The role of impaired de novo Coenzyme A biosynthesis in pantothenate kinase-associated neurodegeneration
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Effects of various doses of pantethine in healthy mice

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Manuscript in preparation
ABSTRACT

Pantothenate kinase-associated neurodegeneration (PKAN) is a neurodegenerative disease with a complex pathogenesis that is so far not understood. Currently, there is no treatment for PKAN and most of the available treatments are directed towards alleviating the symptoms associated with the disease. Recently we have established a *Drosophila* model for PKAN and by using this model we have identified a novel compound, Pantethine, which can rescue the disease symptoms in the *Drosophila* model for PKAN. As a step forward, it will be essential to test the potential of pantethine in a mammalian/mice model with an ultimate goal of finally be able to treat PKAN patients. The aim of the present study was to evaluate the toxicity of orally administered pantethine in wild type C57BL/6J mice. Increasing concentrations of pantethine were administered orally to different groups of mice. Daily activity, general performance, total water intake and body weight were monitored for 3 weeks. Histological analysis was done for the assessment of toxic affects of pantethine in various organs. Oral administration of pantethine up to 15mg/ml of drinking water was tolerable without any adverse effects in the wild type mice. Pantethine dose of 45mg/ml of drinking water resulted in decrease in body weight of 40 %. In summary, we show that pantethine is tolerable in wild-type mice and can be used for further tests in the PKAN disease mouse model.
INTRODUCTION

Pantothenate kinase associated neurodegeneration (PKAN) is a neurodegenerative disease characterized by iron accumulation in specific regions of the brain [1]. PKAN is associated with gene mutations in the PANK2 gene, which encodes the mitochondrial pantothenate kinase (PANK) that catalyzes the first rate limiting step in the de novo biosynthesis of Coenzyme A (CoA) [2]. Although the causative gene of PKAN is known, the pathogenesis is largely unknown and there is no treatment to delay the progression of this painful disorder. Current treatments consisting of pharmacological and surgical interventions are focused on alleviating the symptoms associated with the disease [1, 3-5]. Most of these treatments result in brief periods of benefit [1] and some of them are still under clinical trial [1]. There is an urgent need to understand the pathogenesis of PKAN in order to design novel therapeutics that can treat PKAN.

In order to identify and test drugs and therapies for treatment of human disease, animal models are highly essential. Therefore, a PANK2-/− knockout mouse was created, but although retinal degeneration and azoospermia was reported, this model failed to recapitulate the typical neurodegenerative symptoms of PKAN [6]. Recently, we have established a Drosophila model for PKAN [7, 8]. These mutant fruit flies carry a mutation in fumble, a structural and functional ortholog of human pantothenate kinase 2 [9, 10]. The mutants are further referred to as dPANK/fbl. dPANK/fbl homozygous mutants show abnormal mitochondria, impaired locomotor function, large brain vacuoles and a decreased life span (Chapter 3). These results show that in contrast to PANK2-/− knock out mice, Drosophila fbl/dPANK mutants do show a neurodegenerative phenotype. It was also demonstrated that in dPANK/fbl mutants, levels of CoA are strongly decreased compared to wild type flies. In a search for compounds that are able to increase the levels of CoA in a pantothenate kinase impaired background, the compound pantethine was identified (Chapter 3). Pantethine addition to the food of the mutant fruit flies significantly rescued the neurodegenerative phenotype and resulted in an increased life span of the mutants compared to untreated mutants (Chapter 3). These results suggest that a treatment based on pantethine may be beneficial for PKAN patients.

Pantethine is a naturally occurring physiological compound, it is not an FDA approved drug, however, in few clinical trials, pantethine was tested and studies suggest that pantethine has beneficial effects in various vascular disease due to its ability to decrease serum cholesterol and hyperlipidaemia [11-18]. Our studies using Drosophila showed that pantethine is a protective compound in a pantothenate kinase impaired background; although our studies also showed that high concentrations of pantethine also induce a decreased life span in wild type flies. This suggests that a high dose of pantethine may have a toxic affect. In order to further investigate the potential of pantethine, it will be highly beneficial to perform pantethine studies in higher organisms other than Drosophila, such as mice. In this respect, the first question that needs to be addressed is whether mice can tolerate...
pantethine and if yes, what doses of pantethine is tolerable in mice? The current study aims towards identification of the tolerable dose of orally administered pantethine and to identify possible adverse effects of pantethine in healthy mice. This study will provide a base for future experiments in which pantethine will be tested as a potential drug for PKAN treatment in mice or in clinical trials.

**MATERIAL AND METHODS**

**Mice care and maintenance:** Wild-type C57BL/6J mice were commercially purchased from Harlan, Zeist (The Netherlands). 25 days old wild-type male littermates were fed ad libitum with standard diet formulated and prepared at the animal facility, University of Groningen. All mice were maintained at standard environmental conditions (temperature of 24°C with 55% relative humidity and a 12 h light cycle). All experiments were evaluated and approved by the committee for animal experimentation of the University of Groningen, The Netherlands according to the guidelines provided by the Dutch Animal Protection Act.

**Oral pantethine supplementation via drinking water:** C57BL/6J mice were divided in four groups which consist of one control group and three test groups. Pantethine (P2125, Sigma, USA) was administered orally to the three test groups via drinking water for 3 weeks to determine the in vivo maximum tolerated dose and toxicity of pantethine. Three different concentrations of pantethine given to the test groups were: 1.6mg/ml of drinking water, 15 mg/ml of drinking water and 45 mg/ml of drinking water. The control group received 0 mg pantethine per ml of drinking water. Drinking water (with or without pantethine) was refreshed three times a week.

**Physiological assays:** Total water intake and body weight gain was measured for 3 weeks since the start of the experiment (when mice were 25 days old). Mice were also monitored every day for activity, external appearance (fur and coat) of the mice or any other abnormal behavioral changes.

**Histological analysis:** Mice that were treated with highest concentration of pantethine (45 mg/ml of drinking water) were also subjected to post mortem analysis to observe various toxic effects of pantethine (if any) on various internal organs. Different tissues were isolated and fixed in formaline solution for 12 hours. Further embedding and histoanalysis was done by Nederlands onderzoek institute vor vogels en bijzonder dieren, Veldhoven, The Netherlands, using standard procedures. In summary, the fixed tissues were dehydrated and embedded in paraffin. Thin sections of 4 µm were cut on the microtome (Richter jung microtome, Germany). Sections were examined and photos were captured using a Leica microscope.

**Statistical analysis:** Statistical analysis were performed using the Student’s t test with
p<0.001 as the level of significance. The results are presented as mean ± standard deviation unless indicated otherwise.

RESULTS

Effect of pantethine during daily oral supplementation

Three different doses of pantethine were orally administered to test for toxicity and daily water intake per mouse was calculated. Daily water intake was comparable between the control group (consisting of 4 mice) and the test groups (consisting of 3 mice) receiving pantethine 1.6mg or 15mg pantethine/ml of drinking water (Figure 1). In contrast, a dose of 45 mg pantethine/ml of drinking water resulted in a severe decrease (more than 3 times) in daily water intake compared to the control group (Figure 1).

Effect of pantethine consumption on normal activity

Control and pantethine-treated mice were observed daily to examine for unusual behavior, external irregularities of their fur and for any other observable abnormalities in their appearance. As compared to the control group, the pantethine-treated mice (1.6mg/ml, 15mg/ml and 45mg) showed a similar cage activity. Also the external appearance of their fur was similar in pantethine-treated and control mice. There were no other signs of abnormal behavioral (such as excessive scratching, limping etc) and there were no signs of other pathological conditions (such as diarrhea, lethargy etc).

Effect of pantethine consumption on body weight gain

The weight of the mice (3 weeks of age) was ~ 10 grams at the start of the experiment (Figure 2). During the experiments, the mice were weighted and the control mice showed a consistent increase in weight and at the end of the experiment (3 weeks later) the wild type mice were on an average 22 grams (Figure 2). Mice treated with 1,6 or with 15 mg pantethine/ml drinking water showed a weight gain comparable with control mice (Figure 1).
However, mice treated with 45 mg pantethine/ml drinking water showed a strong decrease in weight gain and after 3 weeks the average weight was 12 grams (Figure 2).

**Table 1: Post mortem macroscopic and microscopic analysis of control and pantethine treated mice**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Abnormalities observed after pantethine feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroscopic</strong></td>
<td>1. Thin abdominal muscles and less abdominal fat.</td>
</tr>
<tr>
<td></td>
<td>2. The reproductive organs (testis, secondary reproductive glands) were less developed.</td>
</tr>
<tr>
<td></td>
<td>3. Slightly swollen spleen.</td>
</tr>
<tr>
<td><strong>Microscopic</strong></td>
<td>1. Liver tissue showed a clear fatty vacuolization (Figure 3)</td>
</tr>
<tr>
<td></td>
<td>2. Thymus section revealed thick outer membrane.</td>
</tr>
<tr>
<td></td>
<td>3. Spleen showed more erythropoieses.</td>
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<tr>
<td></td>
<td>4. Reproductive organs:</td>
</tr>
<tr>
<td></td>
<td>• Testis were relatively smaller in size</td>
</tr>
<tr>
<td></td>
<td>• Normal spermatozoa formation was observed although a delay in progression of spermatogenesis through different stages of maturation was present.</td>
</tr>
<tr>
<td></td>
<td>• A defect in in progression of the spermatogonium stage to the secondary spermatozoa stage.</td>
</tr>
<tr>
<td></td>
<td>• Secondary reproductive glands: epididymus was normal; however, there was less epididymal secretion in the lumen.</td>
</tr>
<tr>
<td></td>
<td>• Glandular vesicularis was rudimentary and under developed (Figure 4) with thick cubical epithelium and almost no secretion in the glands.</td>
</tr>
</tbody>
</table>

**Histological analysis of internal organs of mice treated with 45mg pantethine/ml drinking water**

Mice treated with 45 mg pantethine/ml drinking water showed normal behavior, however, a remarkable decrease in weight gain was observed in this cohort. In order to investigate the effects of pantethine in more detail these mice were subjected to post mortem analysis of the internal organs and the major macroscopic and microscopic findings of the post mortem analysis are summarized in table 1. This macroscopic and microscopic analysis revealed that most of the organs skin, oesophagus, salivary glands, brown fat, lungs, myocardium, spleen (macroscopic), kidney, nerve plexus hilus of kidney and stomach revealed no significant difference between control and pantethine fed group. However, there were
some remarkable differences in the pantethine fed group as compared to the control group. Clearly, the pantethine fed mice showed less fat tissue. In addition, the liver tissue revealed clear greasy vacuolization in the hepatocytes (Figure 3). Another significant difference was that the pantethine fed mice showed a severe delay in the development of the reproductive body organs (like testis and secondary reproductive glands) (see table 1, microscopic analysis section 5 and figure 4).

DISCUSSION

Recently, we have shown that pantethine rescues PKAN disease-associated phenotypes of dpANK/fbl mutant flies [8]. Here our aim was to investigate whether mice tolerate specific doses of pantethine provided via the drinking water. We demonstrate that pantethine is well tolerated, without any observable side effects, up to a dose of 15 mg per ml of drinking water that is equivalent to daily intake of 4057 mg of pantethine/kg body weight. Furthermore, our data show that although mice treated with the highest tested dose (45 mg/ml) did not show abnormal behavior or signs of illness, these mice were reduced in size, contained

Figure 3: High dose of pantethine (45mg per ml of drinking water) results in a fatty vacuolization of liver.

Left panel shows post mortem liver tissue from the control mice (A, A', A'') and right panel shows the post mortem liver tissue from the pantethine treated mice (B, B', B'') containing clear vacuoles as indicated by the arrows in B''. Images A, B are taken at 10X, A', B' at 40X and A'', B'' at 100X magnification.

Figure 4: High dose of pantethine (45mg per ml of drinking water) results in a rudimentary and under developed glandular vesicularis (B, B' as shown by the dotted line) as compared to control mice (A, A'). Image A and B depicts the macroscopic observation and image A' and B' shows the microscopic histosection of the glandular vesicularis at 100X magnification.
less fat, their reproductive organs were smaller in size as compared to the other cohorts and fatty vacuolization was observed in the liver. In various previous studies, it was demonstrated that pantethine possesses serum lipid modulatory affect leading to fat redistribution among various tissues [11-19]. Based on these studies, pantethine was tested in clinical trials for its potential to treat dyslipidemia and fatty liver syndrome. These studies showed that pantethine supplementation can significantly reduce the serum lipid content of triglycerides, total cholesterol and low-density lipoprotein-cholesterol [11, 14, 19]. Therefore, it was suggested that pantethine can offer an effective therapeutic option for the treatment of patient populations with total serum Cholesterol levels >200 mg/dl and/or serum triacylglycerol levels >150 mg/dl [11, 14, 19]. Another study investigated the role of pantethine in fatty acid redistribution and its effect in fatty liver syndrome. In these studies a fat redistribution was observed from the liver and visceral organs to the subcutaneous tissue and this might be beneficial in treatment of fatty liver syndrome [19]. It is highly likely that in mice treated with high concentrations of pantethine fatty acid redistribution occurs which ultimately lead to lean mice with low fat content and fatty vacuolization in the liver. Another finding in mice treated with high concentrations of pantethine was the underdevelopment of some of the reproductive organs. However, currently it remains inconclusive whether under-development of the reproductive organs is due to an overall delay in the physiological development of mice (evident from lack of weight gain and small size) or due to the direct toxicity of pantethine on the reproductive organs.

Remarkably, apart from the abnormalities in the development of reproductive organs and lack of weight gain there were no gross or microscopic abnormalities observed in the other major body organs. Based on the above results, we concluded that up to 15 mg pantethine / ml drinking water is a tolerable dose and this can be used for follow up studies. This dose is equivalent to a daily intake of 4057 mg of pantethine/kg body wt and this is a very high dose compared to the dose that was used in the clinical trials [11, 13, 14, 17] or the dose that was recommended for the food supplementation in US [20], which was only 15 mg/kg body wt. It should be stated that because in our studies pantethine supplementation was only tested for 3 weeks we cannot exclude the presence of long-term toxicity of pantethine.

A next step will be to test whether pantethine supplementation can rescue the reported phenotype of the PANK2-/- or PANK1-/- mice. The PANK1 (-/-) mice were viable and fertile, however their liver CoA content was reduced by more than 40% [21]. These PANK1 knock out mice were also unable to switch their metabolism from the glucose utilization and fatty acid synthesis to gluconeogenesis and β-oxidation of fatty acids during fasting thereby resulting in accumulation of triglycerides and long chain fatty acids and hypoglycemia [21]. PANK2 (-/-) mice showed signs of reduced weight gain (20% less than the wt), retinal degeneration and male infertility due to azoospermia [6]. Although these mice models do not exactly phenocopy PKAN characteristics, pantethine supplementation to these mice, will reveal whether pantethine may work protective in a mammalian model suffering from an impaired pantothenate kinase activity.
REFERENCES


