MPS I: Early diagnosis, and treatment of bone disease

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Citation for published version (APA):
Chapter
Adverse effects of genistein in a Mucopolysaccharidosis type I mouse model

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JIMD Rep 2015; in press.
ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder characterized by diminished degradation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). Patients present with a variety of symptoms, including severe skeletal disease. Current therapeutic strategies have only limited effects on bone disease. The isoflavone genistein has been studied as a potential therapy for the mucopolysaccharidoses because of its putative ability to inhibit GAG synthesis and subsequent accumulation. Cell, animal and clinical studies, however, showed variable outcomes. To determine the effects of genistein on MPS I related bone disease, wild type (WT) and MPS I mice were fed a genistein supplemented diet (corresponding to a dose of approximately 160mg/kg/day) for 8 weeks. HS and DS levels in bone and plasma remained unchanged after genistein supplementation, while liver HS levels were decreased in genistein-fed MPS I mice as compared to untreated MPS I mice. Unexpectedly, genistein-fed mice exhibited significantly decreased body length and femur length. In addition, 60% of genistein-fed MPS I mice developed a scrotal hernia and/or scrotal hydrocele, manifestations which were absent in WT or untreated MPS I mice. In contrast to studies in MPS III mice, our study in MPS I mice demonstrates no beneficial but even potential adverse effects of genistein supplementation. Our results urge for a cautious approach on the use of genistein, at least in patients with MPS I.
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INTRODUCTION
Mucopolysaccharidosis type I (MPS I, OMIM 252800) is a lysosomal storage disorder (LSD) caused by α-L-iduronidase (IDUA, EC 3.2.1.76) deficiency, resulting in impaired degradation and subsequent accumulation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). Patients may present with cardiac and pulmonary disease, inguinal and umbilical hernia, corneal clouding and progressive central nervous system (CNS) disease which significantly limits life expectancy 1. In addition, skeletal dysplasia, generally referred to as dysostosis multiplex, is a striking feature and a major cause of morbidity. Patients show progressive loss of range of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression 1-2. Current therapies, including haematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy, effectively treat many features of MPS I, but have limited effects on bone disease 3,4. Several factors contribute to this lack of effect. Firstly, the inability of the relatively large enzyme to traffic through the poorly vascularized matrix of growth plates and other cartilaginous tissue to target cells 5,6. Secondly, cartilage cells are derived from mesenchymal stem cells, which are not transplanted in sufficient amounts by HSCT 7. Thirdly, therapy is started after the onset of irreversible bone lesions, which may already exist before birth 8,9. An alternative treatment strategy for LSDs is substrate reduction therapy, which aims to reduce the synthesis of the accumulating material, thereby preventing or halting lysosomal storage. This approach has been used successfully in Gaucher disease and Niemann Pick disease type C 10.

The isoflavone genistein has several biological activities. It is an antioxidant, has estrogenic activity and inhibits the activity of tyrosine kinase receptors including the epidermal growth factor receptor (EGFR) 11,12. Genistein has been shown to reduce GAG synthesis in MPS fibroblasts, at least partly via the latter mechanism 13. An in vivo study with a high dose of genistein in MPS IIIB mice, showed reduced GAG levels in brain and impressive amelioration of neurological symptoms 14. Although genistein appears to be well tolerated in high doses, adverse effects, which are associated with its potential antiproliferative and estrogenic actions, have been reported and may include hepatotoxicity and hormonal disbalance 15,16. The only study on the effects of genistein on MPS related bone disease showed increased range of joint motion in genistein treated MPS II patients, suggesting that genistein at least reaches the surrounding connective and muscle tissue of the joints 17. Because genistein can reach bone tissue 18, we fed MPS I mice a high dose genistein diet to evaluate its potential for the treatment of MPS I related bone disease.
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MATERIALS AND METHODS

Animals

MPS I mice (B6.129-Idua<sup>min2ck/J</sup>, 19) were purchased from Jackson Laboratory and maintained as a heterozygote line on an inbred C57BL/6J background at the Academic Medical Centre. The mice were housed at 21 ± 1°C, 40-50% humidity, on a 12 hours light-dark cycle, with <i>ad libitum</i> access to water and food. Genotypes were identified by PCR using a protocol provided by Jackson Laboratory.

An AIN93M diet with 3.7% sunflower oil and 0.3% rapeseed oil instead of 4% soy oil was produced (Research Diet Services B.V.). For the genistein-fed group, genistein aglycone (kind gift from Axcentua) was added to the diet in a concentration of 0.1% (w/w). Assuming a food intake of 0.16g food per gram of body weight per day, this diet results in a dose of 160mg/kg/day, which is similar to other studies on the effects of a high dose of genistein in MPS mice 14,21. One week before weaning, mice received the soy-free diet. At 3 weeks of age, male wildtype (WT) and MPS I mice were weaned on the soy-free diet or the genistein-supplemented diet (n=10 per group). Every week, mice were weighed and examined for general pathological manifestations. If scrotal abnormalities were observed, scrotal hydrocele and/or scrotal hernia were objectified by macroscopic inspection, transillumination and/or ultrasound by a skilled animal technician and macroscopic inspection of the scrotum and abdominal and pelvic cavity after sacrifice. At 11 weeks of age, mice were anesthetized with an intraperitoneal injection of 100mg/kg pentobarbital and euthanized by exsanguination via cardiac puncture. Body length and femur length were measured, and blood and tissues collected. All animal experiments were approved by the animal institutional review board at the Academic Medical Centre, University of Amsterdam.

Tissue processing

Blood was collected into EDTA tubes, kept on ice and centrifuged at 240g for 10 minutes. Plasma was collected and stored at -80°C until analysis. Mouse livers were snapfrozen in liquid nitrogen and stored at -80°C. Before analysis, mouse livers were homogenized in PBS and protein concentration was determined using Pierce® BCA Protein Assay Reagent A (Thermo Scientific) as described by the manufacturer. Mouse humerae were collected and placed in 0.9% NaCl containing Complete mini protease inhibitor cocktail (Roche Applied Science) at 4°C for >1 day. Next, soft tissue was removed and humerae were stored at -80°C. Before analysis, humerae were homogenized in PBS, sonificated twice for 15 seconds on 40 joules/watt/second using a Vibra Cell sonicator (Sonics & Materials Inc.) and centrifuged for 1 minute at 400g. Protein concentration of the supernatant was determined as described above.
GAG analysis

GAG levels in mouse plasma, liver and humerus homogenates were determined using HPLC-MS/MS, as described previously \(^2\), with one modification for tissue samples: 12.5µg protein of liver or humerus homogenates were used. Genistein supplementation did not alter HS or DS disaccharide composition, therefore only values of the most abundant HS derived disaccharide D0A0 and DS derived disaccharide D0a4 are given.

Genistein measurement

Genistein levels were determined by ultra-high performance liquid chromatography (UHPLC) with a protocol modified from Seppen et al. \(^2\). 40µl of plasma or humerus homogenate was acidified with 5µl 1M 4-morpholineethanesulfonic acid. Genistein was hydrolyzed by adding 2.5µl β-glucuronidase (Sigma Aldrich) and 2.5µl sulfatase (10mg/ml in PBS, Sigma Aldrich) and incubated at 37°C for 20 hours. Protein was precipitated by adding 100µl methanol and samples were centrifuged at 8000g for 2 minutes. The supernatant was vaporized using a Techne® Dri-block® heater (Bibby Scientific) and the residue dissolved in 20% v/v acetonitrile. UHPLC was performed on a Dionex 3000 Ultimate UHPLC, equipped with a Polar advantage C18 column (Thermo Scientific), variable wavelength detector set at 260nm, 5µl injection and a flow rate of 0.5ml/min. The following program was used with solvent A (A, 0.1% v/v formic acid in water) and solvent B (B, 0.1% v/v formic acid in acetonitrile): Start 30% B, t=5 55% B, t=5.1 100% B, t=6.1 30% B, t=10 30% B. Linear gradients were employed and t is in minutes. Chromeleon Dionex software (Thermo Scientific) was used to integrate the chromatograms and genistein level was calculated using a calibration curve of genistein dissolved in dimethyl sulfoxide, diluted in mouse plasma.

Morphology of testes

Morphology of testicular cells and structures were analyzed by routine hematoxylin/eosin (HE) staining.

Statistical analysis

Statistical analysis was performed using Mann-Whitney-U tests for nonparametric analysis with SPSS Statistics software version 21 (IBM Corp.). Significance was assumed where \(p\) values were <0.05.

RESULTS

Genistein decreases GAG levels in liver, but not in bone or plasma

As expected, analysis of GAG levels revealed significantly higher levels in MPS I plasma and
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Genistein supplementation did not affect GAG levels in WT or MPS I bone or plasma (fig. 1A and B). HS levels in liver from genistein-fed MPS I mice were 25% decreased ($p<0.01$), as compared to untreated mice. No difference in liver DS levels was observed between genistein-fed and untreated MPS I mice (fig. 1C).

Figure 1. GAG levels in bone (A), plasma (B) and liver (C) in control and genistein supplemented mice. The results are expressed as milligrams GAGs per gram of protein for liver and bone, or as milligrams GAGs per liter of plasma. All values are mean ± standard deviation of 10 mice. Each sample was analyzed in duplicate, **$p<0.01$, ND not detectable, G genistein supplementation.

Figure 2. Weight (A), body length (B) and femur length (C) in control and genistein supplemented mice. All values are mean ± standard deviation of 10 mice, *$p<0.05$, **$p<0.01$, ***$p<0.001$, G genistein supplementation.
Genistein was present in plasma, but undetectable in bone
Genistein levels in plasma of genistein-fed MPS I mice were 782nM ± 503. In the control group, genistein concentration in plasma was below the limit of quantification (data not shown). Genistein levels in bone were below detection level in all MPS I mice (data not shown).

Genistein causes decreased skeletal growth and scrotal hernia/hydrocele in MPS I mice
Total body weight of MPS I mice was 11% increased \((p<0.05)\), as compared to WT mice (fig. 2A). Total body weight of genistein-fed MPS I mice was decreased by 16% \((p<0.001)\) (fig. 2A), as compared to untreated MPS I mice. Following genistein supplementation, total body length was 4% \((p<0.05)\) and 6% \((p<0.001)\) decreased in WT and MPS I mice (fig. 2B), respectively, as compared to untreated mice. Femur length of MPS I mice was 4% decreased \((p<0.05)\), as compared to WT mice (fig. 2C). Femur length of genistein-fed mice were 3% \((p<0.01)\) and 7% \((p<0.001)\) decreased in WT and MPS I mice (fig. 2C), respectively, compared to untreated mice. Surprisingly, 60% of the genistein-fed MPS I mice had an enlarged scrotum (fig. 3) with redness of the overlying skin and the mice showed a wide based gait. None of the WT or untreated MPS I mice showed scrotal abnormalities. The scrotal enlargements were observed to be due to either scrotal hernia and/or scrotal hydrocele. HE staining of

*Figure 3.* Scrotum of a MPS I mouse on control diet (A) and a MPS I mouse on genistein diet (B). 60% of MPS I mice with genistein had either scrotal hydrocele and/or scrotal hernia, as depicted in (B). All other mice had scrotas as depicted in (A).
testes revealed no morphological changes of testicular cells and structures in genistein-fed or untreated mice (results not shown).

DISCUSSION

Skeletal disease is one of the most prevalent and incapacitating disease manifestations in patients suffering from the MPSs and frequently results in the need for multiple surgical interventions. Current disease modifying therapies for the management of MPS I ameliorate a number of clinical signs and symptoms, but have a limited effect on the progression of skeletal disease. Therefore, therapeutic strategies targeting bone disease are urgently needed.

The isoflavone genistein inhibits the activity of tyrosine kinase receptors, thereby modulating the expression of several genes, including some involved in GAG synthesis. Genistein is being investigated for its potential benefit in the treatment of CNS disease in MPS III, as it passes the blood-brain barrier and an in vivo study showed that genistein may reduce GAG accumulation in the brain and corrects behavioural abnormalities in MPS III mice. As prevention of GAG accumulation in cartilage and bone might halt the progression of skeletal disease in the MPSs, we investigated the effects of genistein on GAG accumulation in MPS I mice in order to evaluate its potential for the treatment of MPS I related bone disease.

In agreement with results of previously published studies on the effects of genistein in MPS mice, GAG levels were significantly decreased in liver of genistein-fed MPS I mice. Surprisingly, we observed no effect on GAGs in bone or plasma. The effect of genistein on GAG levels in plasma or bone of MPS III mice has not been described in previous studies. In rats, genistein is known to reach bone in approximately 17 times lower quantities when compared to plasma levels, 2 hours after administration of 4mg/kg genistein (in the current study we used 160 mg/kg/day oral supplementation). Although plasma genistein concentrations in our study were comparable with, or even higher than the concentrations reported in other studies on the effects of genistein in mice, genistein concentrations in bone in our study were below detection level. These results suggest that the treatment duration and/or dosage of genistein may have been insufficient to treat bone. In our study, mice were treated from 3 weeks of age, for a period of 8 weeks as, at 11 weeks of age, the growth plate is almost closed and skeletal development almost completed. Therefore, we do not expect any additional effect on bone with a longer supplementation period. Earlier initiation of genistein supplementation is not feasible as mice are weaned at 3 weeks of age.

Our observation that genistein supplementation led to decreased body weight in mice was not surprising. Although not decreased previously in MPS III mice, the effect of genistein on decreasing lipid deposition and body weight has been reported earlier in mice.
and rats and may be due to the estrogenic effect of increasing lipoprotein lipase activity, or decreased food intake. Genistein-fed mice in our study, however, also exhibited decreased skeletal growth, including decreased femur length, which has not been described previously. In addition, 60% of genistein-fed MPS I mice developed a scrotal hernia and/or a scrotal hydrocele. The cause of the decrease in skeletal growth and the observed scrotal hernia/hydrocele is unclear. Increased levels of DS, an abundant GAG in connective tissue, may have contributed to the development of adverse effects, as our recently published study showed that genistein can increase HS and DS storage in MPS I chondrocytes and fibroblasts (but not in osteoblasts). In the present study, GAG levels were analyzed in complete bones and chondrocytes only make up a small portion of the entire bone. This might explain the fact that no increase in GAG levels was observed in bones of genistein treated MPS I mice, while these animals did show a decrease in femur length. Increased GAG storage in certain cell types might further stimulate the pathophysiological mechanisms causing bone and connective tissue disease in MPS I. In addition, patients with MPS I already frequently display hernia and hydrocele. Genistein may thus lead to a more severe MPS I phenotype, at least in mice. Therefore, increasing the dose of genistein or prolonging the supplementation period, likely will not result in amelioration of bone disease in MPS I mice. The only study in patients on the effects of a high dose, i.e. 150 mg/kg/day, of (synthetic) genistein (at least one year treatment of 22 MPS III patients), concluded that genistein is safe, but did report elevation of liver enzymes, breast development in boys or young girls, menstrual irregularities, and bilateral deep vein thrombosis in a 20 year old woman with several other risk factors for thrombosis. This study did not report on growth rate.

CONCLUSION

In conclusion, our study suggests that high doses of genistein might lead to decreased growth and increased incidence of scrotal hydrocele and hernia, at least in MPS I. These data, in combination with other potential adverse effects reported in previous studies, support a cautious approach to the introduction of high doses of genistein in patients with various MPSs and underscores the need for well-designed and controlled clinical trials allowing the collection of all potential adverse effects over longer periods of time.

ACKNOWLEDGEMENTS

We thank Ronald Wanders, Henk van Lenthe, Wim Kulik and Jos Ruijter for technical assistance and helpful discussions. We thank Axcentua for providing genistein. This work was financial supported by the foundation ‘Steun Emma Kinderziekenhuis AMC’ and the ‘WE Foundation’.
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