenomenon.

In conclusion, we have clearly demonstrated for the first time that oral bioavailability of total genistein was inversely proportional to elevated plasma estrogen levels in female rats, which is partially mediated via changes in hepatic phase II

metabolism of genistein. Therefore, plasma estrogen levels play an important role in oral bioavailability of total genistein in female rats through regulation of hepatic disposition of genistein and efficient enterohepatic recycling of genistein conjugates.

■ ASSOCIATED CONTENT

Supporting Information

Gender-dependent bioavailability of genistein. Plasma concentrations of genistein, genistein glucuronide, and genistein sulfate in male and female SD rats after single oral dose of genistein. Vaginal smears showing four different phases of estrus cycle. Plasma concentrations of genistein, genistein glucuronide, and genistein sulfate in female rats in "proestrus" and "metoestrus" phase of estrus cycle as well as female ovariectomized (circles) SD rats after single oral dose of genistein. Comparison of pharmacokinetic parameters for genistein. Comparison of pharmacokinetic parameters for genistein, genistein glucuronide, genistein sulfate, and total genistein in female SD rats at different estrus cycle phases and in female ovariectomized SD rats with or without estrogen supplementation. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AUC, area under the curve; Pro, "proestrus" phase (or elevated plasma estrogen levels) group of estrus cycle in female rats; Meto, "metoestrus" phase (or basal plasma estrogen levels) group of estrus cycle in female rats; Ova, "ovariectomized" (or undetectable levels of plasma estrogen) group of female rats with no significant estrogen; Ova+E, "ovariectomized + estrogen" (or undetectable levels of plasma estrogen) group of female rats with exogenous dose of estradiol 10 μg/kg/day; UPLC-MS/MS, ultra performance liquid chromatography coupled with tandem mass spectrometry; UGT, uridine diphospho-glucuronosyl transferase enzymes; HBSS, Hank's Balanced Salt Solution; MSDR, male Sprague−Dawley rats; FSDR, Female Sprague−Dawley rats

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