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CONSUMING GENISTEIN IMPROVES SURVIVAL RATES IN THE ABSENCE OF LAXATIVE IN ΔF508-CF FEMALE MICE

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ABSTRACT

Genistein is a naturally occurring isoflavone found in soy. Mice homozygous for the ΔF508 mutation are characterized with severe intestinal disease and require constant laxative treatment for survival. This pathology mimics the intestinal obstruction (meconium ileus) seen in some cystic fibrosis (CF) patients. We therefore tested whether dietary supplementation with genistein would reduce the dependence of the ΔF508 CF mouse model on laxatives for survival, thereby improving mortality rates. At weaning (21 days), we maintained homozygous ΔF508 mice on three diet regimens for a period of up to 65 days; normal diet, normal diet + Colyte or genistein diet. Survival rates for males were as follows: standard diet (38%), standard diet plus Colyte (83%) or genistein diet (60%). Survival rates for females were as follows: standard diet (47%), standard diet plus Colyte (71%), or genistein diet (87%). Average weight of male mice fed genistein diet increased by ~2.5 g more compared to those with Colyte treatment. Genistein diet did not change final body weight of females. Expression of SGLT-1 increased 2-fold with genistein diet in females (no change in males). Expression of GLUT2 and GLUT5 was comparable between all diet groups. Genistein diet reduced the number of goblet cells per micometer of crypt depth in female, yet was without effect in males. We conclude that supplementation of diet with genistein for ~45 days increases the survival rate of female ΔF508-CF mice precluding the requirement for laxatives, and only improves weight gain in males.

Keywords: Genistein, ΔF508-CF, Mouse, Survival

Abbreviations:

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulatory protein: SGLT-1, sodium-dependent glucose cotransporter-1; GLUT2 or 5, facilitated glucose transporter solute carrier-2 or -5.

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INTRODUCTION

Cystic fibrosis (CF), the most common recessive lethal genetic disorder in Caucasians is a result of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The consequences of these mutations are multifarious due to the defective function of the ubiquitous CFTR protein; chronic respiratory tract infections, pancreatic exocrine insufficiency and intestinal obstruction. Clinically this manifests as meconium ileus in some CF newborns and distal intestinal obstructive syndrome (DIOS) in older CF patients [1, 2]. The CF mouse demonstrates intestinal complications comparable to those seen in CF patients; intestinal obstruction attributed to formation of mucus plugs, along with dysfunctional absorptive and secretory functions [3-5], which results in increased morbidity of the mice [6]. The intestinal impactions observed in the CF (ΔF508 homozygous) mouse necessitate a constant laxative treatment (Colyte) for survival [7-9].

Genistein is a naturally occurring isoflavonic phytoestrogen found in high concentrations in soy products, legumes and grains [10]. Epidemiologic studies have suggested multiple health benefits for humans consuming soy-based foods, including reduced risk of cancers [11, 12], reduced incidence of coronary artery disease [13, 14], reduced cholesterol levels [15] and reduced blood pressure [16]. The cellular targets and mechanisms underlying the multiple health benefits of soy products are relatively unclear in large part due to its tissue-specific and sex-specific effects.

Genistein has been shown to increase the open probability (Po) of the most common cystic fibrosis (CF) disease-associated mutation, ΔF508-CFTR, to levels comparable to those seen in Wt-CFTR [17, 18]. These data suggest that genistein may

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have therapeutic potential for treatment of CF. Moreover, we have shown that genistein can improve ΔF508-CFTR in *in vitro* cell systems with an EC50 of 5 μM [19, 20] which is well within the physiological range attainable by dietary modifications. Genistein is readily absorbed across the intestines and can readily reach micromolar concentrations in the serum [21]. Indeed, mice consuming 750 ppm genistein generate plasma genistein concentrations of ~2 μM [22]. We have previously examined the effect of chronic dietary genistein (for 4-weeks) on intestinal function in wild-type mice [23]. Our previous work demonstrated that consumption of 600 mg genistein/kg diet increased serum levels of genistein comparably in wild-type males and females (~5 μM) resulting in a significant increase in basal jejunum transepithelial short circuit current (a measure of chloride secretion) [23]. These levels are comparable to a soy milk diet in humans that also results in plasma genistein concentrations of ~2 μM [24].

Data presented here describes the effect of long-term (4-weeks) dietary supplementation with genistein on survival of the CF mouse. We hypothesized that chronic exposure to elevated levels of dietary genistein would increase survival of the CF mouse.

MATERIALS AND METHODS

CF mouse model: The ΔF508 (CF) mice used in this study were generated by targeted replacement of the wild-type exon 10 allele with the ΔF508 mutant allele. This manipulation also resulted in the neomycin phosphotransferase gene inserted in intron 10 as previously described [25]. Mice were genotyped from tail clip DNA, using primers and methods previously described in detail [25, 26]. CF mice were randomly assigned to one of two diet groups; 600 mg genistein/kg diet or standard (normal) diet either with or without Colyte, and maintained on one of these three diets for 65 days or until demise. Specialized genistein-containing diet was prepared by Dyets Inc. (Bethlehem, PA.) [23]. Mice were housed individually, in an animal care facility with 12:12-hour light-dark cycle and fed/watered ad libitum until day 65. Body weight was measured regularly during the diet study and general health monitored biweekly. CF mice were cared for in accordance with Case Western Reserve University Institutional IACUC, guidelines.

Histology: Freshly isolated segments of jejunum were fixed in sucrose overnight and then embedded in paraffin. Sections (8 μM) were stained for mucin, using Alcian Blue/Periodic Acid Schiff using standard methods. Jejunum morphology (villi length, numbers of goblet cells/villi, crypt depth and number of goblet cells/crypt) measurements were made using Axiovision (Carl Zeiss). Averages of measurements were taken from 10 separate images of jejunum/mouse, and data are presented as the average of multiple mice in each group.

Western blot analysis: At collection, jejuna were immediately snap frozen in liquid nitrogen and stored at -80°C. Jejuna were later prepared for western blot analysis by homogenization, and the western blot protocol used was similar to that described previously [27-29]. Blots were incubated with primary antibody to GLUT2 (1:500, ~60kDa, Santa Cruz, CA), GLUT5 (1:200, ~45-60kDa, Santa Cruz, CA), or SGLT-1 (1:200, ~61kDa, Cell Signaling, Danvers, MA) overnight at 4 ºC. After washing, blots were incubated with secondary antibody, anti-rabbit IgG (H+L) Dylight (1:15,000, Thermo Scientific, Rockford, IL), for 1 hour at room temperature. To re-probe for actin, blots were incubated with anti-GAPDH primary antibody (1:4000, ~40kDa, Thermo Scientific, Rockford, IL) for 1 hour at room temperature. Blots were washed and then re-incubated with the appropriate secondary antibody anti-mouse IgG (H+L) (1:15,000, Dylight, Thermo Scientific Rockford, IL). Images of membranes were taken with all proteins of interest normalized to Actin. Band density was analyzed using Odyssey-Clx (LI-COR, Lincoln, NE) and Image Studio (LI-COR, Lincoln, NE).

Statistics: Data are expressed as mean ± standard error of mean (SEM). Numbers in parentheses are numbers of tissues used from separate individual mice. Kaplan-Meier survival curves were evaluated using a log-rank test. Comparisons between diet groups were performed using one-way ANOVA with post-hoc Neuman-Keuls test using GraphPad (GraphPad Software, Inc., La Jolla, CA.).

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RESULTS

Physical characteristics of the mice

The effect of *ad libitum* feeding of either genistein-containing diet, standard diet with Colyte or standard diet alone on growth of ΔF508 male and female mice was ascertained. Mice entered the diet study at weaning (day 20) and were randomly separated into one of the three diet groups. During the diet study (from day 20 to 65) all mice maintained a steady increase in weight gain (**Figure 1**). Average weight of male mice on Colyte increased from 5.79 ± 0.45 g to 18.66 ± 0.64 g (n = 8), and the average weight of male mice fed genistein diet increased from 6.55 ± 0.33 g to 21.96 ± 0.68 g (n = 14). Genistein diet significantly increased both rate of weight-gain and final body weight (compared to Colyte treated, **Figure 1A**). Average weight of female mice on Colyte increased from 5.50 ± 0.35 g to 16.65 ± 0.59 g (n = 9), and the average weight of female mice fed genistein diet increased from 6.01 ± 0.37 g to 17.77 ± 0.35 g (n = 15). While average weight of females on genistein was significantly greater than females on Colyte from 30-55 days of age, genistein diet did not change final body weight of females (**Figure 1B**).

Mice homozygous for the ΔF508 mutation (CF mice) are characterized by severe intestinal disease and therefore require constant laxative treatment for survival. We therefore tested whether dietary genistein would reduce the dependence of the ΔF508 CF mouse model for laxative treatment for survival. Survival rates for males were as follows: fed standard diet (38%, 8/21), fed standard diet plus Colyte (83%, 35/42) or fed genistein diet (60%, 9/15). Thus, for male ΔF508-CF mice, genistein diet did not improve survival rate compared to those on Colyte (**Figure 2A**). Survival rates for

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females were as follows: fed standard diet (47%, 9/19), fed standard diet plus Colyte (71%, 27/38), or fed genistein diet (87%, 13/15). Thus, genistein diet significantly increased survival rate of female ΔF508 mice compared to the other diet groups (**Figure 2B**).

Jejunum morphology

In theory, modifications in goblet cell numbers and presumably mucin production could have effects on intestinal luminal fluidity and impaction. We predicted genistein diet could have beneficial effects i.e. reduce goblet cell number, which would contribute towards the lack of impaction of genistein fed mice. Histological sections were stained using Alcian Blue/PAS and analyzed for crypt depth and villi length and the numbers of goblet cells. There was no change in villi length among the female groups (F-Std = 339.10 ± 29.60 μm, F-Col = 382.70 ± 40.38 μm, F-Gen = 405.93 ± 21.01 μm, n = 6/group), and no change between the male groups (M-Std = 314.50 ± 9.80 μm, M-Col = 380.26 ± 9.80 μm, M-Gen = 347.73 ± 20.54 μm, n = 6/group). The total number of goblet cells per villus was comparable between the female groups (F-Std = 8.83 ± 1.44, F-Col = 6.48 ± 1.19, F-Gen = 8.19 ± 0.81, n = 6/group). In males, the total number of goblet cells significantly increased 1.37-fold ($P < 0.05$) with genistein (M-Std = 10.83 ± 1.14, M-Col = 11.63 ± 0.66, M-Gen = 15.98 ± 1.50, n = 6/group). The normalized data, expressing the number of goblet cells/μm of villus length is shown in **Figure 3B**. The number of goblet cells per μm villus length was comparable between the female groups and in males, the total number of goblet cells/ μm villus length significantly increased 1.5-fold ($P < 0.05$) with genistein.

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There was a genistein-mediated 1.28-fold significant increase ($P<0.05$) in crypt depth in females (F-Std = 101.03 ± 5.05 µm, F-Col = 106.50 ± 6.26 µm, F-Gen = 135.85 ± 10.80 µm, n = 6/group), and no change between the male groups (M-Std = 129.86 ± 13.54 µm, M-Col = 98.46 ± 5.82 µm, M-Gen = 138.39 ± 24.18 µm, n = 6/group). The total number of goblet cells per crypt was comparable between the female groups (F-Std = 4.97 ± 0.12, F-Col = 4.68 ± 0.51, F-Gen = 4.47 ± 0.68, n = 6/group), and between the male groups (M-Std = 5.07 ± 1.62, M-Col = 3.50 ± 0.23, M-Gen = 4.22 ± 0.43, n = 6/group). The normalized data, expressing the number of goblet cells/µm of crypt depth is shown in **Figure 3C**. In females, there were 27% less goblet cells per µm crypt length in the genistein-fed group ($P < 0.05$), whereas no difference was noted between the male groups.

Expression of key proteins involved in absorption across jejunum

Small intestinal absorption across the villi intestinal epithelial membrane requires the activity of following; sodium-coupled glucose and galactose transport (mediated by SGLT-1), facilitated fructose transport (mediated by GLUT5) across the luminal membrane, along with the facilitated transport of all monosaccharides across the basolateral membrane (mediated via GLUT2). Interestingly, we show that dietary genistein significantly increased expression of SGLT-1 (2-fold) in female mice, but was without effect in males (**Figure 4A**). Our results demonstrate that total protein expression of GLUT5 and GLUT2 was unchanged by genistein-diet for both male and female CF mice (**Figure 4B,C**).

DISCUSSION

This study provides the first evidence that chronic dietary consumption of genistein (600 mg genistein/kg diet), for a period of 4-weeks, increases survival in female ΔF508-CF mice and increases body weight of male ΔF508-CF mice. We conclude that the increased survival rates of female ΔF508-CF mice following 4 weeks on a genistein-rich diet is attributed to at least the following mechanisms; (1) Significantly less goblet cells/crypt depth within jejunum. (2) Significantly increased levels of jejunum SGLT-1 expression.

Genistein, a naturally occurring flavonoid, is found naturally in soy and plants and thus digested in an average daily diet. Previous studies have suggested that genistein concentrations in plasma with soy-rich diets can reach micromolar levels [30]. Indeed, serum concentrations of genistein in the low micromolar range have been obtained in rats and mice after consumption of genistein-containing diets [31, 32] resulting in functional changes in tissues: improved basal transepithelial chloride secretion across freshly excised jejunum in wild-type female mice [23] and improved basal transepithelial chloride secretion across freshly excised jejunum from *ob/ob* female and male mice [28].

For increased survivability ΔF508-CF mice are routinely placed on laxatives [8]. In the current study, survivability for male and female ΔF508-CF mice over a period of 45 days post weaning, fed normal standard diet without laxative treatment resulted in low survivability rates; 38% survival for males and 47% survival for females (**Figure 2**).

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When subgroups of mice were provided normal standard diet with addition of Colyte to drinking water, survivability increased in males to 83% (an improvement of 2.18-fold compared to without Colyte) and in females to 71% (an improvement of 1.51-fold compared to without Colyte). Consuming a genistein-containing diet (in the absence of Colyte) only increased male survivability to 60% (a 1.57-fold increase compared to without Colyte), thus, not as beneficial as Colyte. On the other hand, female mice fed a genistein diet surpassed their male counterpart survivability rates, and even that of either sex on a laxative diet, with a survivability of 87% (a 1.85-fold increase compared to without Colyte). Thus, genistein diet generates sex-dependent improvements in survival rates of females compared to males.

This study provides several notable pieces of evidence supporting the beneficial role of genistein diet on CF mice. (1) Increased weight in CF male mice: It is interesting that consumption of the genistein-diet results in a significantly greater increase in weight gain for the males (12.9 g gained on Colyte, compared to 15.4 g gained on genistein diet). While genistein diet increased weight gain at earlier time points in female mice, this treatment diet had no effect on the final weight gain in females. We acknowledge that increased weight gain in males is in the absence of positive effects on survival rate. Genistein-mediated effects on muscle weight have previously been reported in male mice; Hirasaka *et al* [33] demonstrated that soy isoflavones (genistein and daidzein) significantly increased weight of gastrocnemius muscle (mediated via inhibition of the ERK signaling pathway) in mice after a period of 3-weeks. Thus, it is possible that the CF mice in this study, gained muscle mass resulting in increased total body weight and future studies will address this. (2) Increased SGLT-1 expression in CF female mice:

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Genistein's remarkable effect on SGLT-1 expression in females is perhaps not surprising; regulation of SGLT-1 expression by PKA and PKC has been demonstrated in intestinal tissue [34] and Epidermal Growth Factor has been shown to have stimulatory effects on PKC-regulated glucose absorption [35]. Moreover, genistein treatment can activate the PKC pathway [36]. Whether, or not, genistein stimulates an increase in expression of SGLT-1 via a PKC-mediated mechanism in female CF mice, remains to be elucidated. (3) Decreased goblet cell numbers in CF female mice: reduced goblet cell numbers and thus reduction in mucus production would feasibly contribute toward the lack of impaction and increased survivability of the females fed genistein diet. Exposure of mouse epithelial cells to genistein has been shown to significantly reduce Muc-1 expression [37], and whether or not the loss of goblet cell numbers in our study translates to a reduction in Muc-1 remains to be seen.

Interestingly, sex-based differences have been documented in patients with cystic fibrosis (CF). Females with CF have a significantly higher mortality than males, resulting in a ~4 year difference in the median age of survival [38, 39]. While the reason for this disparity in survival age is presently unknown, our studies indicating a genistein-mediated increase in survival have relevance to the CF female clinical population.

This study provides the first evidence that increased consumption of dietary genistein, a naturally occurring isoflavone, increases survival of female ΔF508-CF mice and increases weight gain in males. The cellular mechanism(s) underlying the genistein-mediated increased growth in ΔF508-CF males are currently unknown. The factors involved for the differential responses of ΔF508-CF males and females to the diet are also unclear. More interestingly, and of particular clinical relevance, we

demonstrate the use of a naturally occurring dietary supplement to improve survival of the CF mouse in the absence of laxatives. These studies provide a basis for the potential adjunct therapeutic use of dietary genistein in CF.

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FIGURE LEGENDS

Figure 1. Effect of dietary genistein on body weight of ΔF508-CF mice. Body weight was measured weekly from weaning (aged 21 days) and monitored up to age 65 days. **A.** Weight of males on Colyte (open squares, n = 8) and genistein diet (solid squares, n = 14). **B.** Weight of females on Colyte (open squares, n = 9) and genistein diet (solid squares, n = 1). Data are mean ± SEM. * indicates significant genistein-mediated difference, $P < 0.05$.

Figure 2. Effect of dietary genistein on survival rates of ΔF508-CF mice. Differing diets were started at weaning (aged 21 days), monitored up to age 65 days. **A**. The percentage of males surviving with each diet were; standard diet (38%, 8/21), colyte diet (83%,35/42) and genistein diet (60%, 9/15). **B.** The percentage of females surviving with each diet were; standard diet (47%, 9/19), colyte diet (71%, 27/38), and genistein diet (87%, 13/15). Data are mean ± SEM. # indicates significant genistein-mediated difference compared to controls, $P < 0.05$.

Figure 3. Effect of dietary genistein on jejunum morphology of ΔF508-CF mice. A. Representative alcian blue stained section from jejunum. **B.** Average number of goblet cells normalized per micrometer of villi length (n = 6/group). **C.** Average number of goblet cells normalized per micrometer of crypt depth (n = 6/group). Note: Std = standard diet, Col = Colyte treated, and Gen = Genistein diet. Data are mean ± SEM. # indicates significant genistein-mediated difference compared to colyte-treated, $P < 0.05$.

Figure 4. Effect of dietary genistein on total expression of SGLT-1, GLUT2 and GLUT5 in jejunum of ΔF508-CF mice. A. Typical western blot demonstrating SGLT-1 expression (normalized to GAPDH) in jejunum from *CF* mice. Average SGLT-1/GAPDH ratio comparing regular standard diet (open bars), standard diet + Colyte (gray bars) and genistein diet (solid bars). n = 4-7/group. **B.** Typical western blot demonstrating GLUT2 expression (normalized to GAPDH) in jejunum from *CF* mice. Average GLUT2/GAPDH ratio comparing regular standard diet (open bars), standard diet + Colyte (gray bars) and genistein diet (solid bars). n = 4-8/group. **C.** Typical western blot demonstrating GLUT5 expression (normalized to GAPDH) in jejunum from *CF* mice. Average GLUT5/GAPDH ratio comparing regular standard diet (open bars), standard diet + Colyte (gray bars) and genistein diet (solid bars). n = 4-8/group Values are means ± SEM. $^{\#}$ denotes statistical genistein-mediated effect, $P < 0.05$.

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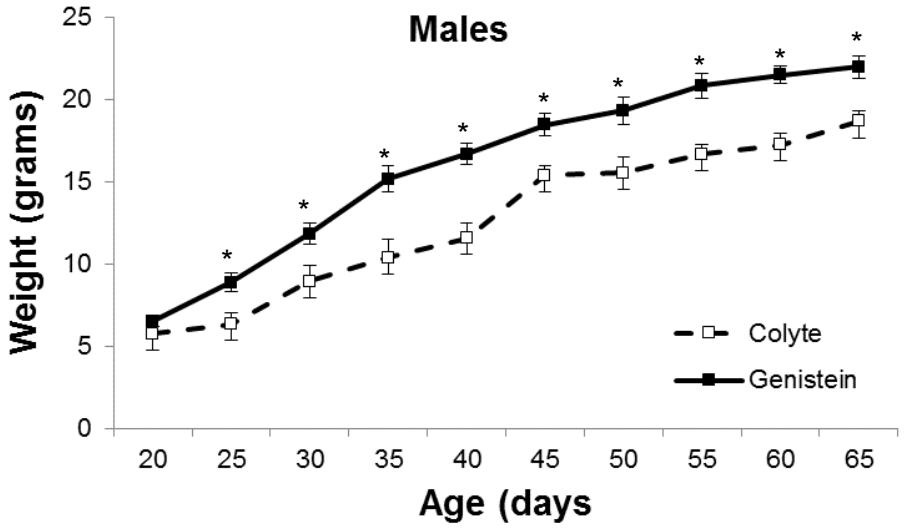
Figure 1

A



B

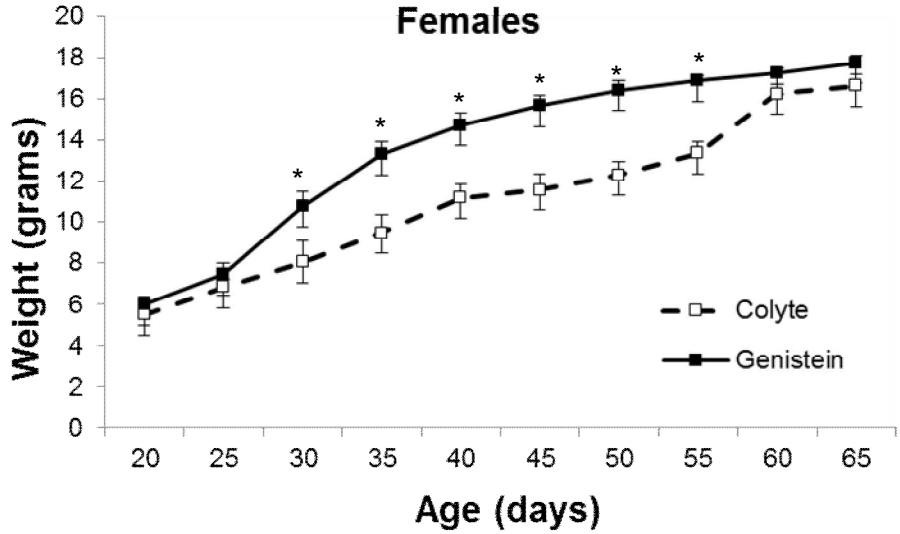


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Figure 2

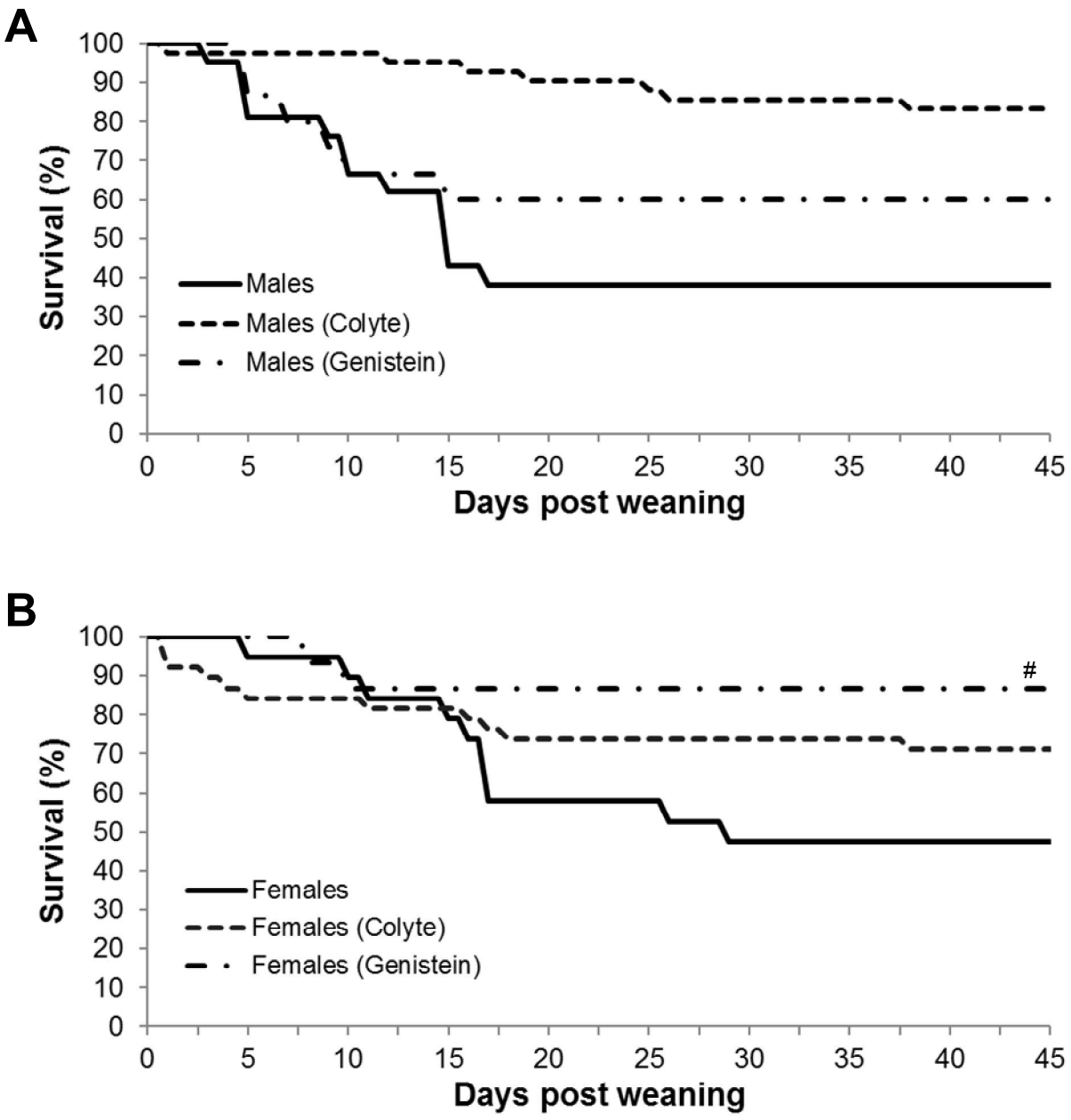

Figure 3

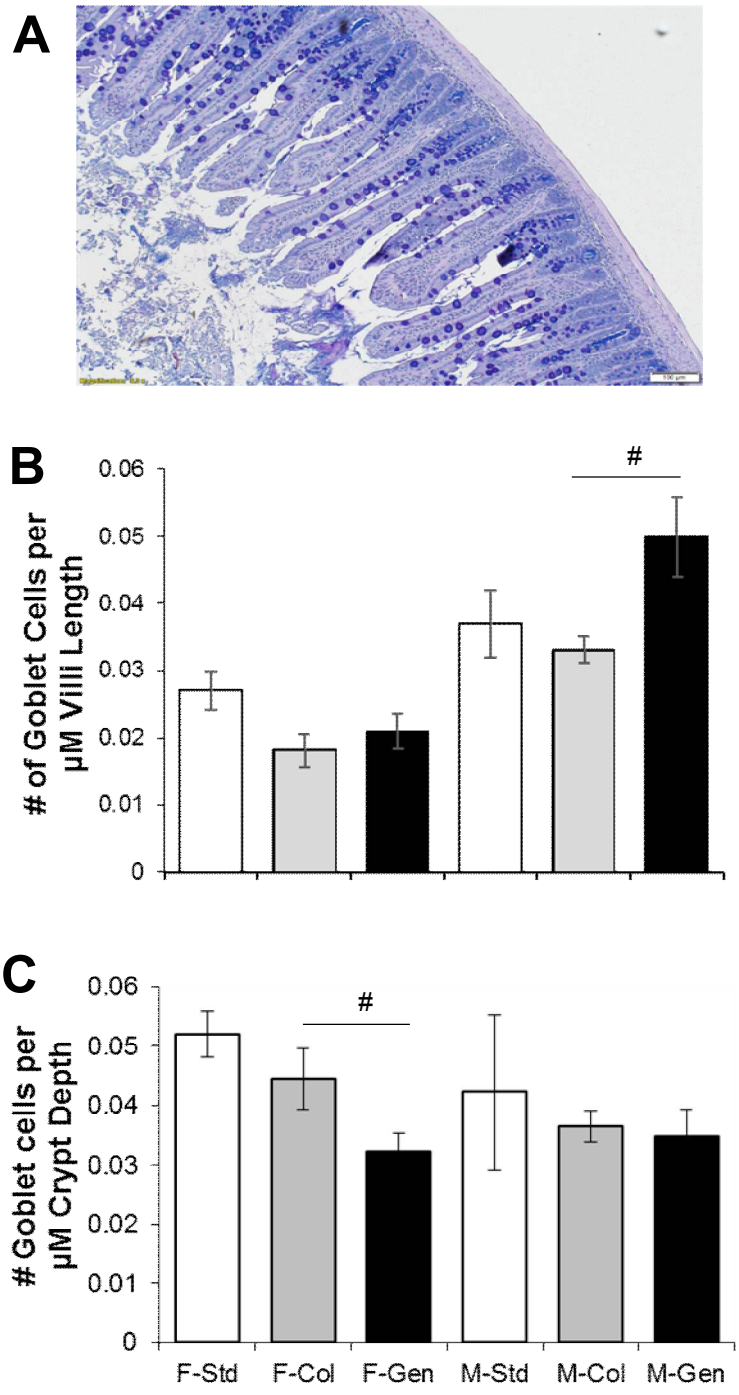

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Figure 4