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Dietary Phytoestrogens Inhibit Experimental Aneurysm Formation in Male Mice

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Abstract

Objective—The purpose of these experiments was to test the hypothesis that dietary phytoestrogens would diminish experimental aortic aneurysm formation.

Materials and methods—Six-week old C57BL/6 mice were divided into groups, fed either a diet with minimal phytoestrogen content or a regular commercial rodent diet with high phytoestrogen content for two weeks. At the eight week, aortic aneurysms were induced by infusing the isolated infra-renal abdominal aorta with 0.4% elastase for five minutes. Mice were recovered and the diameter of the infused aorta was measured at post-operative days 3, 7 and 14. Abdominal aorta samples were collected for histology, cytokine array and gelatin zymography after aortic diameter measurement. Blood samples were also collected to determine serum phytoestrogens and estradiol levels. Multiple-group comparisons were done using ANOVA with post hoc Tukey tests.

Results—Compared to mice on minimal phytoestrogen diet, mice on regular rodent diet had higher levels of serum phytoestrogens (male 1138±846 ng/dL, female 310±295 ng/dL). These serum phytoestrogen levels were also much higher than their own endogenous estradiol level (109 fold higher for males, and 35.5 fold higher for females). While aortic diameters of female mice were unaffected by the phytoestrogen concentration in the diets, male mice on regular rodent diet (M+ group) developed smaller aortic aneurysms than male mice on minimal phytoestrogen diet (M– group) on post-operative day 14 (M+ 54.8±8.8% versus M– 109.3±37.6%, P<0.001). During aneurysm development (post-operative days 3 and 7), there were fewer neutrophils, macrophages and lymphocytes in the aorta from the M+ group than from the M– group. Concentrations of multiple pro-inflammatory cytokines (MMPs, IL-1β, IL-6, IL-17, IL-23, MCP-1, RANTES, INF-γ, TNF-α) from aortas of M+ group were also lower than those from the aortas of M– group. Zymography also demonstrated that M+ group had lower levels of aortic MMP-9s than M– group on post-operative day 14 (P<0.001 for pro-MMP-9, P<0.001 for active MMP-9).

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Disclosures

None

Conclusions—These results suggest that dietary phytoestrogens inhibit experimental aortic aneurysm formation in male mice via a reduction of the inflammatory response in the aorta wall. The protective effect of dietary phytoestrogens on aneurysm formation warrants further investigation.

Keywords

phytoestrogen; aortic aneurysm; mouse AAA model; aneurysm phenotype; inflammatory cytokine; MMP

Introduction

Abdominal aortic aneurysms (AAAs) are a gender-related disease with a prevalence of male to female ratio 4:1. Estrogens play a protective role in AAAs development (1–3). Phytoestrogens are plant-derived chemicals that are strikingly similar to estrogens both in structure and function. Therefore, the potential benefits and risks of phytoestrogen exposure have already attracted much attention (4–9). Major sources of phytoestrogens include soybeans, alfalfa, and flaxseed. Phytoestrogens are selective estrogen receptor modulators (SERMs) and have anti-inflammatory, anti-oxidant, and anti-proliferative properties (10–14). Animal experiments have demonstrated that phytoestrogens can reduce plasma cholesterol and attenuate atherosclerosis (15–17). However, little is known regarding phytoestrogens' effects on aortic aneurysm formation. Therefore, we hypothesized that dietary supplementation with phytoestrogens might reduce inflammation in the aortic wall and thus inhibit aneurysm formation in an experimental model.

Materials and methods

1. Experimental design

Experiment 1 (Gender-based study): Thirty-two (n=16 male, 16 female) 6-week old wild type C57BL/6 mice (Jackson Lab, Bar Harbor, Maine) were divided into four groups of 8 mice based on dietary phytoestrogen exposure to determine the influence of phytoestrogen content on aortic aneurysm formation. Thus, four groups were evaluated: (1) male mice fed with minimal phytoestrogen diet (M–), (2) male mice fed regular diet (M+), (3) female mice fed minimal phytoestrogen diet (F–), and (4) female mice fed regular diet (F+). The isoflavone content, one of the major classes of phytoestrogens, ranged from non-detectable to 20 mg/kg for minimal phytoestrogen diet (2016 Teklad Global 16% Protein Rodent Diet), while the regular diet (7012 Teklad LM485 Mouse/Rat Diet) had between 300 to 500 mg/kg. The other ingredients in the both diets were similar (see www.harlan.com). Both rodent diets were commercially available and obtained on April 2011. Two weeks after mice were placed on the diets, AAAs were induced surgically (18–19).

Briefly, infra-renal abdominal aorta was isolated and infused in situ with porcine pancreatic elastase (0.4 u/ml, Sigma) for 5 minutes at a pressure of 100 mmHg. Elastase solution was evacuated and the mice were allowed to recover. Mice abdominal aortic diameters (n=8/group) were measured immediately after infusion to ensure similar dilation. On post-operative day 14, the infra-renal abdominal aorta was dissected and maximal aortic diameter was measured using video microscopy with NIS-Elements D.3.10 software attached to the microscope (Nikon SMX-800, Melville, NY). Aortic dilation was determined using $(\text{maximal aortic diameter} - \text{internal control diameter}) / \text{maximal aortic diameter} \times 100\%$. The internal control diameter was the diameter of un-infused infra-renal aorta just above the infused section. A dilation of 50% or more was considered to be positive for AAA formation. All measurements were performed while the animal was alive. Blood samples were collected immediately after aorta diameter measurement. Aorta samples were

harvested for protein analysis (n=5/group) and histological studies (n=3/group). Serum samples were used to determine phytoestrogens and estradiol levels.

Experiment 2 (Time course study): Six-week-old wild type C57BL/6 male mice (n=24) were divided into 2 groups based on diet. As above, one group was fed with the minimal phytoestrogen 2016 diet, while the other group was maintained on the phytoestrogen-rich 7012 diet. At 8 weeks of age, all mice were underwent elastase infusion. To evaluate AAA formation at earlier time points, six mice in each group were evaluated at post-operative day 3 and 7. After aortic diameters were measured (n=6/group), samples were collected for histology (n=3/group) and protein analysis (n=3/group).

All experiments were conducted in accordance with standards approved by the Animal Care and Use Committee of University of Virginia (IACUC #3848).

2. Mass spectrometry

Mass spectrometry was performed on serum samples to identify and quantify four common phytoestrogens in serum. Four standards were used in the test (Sigma, St Louis, MO. Purity: diadzein 97%, genistein 98%, equol 99% and coumestrol 95%). The test used a LC-MS system, which consisted of a Thermo Electron TSQ Quantum Access MAX mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75µm id Phenomenex Jupiter 10 µm C18 reversed-phase capillary column. Samples were prepared as published (20). Dry samples were re-suspended using 5% ethanol/water to a volume equal to 25% of the plasma volume processed. Samples were vortexed and spun at 14 K rpm. A 5µL aliquot of each extract was injected and four compounds eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.5µL/min over 0.5 hour. Nanospray ion source was operated at -3 kV (negative ions). Data was analyzed using the peak area (MPM) for each compound in each sample and comparing it to areas obtained for standard curves generated at the start and end of the sample set. The limit of detection (LOD) and limit of quantification (LOQ) was 0.5 fmol/ml and 5 fmol/ml, respectively.

3. Radioimmunoassay

To determine circulating estradiol concentration in the serum, a 200 µl aliquot of each serum sample was used for radioimmunoassay with commercial kit from Siemens Healthcare Diagnostic INC. (Tarrytown, NY). TKE21 kit was used to determine the serum estradiol levels according to the manufacturer's instructions at the Ligand Assay and Analysis Core Facility at the University of Virginia Center for Research in Reproduction.

4. Histology and immunohistochemical stainings

Aortic specimens were fixed in 4% buffered formaldehyde for 24 hours, transferred to 70% ethanol, and subsequently embedded in paraffin. Aortic cross-sections were stained with Verhoeff-Van Gieson and picosirius red staining for elastin and collagen, respectively. Immunohistochemical stainings for neutrophils, migratory macrophages, CD3⁺ T cells and smooth muscle actin (αSMA) were performed as published (21–23). For grading, integrated optical density value of positive staining area of each section was randomly selected and measured. Four randomly selected 20× fields per aneurysm section were assessed for the density of staining by blinded observers.

5. Angiogenesis and cytokines arrays

Protein from aortic samples were isolated and pooled protein (30 µg) from each group was used in proteome profiler mouse cytokine array and proteome profiler mouse angiogenesis array according to manufacturer's instruction (R & D Systems, cat# ARY 006, ARY 015). All samples were run in duplicate and mean value was used to represent each cytokine level.

6. Zymography

Using aortic protein isolated from the samples, gelatin zymography was performed to determine the amounts of MMP-2 and MMP-9. Precast zymography gels (Invitrogen) were loaded with 3µg of tissue protein from each aortic sample diluted into 2× Tris-glycine SDS sample buffer and electrophoretically separated under non-reducing conditions. The gels were renatured for 30 min in renaturing buffer (Invitrogen) and incubated in developing buffer (Invitrogen) for 24 hours at 37 °C rocker. Then, the gels were stained in Simply Blue Safe Stain (Invitrogen). Pro and active forms of MMP-9 and MMP-2 appeared as clear band against the blue background. Quantification was determined according the optical density using Bio-Rad Image Lab Software version 4.0.

7. Statistical analysis

The overall difference among groups, together with gender and diet factor were determined using two-way ANOVA. Post hoc Tukey's test were used for multiple comparisons between groups. (PRISM 5, GraphPad Software, La Jolla, CA). Data are presented as mean ± standard deviation. P value less than 0.05 was considered statistically significant.

Results

1. Reduced AAA diameter in male mice on phytoestrogen-rich diet

In minimal phytoestrogen diet groups, aortic dilation was significant greater in male mice compared to females (M− 109.3 ± 37.6% versus F− 52.9 ± 11.5%, $P<0.001$); In phytoestrogen-rich diet groups, this difference was negated. The aortic dilation of male mice was similar to that of female mice (M+ 54.8 ± 8.8% versus F+ 67.1 ± 17.4%, $P=0.68$). Comparing the two male groups on different diets, mice on phytoestrogen-rich diet had significant smaller aortic dilation than male mice on minimal phytoestrogen diet (M+ 54.8 ± 8.8% versus M− 109.3 ± 37.6%, $P<0.001$). M+ group also had decreased incidence of AAA (75%) compared with M− group (100%). There were no statistical differences between two female groups either in aortic dilation or in AAA incidence (Figure 1A, 1B). Aortic dilation increased over time following elastase perfusion. Aortic dilations were similar in male mice fed minimal phytoestrogen diet or phytoestrogen rich diet at days 3 and 7. Between days 7 and 14, dilation progressed in male mice fed minimal phytoestrogen diet, while further dilation was halted in male mice fed phytoestrogen rich diet (Figure 2).

2. Significant higher level of serum phytoestrogens in mice on phytoestrogen-rich diet

At post-operative day 14, phytoestrogen levels were non-detectable in both groups of mice on minimal phytoestrogen diet (M− and F−), while the other two groups on phytoestrogen-rich diet had much higher levels of total phytoestrogen in their sera (M+ 1138±846 ng/dL, F+ 310±295 ng/dL). The predominant phytoestrogen was equol (M+ 87.3%, F+ 65.5%). The total serum phytoestrogen levels of M+ and F+ groups were significantly higher than their own endogenous estradiol levels, respectively (M+ 109 fold, F+ 35.5 fold). (Table 1)

3. Decreased inflammatory cell infiltration in the early stages of AAA formation in male mice on phytoestrogen-rich diet

Compared with M− group, male aortas from mice fed phytoestrogen rich diet (M+) had fewer lymphocytes on post-operative day 7 ($P=0.02$). The phytoestrogen rich diet group also had fewer neutrophils on post-operative day 7 and fewer macrophages on post-operative day 3 (Figure 3A, 3B). By post-operative day 14, aorta samples of M− group demonstrated obvious elastin fragmentation and disorganization, increased collagen deposition in the media and adventitia of aorta; while the elastin and collagen contents in M+ group were relatively intact, similar to the female groups (Figure 4 A, B). Male groups still contained

more inflammatory cells in the aorta compare with the female groups on post-operative day 14. However, no diet-related differences in inflammatory cell staining were found at this time point (Figure 4 C, D, E). There were no differences in smooth muscle actin- α staining among groups.

4. Lower inflammatory cytokine levels in the aorta of male mice on phytoestrogen-rich diet

Several dozen cytokines of interest and MMPs were analyzed in the study. According to the arrays result, the following matrix metalloproteinases and cytokines were found more closely related with aneurysm formation in this animal model: Matrix metalloproteinases-9 (MMP-9), tissue inhibitors of metalloproteinases-1 (TIMP-1), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-17 (IL-17), interleukin-23 (IL-23), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor- α (TNF- α) and interferon- γ (INF- γ) were all altered. The array analysis indicated that most of the above MMPs and inflammatory cytokines were up-regulated in M- group at post-operative day 3, 7 or 14, while M+ group had relatively lower levels of the above proteins in their aortas (Table 2, Figure 5 and Figure 6).

5. Less MMP-9s in the aorta of male mice on phytoestrogen-rich diet

MMPs are known to be critical in blood vessel matrix degradation and AAA formation. Therefore, gelatin zymography was used to further evaluate MMPs activities. On post-operative day 14, the male group fed phytoestrogen rich diet (M+) had much lower MMP-9s levels in their aortas than male group fed minimal phytoestrogen diet (M-) ($P < 0.001$ for pro-MMP-9, $P < 0.001$ for active MMP-9). Within the two groups of mice fed minimal phytoestrogen diet (F- versus M-), the female group also had less MMP-9s compared with the male group ($P = 0.001$ for pro-MMP-9, $P < 0.001$ for active-MMP-9) (Figure 7). MMP-2s had similar trends at post-operative day 14 as MMP-9s, but the differences were not significant. The present study found no differences in MMPs levels among all groups on post-operative day 3 and 7 (data not shown).

Discussion

In the present study, we tested four phytoestrogens that are most commonly found in a commercial rodent diet. Our study showed that mice on the regular commercial rodent diet had much higher levels of serum phytoestrogens than mice on the minimal phytoestrogen diet. The phytoestrogens in our study were mainly in the form of equol (87.3 % in male, 65.5% in female). Equol is the metabolite of daidzein with a longer half life and greater biological activity. We also tested endogenous serum estradiol levels in order to compare it with phytoestrogen levels in the blood. Our results showed that the total phytoestrogen levels in mice on the regular commercial rodent diet were alarmingly higher than their own endogenous estradiol levels (109 fold for male, 35.5 fold for female). Phytoestrogens come mainly from soybean in the diet. Phytoestrogen concentration in soybean varies from harvest to harvest, so does the phytoestrogen content in each batch of rodent diet. Unfortunately, not all investigators doing experimental AAA studies are aware of the influence of phytoestrogens on AAA formation. Our study demonstrated that it is critical to use minimal phytoestrogen diet in experimental AAA investigations.

Gender-related differences in aneurysm formation have been well documented in both human and animal studies. Male gender is a well-known risk factor for AAA development (24–26). Experiments with rodent AAA models have demonstrated that male animals develop a positive AAA phenotype compared with female animals (19, 27–28). In the present study, gender-related differences in AAA phenotype between the two groups of mice

on minimal phytoestrogen diet (M- versus F- group) were clearly demonstrated ($109.3 \pm 37.6\%$ versus $52.9 \pm 11.5\%$, $P < 0.001$). However, gender-related differences were obviated with phytoestrogen rich diets, as both male and female mice had similar aortic dilation. Both male and female mice on phytoestrogen rich diet had decreased aortic dilation in comparison with male mice on the minimal phytoestrogen diet. Male mice on phytoestrogen-rich diet (M + group) had a much lower aortic dilation than male mice on minimal phytoestrogen diet (M - group) ($54.8 \pm 8.8\%$ versus $109.3 \pm 37.6\%$, $P < 0.001$), while the aortic dilation of both female groups were low and similar. These results suggested that the phytoestrogen-rich diet blunted the aortic dilation that is normally seen in the male mice in the gender-related AAA study. Since female mice are protected against AAA formation, it is suspected that phytoestrogens would not have a further impact on AAA protection.

Statistic analysis showed that the interaction of gender and diet factor accounted for 31.33% of the total aortic dilation variance ($F = 19.56$, $P = 0.0001$). This interaction indicates that the male and female mice had different responses to the same diet factor. The gender factor in this experiment accounted for approximately 12.97% of the total variance in aortic dilation ($F = 8.094$, $P = 0.0082$). The effect is considered very significant. The diet factor in the experiment accounted for about 10.83% of the total variance ($F = 6.760$, $P = 0.0147$) and the effect is considered significant.

When evaluating aneurysm formation over time, our results showed that phytoestrogen-rich diet did not make a significant difference in aortic dilation during the early stages of AAA (post-operative day 3 and 7). Male mice on minimal phytoestrogen diet gained much of their aorta dilation during the second week ($P = 0.012$), which is one of the characteristics of the elastase-induced model (18). On the other hand, the aortic diameter of male mice on phytoestrogen-rich diet remained stable over the second half of the experimental model ($P = 0.999$), indicating the importance of diet. Diet factor in the time course experiment accounted for approximately 4.409% of all the variance ($F = 22.21$, $P < 0.0001$). However, the above results do not mean that dietary phytoestrogens affect only the later stage of aneurysm formation. Aorta dilation in the early stages of the model reflect mainly the mechanical pressure and elastase digestion on the blood vessel; while the later stages of the AAA model include aortic wall degradation caused by inflammation, which mimics the human aneurysm formation. The logical implication of this observation was that dietary phytoestrogens might reduce aneurysm formation through anti-inflammation properties.

In the elastase induced AAA model, the inflammation in aorta wall usually peaks around 5–7 days after surgery, then elastin and collagen disruption and remodeling of the aorta occurs. Our histology demonstrated that there was more inflammatory cell infiltration in the aortas of male mice on minimal phytoestrogen diet on post-operative day 7 compared to male mice fed a diet rich in phytoestrogens. On post-operative day 14, the histology showed increased elastin fragmentation and collagen deposition in the animals on minimal phytoestrogen diet. In contrast, male mice on the phytoestrogen-rich diet experienced less inflammation during the early stage of this model, indicating phytoestrogen's anti-inflammatory effects. Also in this model, aortic dilation usually peaks around post-operative day 14, then, the aorta begins to heal. In the present study, there were still more inflammatory cells left in male samples than in female samples on post-operative day 14. Endogenous estrogen is believed to account for this gender-related difference. We are not sure why there were no diet-related differences at this time point, but hypothesized that the anti-inflammatory effects of phytoestrogen may be detected more easily at the peak of inflammation. By day 14, the acute injury is basically resolving, so gender-related differences at this particular point might have more influence on the recovery process, not in the formation of AAA.

To explore the mechanisms behind diet-related phenotypic differences, multiple MMPs and cytokines in aortic samples were examined. Although the function of each of these pro-inflammatory molecules in aneurysm formation have been studied extensively (27–37), little is known regarding how these inflammatory biomarkers in aorta are regulated in the presence of dietary phytoestrogens. In our study, each of these inflammatory cytokines in male mice on minimal phytoestrogen diet (M–) was up-regulated at different time points. These findings demonstrate that phytoestrogens impact AAA formation process through not one, but multiple cytokine pathways.

Of all the MMPs and cytokines evaluated in the present study, the most interesting ones were MMP-9/TIMP-1 pair. First, the results demonstrated that the rodent diets in the study have a significant impact on MMPs levels in the aorta. For pro-MMP9, diet factors accounted for approximately 22.55% of the total variance ($F=9.90$, $P=0.0071$), which was considered very significant. The diet factor for active-MMP9 was about 30.35% of the total variance ($F=19.89$, $P=0.0005$, extremely significant). The diet factor of pro-MMP2 and active-MMP2 was 24.6% ($F=5.66$, $P=0.0321$, significant) and 17.73% ($F=3.75$, $P=0.0734$, not significant), respectively. Second, on post-operative day 14, male mice on minimal phytoestrogen diet (M–) had significantly higher levels of MMP-9 activity, but the increase of TIMP-1 was not in proportion to the increase in MMP-9. This change in MMP/TIMP-1 ratio was consistent with the aortic remodeling time frame and the positive aneurysm phenotype of M– group. Considering MMPs' critical role in matrix degradation, the high level of MMP-9 in the second week may play an important role in the aorta dilation in M– group.

The anti-inflammatory mechanisms of phytoestrogens are complicated. It could be estrogen receptor(ER)-dependent or ER-independent, or both. Phytoestrogens have the ability to bind both ER- α and ER- β , but with a preference for ER- β . These exogenous phytoestrogens may compete with endogenous estrogens on the same ERs. Although phytoestrogens' affinities for ERs are weak, they can be present in large amounts in the blood. Depending on their concentrations, they can elicit estrogenic or anti-estrogenic effects (38–40). The ER-independent anti-inflammatory effect probably relies on phytoestrogen's antioxidant, anti-proliferative and anti-angiogenic properties. Although the present study demonstrated that phytoestrogens reduced the inflammation in the aorta of male mice, the results did not indicate if this anti-inflammatory effect was ER dependent or ER-independent. Experiments with ER knock-out animals would be helpful to detail the mechanisms. Since MMPs play such an important role in AAA formation, using MMP-9 and TIMP-1 knockout mice in future experiments could also help us understand the relationship between phytoestrogen and MMPs. Other AAA animal models (e.g. angiotensin II model) would be useful to investigate the different mechanisms at play in a model with advanced inflammation.

In summary, the present study showed that dietary phytoestrogens inhibit male experimental aneurysm formation by reducing the inflammatory response in the aorta of male mice. Further studies should reveal more details of the mechanisms involved. Since phytoestrogen's effect may be tissue specific, their selectivity may provide beneficial effect for AAA prevention without the side effects of traditional hormone therapy. Further studies should examine the potential of dietary phytoestrogens for AAA risk reduction in humans.

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(A).

(B).

Figure 1.

(A). Comparison of infra-renal aorta diameter. On post-operative day 14, the dilation of male mice on minimal phytoestrogen diet (M-) was more obvious than the other groups (M+, F-, F+).

(B). Comparison of infra-renal abdominal aortic dilation on post-operative day 14. N=8 mice/group. Male mice on minimal phytoestrogen diet (M-) had larger dilation than those of other groups.

Figure 2.

Comparison of infrarenal abdominal aorta dilation of male mice on different diets. Male mice on phytoestrogen-rich diet (M+) failed to dilated during the 2nd week compared with male mice on minimal phytoestrogen diet (M-). D0-pre means day 0 before infusion. D0-post means day 0 after infusion. D 3,7 and 14 mean day 3, day 7 and day 14 after infusion, respectively.

(A):

(B):

Figure 3.

(A): Representative immunohistochemistry samples of post-operative day 3 & 7 demonstrating macrophage, neutrophil and lymphocyte infiltration in aorta. Macrophage and neutrophil were red, CD3⁺ T cell was brown in their respective staining. On postoperative day 3 and 7, there were more inflammatory cells in M- group than in M+ group (marked by arrows).

(B): Quantification of immunohistochemistry evaluating neutrophil, macrophage and T cell in aorta samples of post-operative day 3 & 7. N=3 mice/group. M- group had more T cells in aorta than M+ group. D3 and D7 mean post-operative day 3 and 7, respectively.

Figure 4. Representative samples of post-operative day 14, demonstrating elastin fragmentation (Van Gieson) and collagen disruption (Picrosirius red) in M- group (A-B). Quantification of immunohistochemistry evaluating neutrophils, macrophages, T-cells, and smooth muscle cells (C-F). N=3 mice/group.

Figure 5.

Cytokine array of mouse aorta samples of post-operative day 3 & 7. Three pooled samples per group. Multiple inflammatory cytokines were up-regulated in M- group compared with M+ group on the same experiment end point. D3,D7 mean post-operative day 3 and 7,respectively. M+ means male mice on phytoestrogen-rich diet; M- means male mice on minimal phytoestrogen diet.

Figure 6.

Cytokine array of post-operative day 14 mouse aorta samples. Five pooled samples/group. IL-6, IL-1 β , IL-23, MCP-1 and MMP-9 were up-regulated in male mice on minimal phytoestrogen diet (M-) compared with M+ group. There were also gender differences in the levels of multiple inflammatory cytokines.

Figure 7.

Gelatin zymography of post-operative day 14 samples (A) demonstrating significant higher level of both pro and active form of MMP-9s in male mice on minimal phytoestrogen diet (B–C). There were similar trends for pro and active form of MMP-2s.

Table 1

Serum phytoestrogens and endogenous estrogen

Group	Gender	Diet	Coumestrol	Diadzein	Genistein	Equol	Total phytoestrogen	Estradiol
F-	Female	7012 Teklad	ND	ND	ND	ND	ND	10.11±1.16
M-	Male	7012 Teklad	ND	ND	ND	ND	ND	11.74±0.77
F+	Female	2016 Teklad	ND	63±99	44±45	203±152	310±295	8.74±1.44
M+	Male	2016 Teklad	ND	85±66	60±40	993±794	1138±846	10.40±1.04

All units were ng/dL; ND= non-detectable; Eight mice per group. In mice on phytoestrogen-rich diet (2016 Teklad), the total serum phytoestrogen levels were much higher than their endogenous estradiol levels (109 fold in male and 35.5 fold in female).

Table 2

Up-regulated cytokine levels in M– group at different time points

Cytokine	Day 3	Day 7	Day 14
TNF- α	0.45	2.58	0.07
INF-r	0.29	5.63	0.17
IL-1 β	1.81	1.48	0.91
IL-6	0.09	1.70	4.15
IL-17	0.82	0.56	0.00
IL-23	0.40	4.36	0.89
MCP-1	0.39	2.36	0.46
RANTES	0.93	2.99	0.27
TIMP-1	0.13	0.66	0.18
MMP9 (pro & active)			7.97

Note: All numbers are the fold of increase compared with M+ group.

M– : male mice on minimal phytoestrogen diet.

M+: male mice on phytoestrogen-rich diet.