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Genistein and Genistein-Containing Dietary Supplements Accelerate the Early Stages of Cataractogenesis in the Male ICR/f Rat

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Abstract

Cataract-related loss of vision affects large numbers of people in today's aging populations and presents a healthcare burden to many nations. The role of dietary supplements within the lens is largely unknown, although benefits from dietary antioxidants are expected. In this study, the effects of genistein as its aglycone, a genistein-containing dietary supplement (Novasoy[®]200), and a genistein-containing food (soy protein isolate, PRO-FAM 932) on the development of lens opacity were examined in the hereditary cataractous ICR/f rat. These studies were carried out in a background diet of semi-purified, isoflavone-free AIN-76A with casein as its protein source. The amount of genistein for the experimental diets was standardized to its concentration (as genistein aglycone as well as simple and complex β -glucoside conjugates) in the soy protein isolate supplement. Also tested was a high-dose genistein diet containing an 11-fold higher amount of genistein aglycone. The composition of each diet was verified by reverse-phase HPLC and blood plasma isoflavone concentrations were determined by LC-tandem mass spectrometry. The development of opacity in each lens was monitored and digitally recorded using slit-lamp examination over the course of the study. Each of the genistein-containing diets caused a significantly more rapid development of fibrous opacification in the anterior cortical region and development of apparent water clefts or vacuoles in the posterior subcapsular region than the AIN-76A control diet; however, the establishment of dense lens opacification was not significantly different between each of the diets. There was also no significant difference observed

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between the low-dose and high-dose genistein aglycone groups. These data suggest that genistein-containing dietary supplements accelerate the early stages of cataractogenesis in the male ICR/f rat, with no dose-dependent effects.

Keywords

Lens; Cataract; ICR/f Rat; Antioxidant; Genistein; Soy; Dietary Supplement

Introduction

Since there are no recognized treatments to reverse lens cataracts once they have begun to form, it is important to assess intervention strategies that could prevent or delay lens cataract formation at an early stage of their development. To develop such strategies requires a deeper understanding of cataractogenesis. Mechanisms that have been associated with cataract formation include light-induced photo-oxidation (Davies and Truscott, 2001), oxidative stress (Vinson, 2006), and protein glycation secondary to uncontrolled diabetes (Sharma and Santhoskumar, 2009; Stevens, 1998). Pre-clinical and observational studies have suggested that supplementation with nutritional factors (vitamin C and the carotenoids, lutein and zeaxanthin) decreases the risk of cataracts (Arnal et al., 2009; Ribaya-Mercado and Blumberg, 2004). However, the results of intervention studies have not been consistent (Age-Related Eye Disease Study Research Group, 2001; Christen et al., 2008; Chylack et al., 2002; Ferrigno et al., 2005) and other dietary and environmental factors may contribute to the risk of lens cataracts. Murine models permit investigation of the development of genetically- and environmentally-induced cataract disease in a laboratory setting. One such model is the ICR/f rat, a hereditary model of cataract disease (Ihara, 1983). The ICR/f rat progressively develops lens cataracts through a continual process that leads to complete opacification within 10 weeks, making this rat a potential model of age-related vision loss (Ihara, 1983).

The use of dietary supplements containing polyphenols, which is widespread throughout the U.S. and many other countries, is believed to improve health and wellness (Barnes et al., 2008). In many countries, herbal materials derived from plants, rich in polyphenols (flavonoids, proanthocyanins, coumestanes, lignans and stilbenes), are commonly used in lieu of conventional medicines (Cassileth et al., 2001; Adlercreutz, 2007). Polyphenolic compounds have many biological activities, among which is the potential to act as antioxidants within the lens (Vinson, 2006), that may augment the effects of other lens antioxidants such as ascorbate, glutathione and uric acid. A polyphenol-rich extract from grape seeds (containing monomers and oligomers of flavanols) has been reported to slow the appearance of full cataracts in the ICR/f rat (Yamakoshi et al., 2002).

Soy protein products contain isoflavones, a class of polyphenols (Coward et al., 1993). Interestingly, soy isoflavones and their metabolites have weak estrogenic activity. The physiologic estrogen, 17 β -estradiol, prevented the formation of lens cataracts in ovariectomized rats treated with the carcinogen N-methylnitrosourea (Bigsby et al., 1999), suggesting that genistein could have a similar effect. Soy-derived isoflavone supplements are used by the everyday consumer in various forms; for example, as the genistein aglycone, as a concentrated extract of soy isoflavones containing genistein in its β -glucoside form (Novasoy[®]200), and as a low fat protein product (PRO-FAM 932 soy protein isolate, SPI) containing mixed isoflavone β -glucosides (Barnes et al., 1994). Based on previous studies examining the effects of polyphenols on cataract development, it was hypothesized that the addition of genistein and genistein-containing supplements within the diets of the ICR/f rat would slow or prevent the progression of cataract formation.

The present study was designed to carefully control the diet by using AIN-76A (a diet which lacks isoflavones and has casein as the sole protein source) so that each diet had an equal amount of genistein (in the form of its aglycone or of its β -glucoside conjugates). This design allowed the examination of genistein's role in the formation of cataracts while observing whether other factors (other isoflavones, as well as the protein fraction) in soy supplements contribute to cataractogenesis. A high-dose aglycone genistein diet was used to test for dose-dependent effects of genistein. The development of cataracts within the lenses of animals on the different diets was monitored using a slit-lamp examination technique.

Materials and Methods

Materials

Genistein, daidzein, dihydrodaidzein (DHD), equol, *O*-desmethylangolesin (*O*-DMA), glycitein, enterodiol, enterolactone, and chrysin standards were purchased from LC-laboratories (Woburn, MA). All HPLC solvents and reagents were purchased from Fisher Scientific Co. (Norcross, GA) and were of HPLC grade. Phenolphthalein β -glucuronide, 4-methylumbelliferone sulfate and β -glucuronidase/sulfatase from *Helix pomatia* was purchased from Aldrich-Sigma Chemical Co. (St. Louis, MO).

AIN-76A diet was purchased from Purina TestDiet (Richmond, IN) and was stored at 4°C prior to use. Three types of supplements were selected for this experiment. PRO-FAM 932 Soy Protein Isolate (SPI) and Novasoy®200, a dietary supplement of soy isoflavone concentrate, both of which were generously provided by Archer Daniels Midland Company (ADM, Decatur, IL). Genistein aglycone (>99% pure by reverse-phase HPLC) was generously provided by DSM (Heerlen, Netherlands).

Experimental diet preparation

Fully formulated AIN-76A contains 20% protein by weight solely as casein. In order to prepare the AIN-76A/SPI diet, casein as the protein source was fully replaced by the SPI. To ensure that the formulated diet would be isocaloric and isonitrogenous to the 20% casein in the control AIN-76A diet, 21.69 g of SPI was added per 100 g of AIN-76A (since SPI is 92.2% protein). From reverse-phase HPLC analysis, SPI contained 0.834 mg of genistein (as various conjugates) per 1 g of powder (Fig. 1, Table 1), resulting in 18.09 mg total genistein in 100 g of AIN-76A/SPI diet for a final concentration of 0.018%. Accordingly, the NovaSoy®200 and genistein aglycone were added to the casein-based AIN-76A to produce equivalent concentrations of genistein in each diet. An additional high-dose diet was prepared by adding genistein aglycone to AIN-76A for a final concentration of 0.2%. All diets were manufactured and pelleted by Purina TestDiet (Richmond, IN). Upon arrival and over the course of the study, diets were stored at 4°C, with samples taken periodically (stored at -80°C) for further analysis of compound stability.

Experimental design

ICR/f rats were obtained from Kiwa Laboratory Animals Co. (Wakayama, Japan). Three males and six females were inbred to establish the colony of ICR/f rats used in this study. All animal procedures were approved by the University of Alabama at Birmingham's Institutional Animal Care and Use Committee and complied with the 1996 Public Health Service Policy on Humane Care and Use of Laboratory Animals. At the time of breeding, animals were placed on an isoflavone-free AIN-76A diet to minimize premature fetal exposure to genistein or other isoflavones. Female and male ICR/f rats were caged together for 15 days, after which the pregnant females were separated and placed into individual cages in preparation for birth. Pups were maintained on the isoflavone-free AIN-76A diet until weaning at 24 days of age. At weaning, male pups were randomized and five animals

were placed on each of the five different diets. Since cataracts developed at different rates in the lens of the animals, each lens could itself be treated as a separate data point. Therefore, since there were five animals in the control group and each of the treatment groups, there were up to ten lenses in which to observe cataract formation.

Animals on the experimental diets were allowed to feed and drink ad libitum. Lens observations were carried out on a twice weekly basis from day 31 through day 56, three times weekly from day 56 through day 72, and daily from day 72 until the end of the study. Prior to beginning each examination, animals were weighed using an Ohaus CS 2000 compact series scale and their eyes were dilated with 2–3 drops of a 0.5% Accu-Tropicamide solution (Accutome, Malvern, PA). Each animal was then anesthetized at the time of lens examination using inhaled isoflurane and placed on the stage of a modified Nikon FS-2 slit-lamp apparatus fitted with a nose cone to maintain anesthesia. The examination was filmed using a Sony SCR-DVD405 digital camcorder. Each lens was filmed individually and the image file saved separately. After each session, all videos were backed-up onto a hard disk for storage and analysis. At the termination of the study, all animals had developed cataracts and were euthanized by inhaled isoflurane anesthesia and exsanguination. Blood plasma, aqueous humor and lens tissue samples were collected from each animal and stored at -80°C for subsequent analysis.

Cataract Scores—Videos collected throughout the study were analyzed to categorize each lens based on the scoring scheme indicated in Figure 2. Each video was visually analyzed and classified to the proper stage of development, at each time point of observations. The assessor was not blinded to the diet group. Stage 0 cataract was defined by having no overtly visible signs of opacity development. Stage 1 was designated by the appearance of a slight fibrous opacification in the lens cortex, with no changes to the posterior regions. The continuance of the fibrous opacification in the anterior cortical region, combined with the appearance of apparent water clefts in the posterior subcapsular region of the lens denoted stage 2. Visible dense opacification in the anterior and posterior cortical regions of the lens, causing the light reflexes of the slit lamp to form an “O”, was the marker that denoted a stage 3 cataract. The final stage (Stage 4) of cataract development was marked by a diffuse and dense opacification throughout the entirety of the lens (Nishida et al. 1992).

Isoflavone analysis

In order to extract genistein for analysis from the supplemental compounds 2.5 mg of genistein aglycone, 20 mg Novasoy[®]200, and 1 g SPI were dispersed into 5 mL aliquots of 80% aqueous methanol containing a 200 $\mu\text{mol/L}$ fluorescein internal standard (Prasain et al., 2003). Extractions were performed in triplicate. The samples were placed on an inverter and allowed to extract for 2 h at 4°C . After the extraction, 1 mL of each sample was placed into a 1.5 mL micro-centrifuge tube, and centrifuged at $7,000 \times g$ for 5 min. The supernatant was aspirated and 200 μL used for reverse-phase HPLC analysis. A six point genistein standard curve was prepared by serial dilutions from a 5 mmol/L genistein stock to produce concentrations of 0, 0.01, 0.05, 0.1, 0.5 and 1 mmol/L; each contained 200 $\mu\text{mol/L}$ fluorescein as the internal standard.

The samples were analyzed on a 220 mm $\times$ 4.6 mm i.d. Aquapore RP-300 7 μm column (Perkin Elmer, Waltham, MA), with a 15 mm $\times$ 3.2 mm i.d. RP-8 NewGuard[®] guard column (Perkin Elmer), on an HP 1100 HPLC (Agilent Technologies, Santa Clara, CA) with a UV-diode array detector. Prior to running the samples, the column was equilibrated with solvent A (90% double distilled H_2O /10% acetonitrile, 0.1% trifluoroacetic acid (TFA)) for 15 min. To analyze samples, isoflavones were resolved using a 30 min linear gradient of 0–

30% solvent B (90% acetonitrile/10% double distilled H₂O, 0.1% TFA), followed by a wash of 100% solvent B for 5 min and re-equilibration with solvent A for 5 min. The flow rate was constant at 1.5 mL/min. All solvents were of HPLC grade, and were filtered before use with a 0.45 µm filter. Representative chromatograms from initial analysis of the genistein-containing material in the dietary supplements are shown in Figure 1. HPLC analysis was conducted on a sample of each diet after formulation (Fig. 1) and also on samples taken throughout the course of the study to ensure that genistein levels were consistent with the pre-formulated concentration and that degradation had not occurred during storage.

Mass spectrometry analysis

Analysis was carried out by LC-multiple reaction ion monitoring mass spectrometry (Coward et al., 1996). Plasma samples (200 µL) were mixed with 250 µL of 100 mM ammonium acetate buffer, pH 5.0, containing *Helix pomatia* β-glucuronidase/sulfatase (1 mg) and incubated at 37°C for 16 h. Glacial acetic acid (50 µL) and the internal standard chrysin (10 µL) were added, and the mixture was extracted twice with two volumes of diethyl ether. The ether layers were combined and evaporated to dryness. The extract was reconstituted in 100 µL of 80% aqueous methanol and an aliquot (5 µL) was injected onto a 50 mm × 2.0 mm i.d. C₁₈ reverse-phase column equilibrated with 10 mM ammonium acetate, pH 7.2, in 25% aqueous acetonitrile at 50°C. The isoflavones were resolved using a mobile phase consisting of a 1 min linear gradient (25–70%) of acetonitrile at flow rate of 750 µL/min. and the eluate passed into a TurboIonSpray interface connected to an AB-Sciex (Concorde, Ontario, Canada) 4000 triple quadrupole mass spectrometer operating in the negative ion mode. The source temperature was set at 400°C and ion spray voltage of 4 kV. Nitrogen was used as nebulizer, secondary and curtain gas. The tandem mass spectrometer was tuned in the multiple reaction monitoring mode to monitor mass transitions *m/z* 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108 (*O*-desmethylanylesin), 269/133 (genistein), 283/184 (glycitein), and the internal standards 175/119 (4-methylumbelliferone), 317/93 (phenolphthalein) and 253/143 (chrysin).

Statistical analysis

The time until a cataract score of 2 was modeled as a function of treatment using a linear statistical model (Kutner, et al., 2005). The factors included in the model were eye (left and right), treatment (AIN-76A/0.018% genistein, AIN-76A/0.20% genistein, AIN-76A/NovaSoy®200, and AIN-76A/SPI), and rat (23 total; 5 in AIN-76A, AIN-76A/0.20% genistein, and AIN-76A/NovaSoy®200; 4 in AIN-76A/0.018% genistein and AIN-76A/SPI). By using this model with the rats nested within treatments, variation among rats was taken into account. In this model, treatment was a between-rat factor and eye was a within-rat factor. Since this analysis did not provide evidence in support of a difference between left and right eyes, an additional analysis was performed using a single outcome for each animal. The cataract scores for the left and right eyes were averaged and time until a total score of 2 was analyzed using a one-way ANOVA. Plasma isoflavones were compared using a one-way ANOVA followed by a multiple comparison of means using the Tukey procedure when there was a significant effect. Statistical significance was set at *P* < 0.05. Analyses were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC).

Results

Animal Breeding

Preliminary studies revealed that breeding of ICR/f rats required careful attention to detail with regard to husbandry conditions. In most cases, older animals (>6–9 months) either failed to produce or terminated their litters. Plastic housing units and shredding paper were provided for the 2–6 month old dams in breeder cages to improve nesting conditions, and

care was taken to ensure the animals were not disturbed by noise, drafts and other unnecessary disturbances. This raised breeding efficiency to create a sufficient number of animals for the experiments in this study.

Diet Formulation

AIN-76A was chosen as the base diet because the replacement of corn oil by soybean oil in an AIN-93G diet (Reeves, 1997; Lien et al., 2001) re-introduces unknown soy elements. AIN-76A was determined to be isoflavone- and polyphenol-free (as verified in Fig. 1). After formulation, samples from each of the diets were analyzed by HPLC to check for genistein content and possible degradation. Chromatograms are shown for the analysis of the supplements and the formulated diet (Fig. 1). Based on the formulated diet HPLC analysis, the amount of genistein (in all forms) in each diet was calculated, and in turn matched to the desired concentrations (Table 1). The composition of the diet was determined by HPLC at the end of the experiment and this verified that degradation during storage did not occur (Table 1). During the course of the study there were no statistically significant differences in body weights between the diets, although the SPI diet was associated with slightly larger body weights (Supplemental Fig. 1).

Plasma concentrations of isoflavones and their metabolites

Observed plasma levels of isoflavones, are summarized in Table 2. As expected, no genistein or other isoflavones (or their metabolites) were detected in the plasma of ICR/f rats on the control AIN-76A diet. For ICR/f rats on both diets in which genistein aglycone was added, genistein was the only isoflavone detected in the plasma. The mean plasma concentration of genistein in rats on AIN-76A/0.20% genistein was 2138.1 ± 720.8 nmol/L, 18.9 times higher than that observed in rats on the AIN-76A/0.018% genistein diet (Table 2). The plasma concentration of genistein in rats fed AIN-76A/Novasoy[®]200 was not significantly different from the rats fed low-dose genistein aglycone (Table 2). On the AIN-76A/Novasoy[®]200 diet, besides genistein, daidzein and its metabolites dihydrodaidzein, O-desmethylanagolensin and equol were also present in plasma (Table 2). In the case of rats fed AIN-76A/SPI, the plasma concentrations of genistein and daidzein and its metabolites were all lower than the AIN-76A/Novasoy[®]200 group (Table 2). It is worth noting that the lignan enterolactone was present in relatively consistent levels in the plasma of rats on all the diets, including the control AIN-76A diet.

Development of opacity

After the completion of the experiment, all videos were visually assessed and each individual lens was classified into stages 0 – 4 (based on the classification schemes depicted in Fig. 2) determinant upon their level of development at each point of observation. The development of lens opacity for each diet group is plotted against time in Figure 3, as the mean cataract score for each group per point of observation after dietary intervention. All the ICR/f rats in this study developed mature lens cataracts by 85 days of age, although one animal each from the AIN-76A/0.018% genistein group and the AIN-76A/SPI group, were removed from further analysis due to abnormal developmental patterns during the early stages of cataract formation. These two groups therefore had a total of four animals instead of five, i.e., the total number of lenses was eight instead of ten.

For the time to a cataract score of 2, no difference was found for the appearance of opacity between the left eye and the right eye ($F=3.75$; $df=1,18$; $P=0.07$) and there was no indication that the effects of the treatments differentially affected the left eye versus the right eye ($F=0.64$; $df=4,18$; $P=0.64$). Therefore, one-way ANOVA was used to compare the treatments using the time to an average score of 2 for the lens from both eyes. All the animals that were classified as having an average score of 2 had a (2,2) left eye/right eye

combination – there were no (3,1) or (4,0) combinations. The means and standard deviations are given in Table 3. Differences among the treatments were statistically significant ($F=13.04$; $df=4,18$; $P=0.0001$). The Tukey multiple comparison procedure indicated that the AIN-76A control diet was associated with a longer time to an average score of 2 than the other four treatments, which were not distinguishable from each other. When treatments were compared using the time to reach full lens opacity, i.e., a score of 4, no differences were found among treatments ($F=0.81$; $df=4,18$; $P=0.53$).

In transition from stage 0 with no visible signs of opacity, to stage 1 with slight opacity of the fiber cells in the outer cortex region, no statistically significant differences among the diets were found. For transition from stage 1 to stage 2, with visible formation of apparent water clefts in the posterior cortical region of the lens, transition occurred earlier ($P<0.05$) for animals consuming the genistein-containing diets (AIN-76A/0.018% genistein 59.8 ± 2.5 days; AIN-76A/0.20% genistein, 59.8 ± 1.1 d; AIN-76A/NovaSoy®200 58.2 ± 2.2 d; AIN-76A/SPI 61.0 ± 4.1 d) compared the control AIN-76A diet (69.4 ± 3.3 d). When transitioning to stage 3, with a dense opacification in the nuclear region of the lens, no statistically significant differences among the diets were found. Finally, for the transition to stage 4, which is signified by an overtly visible cataract, no statistically significant differences among the diets were found.

Discussion

This study demonstrated that the addition of genistein and genistein-containing dietary supplements to AIN-76A diet accelerates the early stages of lens cataract formation in the male ICR/f rat. Although these data are in contrast to the beneficial effects of isoflavones and other polyphenols reported (Supplemental Table 1), variables such as the timing of assessment for cataract formation, the animal strain used, base diet formulation, and dose of the supplemented compound, along with the particular chemical form of the supplement, may have influenced the particular results of this study.

The focus on the early stages of cataract development, along with the later stages, is a crucial difference between the present study and others conducted with both the ICR/f rat and other rodent models. Using the data generated from this current study, if the final stage of cataract formation is used as the endpoint, the effects of the diets were quite small – at most, there was a 4-day difference between the control AIN-76A diet and the AIN-76A/0.018% genistein-supplemented diet. More meaningfully, substantial differences induced by the diets occur at earlier stages of lens cataract development. In the transition from stage 1 to stage 2, lenses in the rats on the genistein-containing diets reached the midpoint (stage 2) of cataract development on average 10 days earlier than animals on the control AIN-76A diet (Fig. 3, Table 3).

Most animal models of cataract disease have to be combined with other stressors to induce cataracts. These include both chemicals, such as diabetes-inducing streptozotocin (Suryanarayana et al., 2005; Suryanarayana et al., 2007; Lu et al., 2008) and selenite (Durunkan et al., 2006), and non-chemical stressors such as x-ray radiation (Nduaguba et al. 2003). Huang et al. (2007) used a galactose-induced lens cataract model and observed that daily gavaging of genistein at 15 mg/kg body weight reduced severity of cataracts in the later stages of their development. Lu et al. (2008) demonstrated that mixed isoflavones reduced the formation of cataracts in the streptozotocin model of diabetes.

The present study examined the effects of genistein in the ICR/f rat, an age-related model of cataract disease (Ihara, 1983). This phenotype is autosomal recessive and although not completely elucidated, genetic factors responsible for cataract development were explored

using an alternative inbred sub-strain of the ICR/f rat, the cataractous and epileptic Ihara epileptic rat (IER) (Yokoyama et al., 2001). A cataract-associated gene, *Cat11* (responsible for the presence of the cataract) was localized to a region on Chromosome 8 containing the *Apoc3* gene, a gene involved in lipid metabolism (Yokoyama et al., 2001). As such, this localization may disrupt proper lipid metabolism resulting in the peak of plasma lipid peroxides observed at day 13 in the ICR/f rat (Yagi et al., 1985) which also corresponds to a developmental stage in the rat eye when the hyaloid artery regresses from the posterior side of the lens (Taniguchi et al., 1999). In addition, at 63 days an elevation of nitric oxide leads to increased lipid peroxide formation (Nagai et al., 2008). These increases in lipid peroxidation make the ICR/f rat a model of lipoprotein metabolism defects, which is a feature of chronic disease risk involved with aging (Holvoet et al., 2008). Another cataract-associated gene, *Cat12* was also observed in the IER strain (on Chromosome 15), but this locus was primarily responsible for the timing of the cataracts' development (Yokoyama et al., 2001). This locus separated the rats into two groups, an early-onset group (EOG), developing cataracts within four months of birth and a late-onset group (LOG), developing cataracts after eight months or longer (Yokoyama et al., 2001). Since all the ICR/f animals in the present study had full cataracts by 85 days of age, it is probable that similar epistatic interactions of *Cat11* and *Cat12* genes occur in this model. However, further studies at the histological and molecular level are needed to fully characterize the ICR/f rat.

The plasma concentrations of genistein were assessed using a single blood sample taken at the time of euthanasia. Rats on the control AIN-76A diet had unmeasurable levels of genistein or any isoflavone metabolites, although interestingly the lignan, enterolactone, was present. Rats on the two genistein aglycone-containing diets only had genistein in their plasma. The mean plasma genistein concentration in animals consuming the AIN-76A/0.2% genistein diet was 19 times that observed in rats consuming the AIN-76A/0.018% genistein diet, consistent with the increased dose. Daidzein and its metabolites, dihydrodaidzein, *O*-desmethylanagolensin and equol, were only present in plasma from animals receiving the Novasoy®200 and SPI supplements containing a mixture of isoflavones (Fig. 1).

These data with genistein treatments suggest that other dietary supplements might have a similar effect. Polyphenols have anti-oxidant properties, and when combined with vitamin C may enhance the anti-oxidant environment of the lens (Patel, et al., 2001; Barnes et al., 2006). However, while vitamin C is an anti-oxidant, it also has oxidant properties. Indeed, in a transgenic mouse model expressing the human vitamin C transporter, and thereby having human-like vitamin C concentrations in the aqueous humor, lens cataract formation was accelerated (Fan et al., 2006). Furthermore, Schey et al. (2000) showed that in the presence of UV light, the dietary supplement hypericin induced oxidative modifications to the alpha crystallins, as well as polymerization. Recent evidence suggests that the use of St. John's Wort (containing hypericin) increases the risk of lens cataracts (Booth and McGwin, 2009). Genistein can form radicals in the presence of lipid peroxy radicals (Patel et al., 2001); therefore, it is possible that genistein could stabilize radical formation induced by UV light on tryptophan residues in lens proteins (Davies et al., 2001). The mechanisms for genistein's action in the ICR/f rat may be entirely different from those operating in galactose- or streptozotocin-induced diabetic animals where cataract formation may result from glycation events and the formation of advanced glycation end products (Huang et al., 2007; Lu et al., 2008).

The results of the present study are consistent with the protective effect of AIN-76A diet versus soy-containing lab chow diet as observed in another animal model of lens cataract disease – the pink-eyed, Royal College of Surgeons (RCS) rat (Hess et al., 1985). RCS rats fed lab-chow diets (containing soy and other proteins) had a cataract incidence of 27–29% up to 12 months of age. In contrast, only 1 of 50 rats fed the AIN-76A diet developed a

mature cataract over the same period (Hess et al., 1985). These data can be interpreted in two ways – one that the AIN-76A diet contains protective agents against lens cataract formation, or that the non-nutritive components coming from the soy diet stimulate lens cataract formation. The present study where the background AIN-76A diet was held constant and isoflavones were introduced at least in part supports the latter concept.

A limitation of the current study was that the individual classifying the lenses into cataract stages was not blinded to the diet group and experimental design. In future, videos could be digitized so that classification is based on pixilation, or, if visual assessment is used, the individual making the assessment should be blinded from the organization of the study. Light scattering techniques could also be used as a method to monitor the development of opacity within the lens. Wegner et al. (2002) used Scheimpflug photography, a light scattering technique, to examine the effects of normal aging of the lens within a Wistar rat model. Such techniques could be beneficial in determining differences that might not be distinguishable when using visible or digitized analyses. In that event, the lens imaging data could also be subjected to analysis using mathematical modeling.

In conclusion, we have determined that supplementation with genistein no matter the chemical form or dose, accelerates the early stages of cataract development in the male ICR/f rat. The present study also demonstrates the need for a more detailed examination of a supplement's effects throughout the entirety of the formation of lens cataract disease.

Research Highlights

- Genistein accelerates the early stages of cataractogenesis in the ICR/f rat
- In the ICR/f rat, genistein had little effect on later stages of cataractogenesis
- Genistein's effect was independent of its chemical form in dietary supplements
- Plasma genistein concentrations reflected the doses delivered in the diets
- At the genistein doses used, there was no dose-dependent effect

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Adlercreutz H. Lignans and human health. *Crit. Rev. Clin. Lab Sci* 2007;44:483–525. [PubMed: 17943494]

- Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E and beta carotene for age-related cataract and vision loss: AREDS report no. 9. *Arch. Ophthalmol* 2001;119:1439–1452. [PubMed: 11594943]
- Arnal E, Miranda M, Almansa I, Muriach M, Barcia JM, Romero FJ, Diaz-Llopis M, Bosch-Morell F. Lutein prevents cataract development and progression in diabetic rats. *Graefes Arch. Clin. Exp. Ophthalmol* 2009;247:115–120. [PubMed: 18766362]
- Barnes PM, Bloom B, Nahin RL. Complementary and Alternative Medicine Use Among Adults and Children: United States, 2007. *Natl. Health. Stat. Report* 2008;10:1–24. [PubMed: 19361005]
- Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J. Agric. Food Chem* 1994;42:2466–2474.
- Barnes S, Prasain JK, Wang C-C, Moore DR II. Applications of LC-MS in the study of the uptake, distribution, metabolism and excretion of bioactive polyphenols from dietary supplements. *Life Sci* 2006;78:2054–2059. [PubMed: 16460766]
- Bigsby RM, Cardenas H, Caperell-Grant A, Grubbs CJ. Protective effects of estrogen in a rat model of age-related cataracts. *Proc. Natl. Acad. Sci. U.S.A* 1999;96:9328–9332. [PubMed: 10430942]
- Booth JN 3rd, McGwin G. The association between self-reported cataracts and St. John's Wort. *Curr. Eye Res* 2009;34:863–866. [PubMed: 19895314]
- Cassileth BR, Schraub S, Robinson E, Vickers A. Alternative medicine use worldwide: the International Union Against Cancer survey. *Cancer* 2001;91:1390–1393. [PubMed: 11283941]
- Christen WG, Liu S, Glynn RJ, Gaziano JM, Buring JE. Dietary carotenoids, vitamins C and E, and risk of cataract in women: a prospective study. *Arch. Ophthalmol* 2008;126:102–109. [PubMed: 18195226]
- Chylack LT Jr, Brown NP, Bron A, Hurst M, Köpcke W, Thien U, Schalch W. The Roche European American Cataract Trial (REACT): a randomized clinical trial to investigate the efficacy of an oral antioxidant micronutrient mixture to slow progression of age-related cataract. *Ophthalmic Epidemiol* 2002;9:49–80. [PubMed: 11815895]
- Coward L, Barnes NC, Setchell KDR, Barnes S. The antitumor isoflavones, genistein and daidzein, in soybean foods of American and Asian diets. *J. Agric. Food Chem* 1993;41:1961–1967.
- Coward L, Kirk M, Albin N, Barnes S. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin. Chim. Acta* 1996;247:121–142. [PubMed: 8920232]
- Davies MJ, Truscott RJW. Photo-oxidation of proteins and its role in cataractogenesis. *J. Photochem. Photobiol. B* 2001;63:114–125. [PubMed: 11684458]
- Durukan AH, Evreklioglu C, Hurmeric V, Kerimoglu H, Erdurman C, Bayraktar MZ, Mumcuoglu T. Ingestion of IH636 grape seed proanthocyanidin extract to prevent selenite-induced oxidative stress in experimental cataract. *J. Cataract Refract. Surg* 2006;32:1041–1045. [PubMed: 16814068]
- Fan X, Reneker LW, Obrenovich ME, Strauch C, Cheng R, Jarvis SM, Ortwerth BJ, Monnier VM. Vitamin C mediates chemical aging of lens crystallins by the Maillard reaction in a humanized mouse model. *Proc. Natl. Acad. Sci. U.S.A* 2006;103:16912–16917. [PubMed: 17075057]
- Ferrigno L, Aldigeri R, Rosmini F, Sperduto RD, Maraini G. Italian-American Cataract Study Group. Associations between plasma levels of vitamins and cataract in the Italian-American Clinical Trial of Nutritional Supplements and Age-Related Cataract (CTNS): CTNS Report #2. *Ophthalmic Epidemiol* 2005;12:71–80. [PubMed: 16019690]
- Hess HH, Knapka JJ, Newsome DA, Westney IV, Wartofsky L. Dietary prevention of cataracts in the pink-eyed RCS rat. *Lab. Anim. Sci* 1985;35:47–53. [PubMed: 3981959]
- Holvoet P, De Keyser D, Jacobs DR. Oxidized LDL and the metabolic syndrome. *Future Lipidol* 2008;3:637–649. [PubMed: 19802339]
- Huang R, Shi F, Lei T, Song Y, Hughes CL, Liu G. Effect of the isoflavone genistein against galactose-induced cataracts in rats. *Exp. Biol. Med. (Maywood)* 2007;232:118–125. [PubMed: 17202592]
- Ihara N. A new strain of rat with an inherited cataract. *Experientia* 1983;39:909–911. [PubMed: 6873248]

- Kutner, MA.; Nachtsheim, CJ.; Neter, J.; Li, W. Applied Linear Statistical Models. 5th edition. Boston: McGraw Hill; 2005.
- Lien EL, Boylea FG, Wrenn JM, Perry RW, Thompson CA, Borzelleca JF. Comparison of AIN-76A and AIN-93G diets: a 13-week study in rats. Food Chem. Toxicol 2001;39:385–392. [PubMed: 11295485]
- Lu MP, Wang R, Song X, Chibbar R, Wang X, Wu L, Meng QH. Dietary soy isoflavones increase insulin secretion and prevent the development of diabetic cataracts in streptozotocin-induced diabetic rats. Nutr. Res 2008;28:464–471. [PubMed: 19083447]
- Nagai N, Ito Y, Takeuchi N. Effect of disulfiram eye drops on lipid peroxide formation via excessive nitric oxide in lenses of hereditary cataract ICR/f rats. Biol. Pharm. Bull 2008;31:981–985. [PubMed: 18451530]
- Nduaguba C, Congdon N, Mahmoud M, Daly M, Lai H, deJuan E. Dietary genistein is protective against X-ray cataract in the rat. Invest. Ophthalmol. Vis. Sci 2003;44 E-Abstract 4322.
- Nishida S, Mizuno K, Matubara A, Kurono M. Age-related cataract in the hereditary cataract rat (ICR/1): development and classification. Ophthalmic Res 1992;24:253–259. [PubMed: 1475073]
- Patel RP, Boersma B, Crawford JH, Hogg N, Kirk M, Kalyanaraman B, Parks D, Barnes S, Darley-Usmar V. Antioxidant mechanisms of isoflavones in lipid systems: Paradoxical effects of peroxyl radical scavenging. Free Rad. Biol. Med 2001;31:1570–1581. [PubMed: 11744331]
- Prasain JK, Jones K, Kirk M, Wilson L, Smith-Johnson M, Weaver C, Barnes S. Profiling and quantification of isoflavonoids in kudzu dietary supplements by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry. J. Agric. Food Chem 2003;51:4213–4218. [PubMed: 12848487]
- Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J. Nutr 1997;127:838S–8341S. [PubMed: 9164249]
- Ribaya-Mercado JD, Blumberg JB. Lutein and zeaxanthin and their potential roles in disease prevention. J. Am. Coll. Nutr 2004;23:567S–587S. [PubMed: 15640510]
- Schey KL, Patat S, Chignell CF, Datillo M, Wang RH, Roberts JE. Photooxidation of lens alpha-crystallin by hypericin (active ingredient in St. John's Wort). Photochem. Photobiol 2000;72:200–203. [PubMed: 10946573]
- Sharma KK, Santhoshkumar P. Lens aging: effects of crystallins. Biochim. Biophys. Acta 2009;1790:1095–1108. [PubMed: 19463898]
- Stevens A. The contribution of glycation to cataract formation in diabetes. J. Am. Optom. Assoc 1998;69:519–530. [PubMed: 9747048]
- Suryanarayana P, Saraswat M, Petrash JM, Reddy GB. *Embllica officinalis* and its enriched tannoids delay streptozotocin-induced diabetic cataract in rats. Mol. Vis 2007;13:1291–1297. [PubMed: 17679931]
- Suryanarayana P, Saraswat M, Mrudula T, Krishna TP, Krishnaswamy K, Reddy GB. Curcumin and turmeric delay streptozotocin-induced diabetic cataract in rats. Invest. Ophthalmol. Vis. Sci 2005;46:2092–2099. [PubMed: 15914628]
- Taniguchi H, Kitaoka T, Gong H, Amemiya T. Apoptosis of the hyaloids artery in the rat eye. Ann. Anat 1999;181(6):555–560. [PubMed: 10609053]
- Vinson JA. Oxidative stress in cataracts. Pathophysiology 2006;13:151–162. [PubMed: 16765571]
- Wegener A, Kaegler M, Stinn W. Age-Related Light Scattering in Rat Lenses Observed in a 2-Year Inhalation Toxicity Study. Ophthalmic Res 2002;34:273–280. [PubMed: 12381887]
- Yagi K, Komura S, Ihara N, Abe H, Konishi H, Arichi S. Serum lipid peroxide levels in rats with inherited cataracts. J. Appl. Biochem 1985;7:202–206. [PubMed: 4055555]
- Yamakoshi J, Saito M, Kataoka S, Tokutake S. Procyanidin-rich extract from grape seeds prevents cataract formation in hereditary cataractous (ICR/f) rats. J. Agric. Food Chem 2002;50:4983–4988. [PubMed: 12166994]
- Yokoyama M, Amano S, Tsuji A, Sasahara M, Serikawa T, Ihara N, Matsuda M, Hazama F, Handa J. Genetic analysis of cataract in Ihara epileptic rat. Mamm. Genome 2001;12:207–211. [PubMed: 11252169]

Figure 1. Representative chromatograms of genistein-containing supplements and post-formulation diet analysis

The following are chromatogram identities: (A) 1 mM genistein standard, (B) genistein aglycone (from DSM), (C) NovaSoy[®]200 (from ADM), (D) Soy Protein Isolate (from ADM), (E) AIN-76A, (F) AIN-76A/0.018% genistein, (G) AIN-76A/NovaSoy[®]200, (H) AIN-76A/SPI, (I) AIN-76A/0.20% genistein. Chromatograms A, B, C, and D are from the original compound analysis before diet formulation and are all on the same scale of 2100 mAU. Chromatograms E, F, G, and H are on a scale of 125 mAU, while chromatogram I is on a scale of 1300 mAU, and are all from the post-formulation analysis of the experimental diets. The peak identities for chemicals analyzed are: (1) daidzin, (2) glycitin, (3) genistin, (4) 6''-O-malonyldaidzin, (5) 6''-O-malonylgenistin, (6) daidzein, (7) genistein, (8) fluorescein.

Figure 2. Cataract stage classification scheme

Views A, C, E, G, and I are taken from the same angle and examine the anterior view of the lens from the reflexes of the slit-lamp, while views B, D, F, H, and J also taken from the same angle utilize the posterior reflexes of the slit-lamp to examine lens health. (A, B) Stage 0: lens with no overtly visible cataract indications in either the anterior or posterior regions, (C, D) Stage 1: slight fibrous opacification in the anterior cortical region of the lens with no overt signs of cataract in the posterior region, (E, F) Stage 2: continuance of fibrous opacification in the anterior cortical region and development of apparent water clefts or vacuoles in the posterior subcapsular region, (G, H) Stage 3: dense opacification apparent in the anterior portion of the lens and dense opacification in the posterior cortical region

causing the light reflexes to form an “O”, (I, J) Stage 4: dense opacification encompassing nuclear and cortical regions as seen in both the anterior and posterior views.

Figure 3. Mean cataract score plotted against the day of observation, for the control and each of the experimental diets

The Mean Cataract Score for each day of observation was determined by summing the stage classifications (Stages 0–4) for each of the lenses in a diet group, and dividing by the total number of lenses in each group. The scores for each diet group are plotted as follows;

AIN-76A (

), AIN-76A/0.018% genistein (

), AIN-76A/0.20% genistein (

), AIN-76A/ NovaSoy®200 (

), AIN-76A/SPI (

). The time to reach a score of 2 in cataract formation is indicated by the point at which each plot comes into contact with the dashed line.

Table 1

Formulated Diet (Post-Formulation and Post-Experiment) Analysis Genistein Concentrations, and Amount Totals per 20 kg of Diet.

<u>Post-Formulation Genistein Analysis</u>		
<u>Diet</u>	<u>Concentration (mg/g of diet)</u>	<u>Amount per 20kg Diet (g)</u>
AIN-76A	0	0
AIN-76A/0.018% Genistein	0.161	3.22
AIN-76A/0.020% Genistein	1.88	37.6
AIN-76A/NovaSoy®200	0.167	3.34
AIN-76A/SPI	0.194	3.88
<u>Post-Experiment Genistein Analysis</u>		
<u>Diet</u>	<u>Concentration (mg/g of diet)</u>	<u>Amount per 20kg Diet (g)</u>
AIN-76A	0	0
AIN-76A/0.018% Genistein	0.151	3.02
AIN-76A/0.20% Genistein	1.87	37.4
AIN-76A/NovaSoy®200	0.167	3.33
AIN-76A/SPI	0.189	3.78

Table 2
Plasma Concentrations (nmol/L) in ICR/f Rats Following Administration of Genistein-Containing Supplements.

<u>Diet</u>	<u>N</u>	<u>Genistein</u>	<u>Daidzein</u>	<u>DHD</u> [#]	<u>O-DMA</u> [§]	<u>Equol</u>	<u>Glycitein</u>	<u>Enterolactone</u>
AIN-76A Control	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	107.3 ± 21.9 ^a
AIN-76A/0.018% Genistein	3	113.4 ± 63.2 ^b	n.d.	n.d.	n.d.	n.d.	n.d.	80.6 ± 29.9 ^{ab}
AIN-76A/0.20% Genistein	4	2138.1 ± 720.8 ^a	n.d.	n.d.	n.d.	n.d.	n.d.	53.7 ± 31.0 ^b
AIN-76A/NovaSoy [®] 200	5	120.9 ± 107.3 ^b	540.4 ± 467.9 ^a	140.9 ± 67.7 ^a	472.3 ± 182.6 ^a	1356.0 ± 445.7 ^a	46.8 ± 24.2 ^a	74.8 ± 16.6 ^{ab}
AIN-76A/SPI	4	72.5 ± 38.9 ^b	189.5 ± 95.1 ^a	35.5 ± 14.6 ^b	310.9 ± 96.3 ^a	475.9 ± 302.1 ^b	9.1 ± 6.6 ^b	62.3 ± 27.0 ^{ab}

n.d., not detected

[#] DHD, dihydrodaidzein

[§] O-DMA, O-desmethylanagolensin

^{a b} Diets with different letters are significantly different from each other based on the Tukey procedure with P=0.05

Table 3

Means, Standard Deviations, and Sample Sizes for to an Average Score of 2 for both Eyes, by Treatment.

Treatment	Mean (Days)	Standard Deviation	N
AIN-76A Control	69.4 ^a	3.3	5
AIN-76A/0.018% Genistein	59.8 ^b	2.5	4
AIN-76A/0.20% Genistein	59.8 ^b	1.1	5
AIN-76A/NovaSoy®200	58.2 ^b	2.2	5
AIN-76A/SPI	61.0 ^b	4.1	4

^{a, b} Treatments with different letters were significantly different from each other based on the Tukey multiple comparison method with P=0.05.