Histological assessment of intermediate- and long-term creatine monohydrate supplementation in mice and rats

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Tarnopolsky, M. A., J. M. Bourgeois, R. Snow, S. Keys, B. D. Roy, J. M. Kwiecien, and J. Turnbull. Histological assessment of intermediate- and long-term creatine monohydrate supplementation in mice and rats. Am J Physiol Regul Integr Comp Physiol 285: R762–R769, 2003; 10.1152/ajpregu.00270.2003.—Creatine monohydrate (CrM) supplementation appears to be relatively safe based on data from short-term and intermediate-term human studies and results from several therapeutic trials. The purpose of the current study was to characterize pathological changes after intermediate-term and long-term CrM supplementation in mice [healthy control and SOD1 (G93A) transgenic] and rats (prednisolone and nonprednisolone treated). Histological assessment (18–20 organs/tissues) was performed on G93A mice [healthy control and SOD1 (G93A) transgenic] and rats (prednisolone and nonprednisolone treated). Histological assessment of intermediate- and long-term creatine supplementation appears to be relatively safe based on data from short-term and intermediate-term human studies and results from several therapeutic trials. The purpose of the current study was to characterize pathological changes after intermediate-term and long-term CrM supplementation in mice [healthy control and SOD1 (G93A) transgenic] and rats (prednisolone and nonprednisolone treated). Histological assessment (18–20 organs/tissues) was performed on G93A mice after 159 days, and in Sprague-Dawley rats after 365 days of CrM supplementation (2% wt/wt) compared with control feed. Liver histology was also evaluated in CD-1 mice after 300 days of low-dose CrM supplementation (0.025 and 0.05 g kg−1 day−1) and in Sprague-Dawley rats after 52 days of CrM supplementation (2% wt/wt) with and without prednisolone. Areas of hepatitis were observed in the livers of the CrM-supplemented G93A mice (P < 0.05), with no significant inflammatory lesions in any of the other 18–20 tissues/organs that were evaluated. The CD-1 mice also showed significant hepatic inflammatory lesions (P < 0.05), yet there was no negative effect of CrM on liver histology in the Sprague-Dawley rats after intermediate-term or long-term supplementation nor was inflammation seen in any other tissues/organs (P = not significant). Dietary CrM supplementation can induce inflammatory changes in the liver of mice, but not rats. The observed inflammatory changes in the murine liver must be considered in the evaluation of hepatic metabolism in CrM-supplemented mice. Species differences must be considered in the evaluation of toxicological and physiological studies.

dietary supplements; hepatitis; drug toxicity; side effects

CREATINE IS A GUANIDINO COMPOUND produced endogenously by the liver and kidneys and is consumed in meat-containing diets (44). It is transported to skeletal muscle, heart, brain, and several other tissues by a sodium-dependent transporter (13). Creatine is an important compound in cellular energy buffering and in the “shuttling” of energy from the mitochondria to the cytosol (45). Studies have found that dietary supplementation with creatine monohydrate (CrM) can increase skeletal muscle (15, 31) and brain (7, 25) total creatine and phosphocreatine concentrations, with an even greater degree of increase seen in organs with low baseline creatine content such as liver and kidney (16). CrM supplementation has become popular among athletes due to a performance-enhancing potential (11, 12, 26, 42, 44), and some human studies have demonstrated benefits in certain pathological conditions (25, 41, 43, 46). There is growing evidence showing neuroprotective effects from CrM in murine studies (9, 27, 28, 32, 35), especially the SOD1 G93A transgenic mouse (a model of familial motor neuron disease/amyotrophic lateral sclerosis (ALS); see Refs. 1, 20, and 39).

There has been some concern regarding the potential for CrM toxicity based on two anecdotal human case reports (21, 34), one animal study in hypertensive rats (8) and the fact that carcinogens can be formed if creatine and sugars are heated to high temperatures (47, 48). In humans, most of the studies that have examined the potential for toxicity have not found evidence of side effects when consumed at “recommended” doses (22, 29, 33, 36, 37, 44). Several recent reviews have concluded that dietary CrM supplementation in humans appears to be relatively safe in the short term; however, they cautioned that the long-term side effects have not been evaluated systematically (19, 44). Encouragingly, more recent studies in humans have not found evidence for CrM-associated toxicity based on blood analysis and side-effect questionnaires in older adults (3, 5), young athletes (29, 44), and in patients with neurological diseases (17, 38, 41, 46).

To date, there have not been any systematic pathology studies in animals treated with CrM, and most of the studies evaluating the efficacy in animal models of disease did not evaluate the potential for side effects (1,
20, 27, 28). Given the widespread use of CrM supplementation among athletes and nonathletes (18), the potential for its use in a variety of disease states (see above), and because there are several ongoing studies in patients with neurological disease, it is important that studies more carefully evaluate the potential for side effects from longer-term CrM supplementation at the tissue and organ level.

One of the theories regarding the potential for toxicity from CrM supplementation is that creatine can increase oxidative stress and potentially form carcinogenic compounds in vitro (47). In contrast, several studies have found that CrM supplementation decreased markers of oxidative stress in animal models of neurodegenerative disease (20, 28) and is a weak, but abundant, antioxidant (24). Other studies have found CrM to have an anti-tumorigenic effect on solid tumor growth in animals (23, 30). If CrM is acting via an increase in oxidative stress, it would be expected that animals with an inherently high level of oxidative stress would show a greater propensity toward CrM-mediated toxicity. The SOD1 G93A transgenic mouse has a mutation in the Cu/Zn-superoxide dismutase gene that results in an increase in oxidative stress (14). As a consequence of the increased oxidative stress, the G93A mouse is a good candidate model to evaluate the potential for enhanced CrM tissue toxicity in addition to the fact that it is a well-studied model of ALS (1, 14, 20).

Our initial goal was to determine whether there was any evidence of histopathological lesions in 22 different organs/tissues obtained from SOD1 G93A mice after 140 days of CrM supplementation. After finding significant hepatic inflammatory lesions in the CrM-treated mice, we sought to determine whether or not these lesions occurred after a lower CrM dose exposure in a nontransgenic murine strain (CD-1) and in a different species (Sprague-Dawley rat). These results indicate that tissues, and results obtained from tissues, of one species may not be representative of other species.

METHODS

Animals

Study 1. For the SOD1 G93A studies, breeding pairs of transgenic mice, BbCg-Tg (SOD1-G93A) 1Gur, were obtained from Jackson Laboratories (Bar Harbor, ME). Offspring bearing the human transgene were identified from blood samples (retro-orbital venous sampling), using PCR amplification of a fragment of the transgene as outlined by Jackson Laboratories (www.JAX.org). Equal numbers of male and female mice were allocated to each group. The lower-copy-number SOD1-G93A mice (used in the current study) develop progressive limb weakness at ~180 days of life that progresses to paralysis within 3–4 wk because of a progressive loss of anterior motor horn neurons in part as a result of excessive oxidative stress and apoptosis of the neurons (39).

Study 2. For the longer-term, lower-dose study, CD-1 female mice were obtained from Charles River Laboratory (Wilmington, MA) at 15 wk of age.

Studies 3 and 4. Young male Sprague-Dawley animals were purchased from Taconic Laboratories (Germantown, NY) at 21 days of age. The rats were Pneumocystis carinii free at the start of the studies and were given a minimum of 1 wk to observe for any illness signs or behavior.

Ethical approval for studies 1, 3, and 4 was obtained from the McMaster University Animal Research Board, and the studies were conducted in accordance with the Canadian Council on Animal Care. The studies were completed at the Central Animal Facility of McMaster University (Hamilton, Canada). Study 2 was completed at Springfield College (Springfield, MA). The procedures followed were approved by the Springfield College Animal Use Review Committee. All studies adhered to the policy outlines in the Guide For the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 80-23, Revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Design

Study 1. The data for the current study were collected during a study comparing the clinical efficacy of CrM compared with riluzole (see below) as therapies for SOD1 G93A mice (see Ref. 39). After being weaned, animals were housed individually with a 12:12-h light-dark cycle. Equal numbers of male and female animals were randomly assigned to one of four groups receiving either standard powdered murine chow (control, n = 16), riluzole (n = 14), CrM (n = 14), or a combination of riluzole and creatine (n = 15). The CrM (Sigma, St. Louis, MO) was mixed with the standard murine chow (Purina) at 2% wt/wt. The riluzole was provided by Rhone Poulenc Rorer, Canada (now Aventis Pharma), and was given in drinking water (100 μg/ml) ad libitum.

The combination (riluzole + CrM) included both treatments at the above doses, and treatment for all groups was initiated when the mice were 40 days old for a total of 159 days of treatment. Each of the treatment groups (CrM, riluzole, riluzole + CrM) in this study showed a significant prolongation in the time to onset of clinical symptoms (paralysis) and a lower symptom score at the time of death at 199 days of life compared with the control animals (39). The average lifespan of these high-copy-number animals is between 200 and 230 days with death from paralysis.

All animals were euthanized using an overdose of pentobarbital sodium at 199 days of life (159 days of treatment) and were immediately dissected; tissues were visualized and palpated for evidence of gross pathology. Portions of liver, kidney, adrenal gland, esophagus, duodenum, jejunum, cecum, colon, urinary bladder, spleen, pancreas, lung, heart, skeletal muscle (red and white gastrocnemius), testes, ovaries, uterus, cervix, thyroid, spinal cord, and brain were immersed in 10% phosphate-buffered formaldehyde (formalin). These samples were dehydrated in increasing concentrations of ethanol and xylene and embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin and cover slipped.

Study 2. Twenty-four CD-1 mice were randomly separated into three equal groups of eight animals and housed in these groups. All groups had ad libitum access to food and water, with the control group receiving no CrM while the experimental groups received CrM (Pfanstiehl Laboratories, Waukegan, IL) added to the water supply to achieve a dose of ~0.025 g·kg⁻¹·day⁻¹ (very-low-dose group) and 0.05 g·kg⁻¹·day⁻¹ (low-dose group). Two animals from each group were randomly killed at 56 days of age. Water consumption was monitored weekly, and the doses above repre-
sent the mean intake per animal over the intervention period. The remaining mice were killed after 300 days of intervention, and the liver, heart, kidneys, and skeletal muscle (biceps femoris, semitendinosus, semimembranosus, and gracilis) were excised and processed as outlined above for histological analysis.

**Study 3.** Equal numbers \( (n = 20) \) of male Sprague-Dawley rats were randomly allocated to receive CrM (2% wt/wt; Traco Laboratories, Champaign, IL) or normal rat chow, and each of these groups was further subdivided into groups receiving methylprednisolone (7 mg·kg\(^{-1}\)-wk\(^{-1}\)) or a placebo injection starting on day 30 of life. Thus there were four treatment groups [CrM + placebo, CrM + methylprednisolone, normal + placebo, normal + methylprednisolone \((all \ n = 10)\)], and these treatments were administered over a 50-day period. Because of the risk of immunosuppression from the prednisolone, the rats were housed in a barrier room free of rodent infections, and the light-dark cycle was 12:12 h. At day 50, the rats were euthanized with pentobarbital sodium, and the liver was extracted and placed in 10% formalin and processed as above.

**Study 4.** Equal numbers \( (n = 6) \) of male Sprague-Dawley rats were randomly allocated to receive CrM in rat chow (2% wt/wt; Traco Laboratories) or normal rat chow over a 365-day period starting on day 30 of life. The rats were housed in pairs with a light-dark cycle of 12:12 h. At day 365, the rats were killed with pentobarbital sodium and were immediately dissected; tissues were visualized and palpated for evidence of gross pathology. Portions of liver, kidney, adrenal gland, esophagus, duodenum, jejunum, cecum, colon, urinary bladder, spleen, pancreas, lung, heart, skeletal muscle (red and white gastrocnemius), testes, thyroid, spinal cord, and brain were immersed in 10% formalin and processed as above.

A summary of each of the treatment groups is presented in Table 1. The tissue sections collected at the complete necropsy in study 1 were reviewed blindly by a veterinary pathologist (Kwicien) and a human pediatric pathologist (Bourgeois) for histological changes. The hepatic sections from studies 1, 2, and 3 were evaluated blindly by the veterinary pathologist (Kwicien) and a human pediatric pathologist (Bourgeois) using a transmitted white light microscope (Olympus BX-60) to look for histological changes. The pathology was graded on an ordinal scale with 1 = no significant pathological changes (i.e., no more than one area with <10 inflammatory cells); 2 (grade I pathology) = small aggregates of 10 or greater inflammatory cells measuring between 30 and 50 \( \mu \)m with or without associated hepatocellular injury; 3 (grade II pathology) = multifocal aggregates of mixed inflammatory cells measuring between 51 and 100 \( \mu \)m with associated hepatocellular injury; and 4 (grade III pathology) = multifocal aggregates of mixed inflammatory cells measuring >100 \( \mu \)m with associated hepatocellular injury. The vast majority of inflammatory cells were lymphocytes with some histiocytes seen in association with necrotic hepatocytes primarily in grade II and III pathology. The interrater correlation coefficient between the two pathologists using this scale in 59 blinded slides was 0.96. The liver sections for study 2 were also evaluated by one of the authors (Keys) using a slightly different grading system (although they were subsequently reanalyzed using the common grading system described above), and the cell diameters were also determined for the mice treated for 56 days. The test/retest coefficient of variation for the cell diameters was <3%. Finally, all of the histological slides from studies 2 and 4 were examined blindly by one of the pathologists (Bourgeois). The histology results from study 2 were based on the common grading system described. Examples of a normal liver section and the three grades of pathology are presented in Fig. 1.

### Statistics

The tissue histology was graded according to the ordinal scale above and was analyzed using the Kruskal-Wallis non-parametric statistical test and Dunn’s multiple-comparison post hoc test (version 3.03; Prism, San Diego, CA). The data in Tables 1–5 reflect the number of animals whose tissue fell within a given pathological category compared with the total number of animals. For the cell diameters, a one-way ANOVA was employed with Tukey’s post hoc test (version 3.03; Prism). A \( P \) value <0.05 was taken to indicate statistical significance.

### RESULTS

#### Study 1

Gross changes were not observed in any of the tissues/organisms from the SOD1 G93A mice. There were a few minor histopathological changes that were considered incidental in some of the tissues and organs (i.e., kidney, 3 samples with small collections of lymphocytic aggregates; lung, 4 samples with scattered lymphocytic aggregates) from the G93A mice (all seen in <5% of the sections (excluding muscle and spinal cord; see below)], with no differences between the treatment groups \( [P \) not significant (NS)] and no sex differences. There was an ~50% reduction in the number of motor neurons in the lumbar region of the G93A mice at 199 days compared with age-matched nontransgenic mice of the same background, and the skeletal muscle showed an abnormal number of internalized myonuclei and scattered atrophy, with no differences between treatment groups (also see Ref. 39). In contrast, hepatic inflammatory changes were observed with a greater frequency in the livers of the G93A mice in the creatine group compared with the placebo and riluzole groups \( (P < 0.01) \). Similar results were found for the grade III lesions, with more in the creatine group compared with the placebo and riluzole groups \( (P < 0.05) \). There were

### Table 1. Summary of the animals and treatments

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Animal Species</th>
<th>Total No.</th>
<th>Creatine Dosage</th>
<th>Duration of Creatine Treatment, days</th>
<th>Cointervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SOD1-G93A mice</td>
<td>59</td>
<td>2% wt/wt diet</td>
<td>159</td>
<td>Riluzole (25%)</td>
</tr>
<tr>
<td>2</td>
<td>CD-1 mice</td>
<td>6 (ST) 18 (LT)</td>
<td>0.025 ≤ 0.05 g·kg(^{-1})-day(^{-1})</td>
<td>56 (ST) and 300 (LT)</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>Sprague-Dawley rats</td>
<td>40</td>
<td>2% wt/wt diet</td>
<td>50</td>
<td>Methylprednisolone (50%)</td>
</tr>
<tr>
<td>4</td>
<td>Sprague-Dawley rats</td>
<td>12</td>
<td>2% wt/wt diet</td>
<td>365</td>
<td>Nil</td>
</tr>
</tbody>
</table>

ST, short-term; LT, long-term.
no grade II or III pathological changes observed in the livers from either of the placebo or riluzole groups (Table 2). When the data from the creatine and creatine + riluzole groups were combined (i.e., all animals on CrM) compared with the riluzole and placebo groups (i.e., all animals not exposed to CrM), there were more grade II and III lesions seen for creatine and creatine + riluzole compared with the combined non-CrM-treated groups ($P < 0.05$).

**Study 2**

There were no remarkable changes seen in the kidney, heart, or skeletal muscle of the mice from any of the CD-1 mice groups. There was a significant increase in the number of inflammatory lesions observed in the liver from the 0.05 g·kg$^{-1}$·day$^{-1}$ supplemented mice compared with the nonsupplemented group, and the results were not different when the histological grading was performed at McMaster University (Bourgeois) or at Springfield College (Keys; Table 3). There was also evidence of mild connective tissue infiltration in most of the CrM-treated mice on both dose levels (data not shown). There was no evidence of inflammation or connective tissue proliferation in the liver sections from the 56-day treated animals.

The hepatocytes (mean of 15 cells/animal) from control livers (56 days) averaged 15.2 ± 2.6 (SD) μm in diameter. Hepatocytes from mice maintained on 0.025 g·kg$^{-1}$·day$^{-1}$ averaged 18.5 ± 1.4 μm in diameter (+22.5%, $P < 0.05$ vs. control). Hepatocytes from mice maintained on 0.05 g·kg$^{-1}$·day$^{-1}$ for 56 days averaged 22.0 ± 1.5 μm in diameter (+45%, $P < 0.01$ vs. control and $P < 0.05$ for 0.05 vs. 0.025 g·kg$^{-1}$·day$^{-1}$).

**Study 3**

There were no gross lesions in the livers of the rats. There were a few grade I and II lesions in some of the livers from the rats (no grade III lesions), with no between-group differences (NS, Table 4).

**Study 4**

There were some grade I and II lesions seen in the livers of the rats, with no difference between the groups (NS, Table 5). There were a few mild foci of inflammatory cells in the lungs of four control and four CrM-treated animals and two control and one CrM-treated animal, with trivial sclerotic changes seen in the kidneys (NS between groups, data not shown). For all of the remaining tissues, there was no evidence of

<table>
<thead>
<tr>
<th>Pathological Category</th>
<th>Placebo</th>
<th>Riluzole</th>
<th>Creatine</th>
<th>Creatine + Riluzole</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>14/16</td>
<td>12/14</td>
<td>3/14</td>
<td>9/15</td>
<td>Creatine &lt; all others$^*$</td>
</tr>
<tr>
<td>Grade I</td>
<td>2/16</td>
<td>3/14</td>
<td>4/14</td>
<td>3/15</td>
<td>NS between groups</td>
</tr>
<tr>
<td>Grade II</td>
<td>0/16</td>
<td>0/14</td>
<td>3/14</td>
<td>2/15</td>
<td>NS between groups</td>
</tr>
<tr>
<td>Grade III</td>
<td>0/16</td>
<td>0/14</td>
<td>4/14</td>
<td>1/15</td>
<td>Creatine &gt; Riluzole, Placebo$^a$</td>
</tr>
<tr>
<td>Total abnormal</td>
<td>2/16</td>
<td>2/14</td>
<td>11/14</td>
<td>6/15</td>
<td>Creatine &gt; Riluzole, Placebo$^b$</td>
</tr>
</tbody>
</table>

NS, nonsignificant. Ratios represent the number of animals out of each group; i.e., 1 animal out of 15 total. $^*P < 0.05$ and $^†P < 0.01$. 

**Table 2. Hepatic pathology in SOD1 G93A mice [study 1 (159 days treatment)]**

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**Fig. 1. Hepatic histological classification scheme to grade pathology.** A: normal murine hepatic pathology. Thin arrow, portal tract; thick arrow, central vein. B: grade I pathology. Thin arrow, small aggregates of 10 or greater inflammatory cells measuring between 30 and 50 μm with or without associated hepatocellular injury. C: grade II pathology. Multifocal aggregates of mixed inflammatory cells measuring between 51 and 100 μm with associated hepatocellular injury (thin arrow). D: grade III pathology. Multifocal aggregates of mixed inflammatory cells measuring >100 μm with associated hepatocellular injury (thin arrow). Hematoxylin- and eosin-stained sections ×200 magnification.

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histopathology in more than one animal and nothing more than minor changes. There was no evidence of neoplasia in any tissue/organ.

**DISCUSSION**

The administration of CrM to both G93A transgenic mice and CD-1 nontransgenic strains of mice resulted in histological evidence of hepatitis with no evidence of pathology in a variety of other tissues and organs. CrM administration to rats did not result in pathology indicative of hepatic toxicity, and there were no changes in any of the other organs examined. These results are of interest, for they demonstrate that alterations in hepatic function could affect glucose and/or urea/ammonia metabolism, or even animal viability if the changes were extensive. Clearly, these hepatic changes must be considered in the design and interpretation of future studies using CrM supplementation in murine models of human medical disorders.

We considered several issues in trying to further understand the observed hepatic inflammatory changes. Initially, it was felt that the changes were unique to the SOD1 G93A mice, perhaps as an interactive effect with the known increase in oxidative stress induced by the mutation (14). It was considered plausible that the increased production of free radicals may have contributed to hepatocellular injury that was exacerbated by CrM. However, given that CrM supplementation reduced indexes of oxidative stress in animal models of neurological disease (9, 20, 27, 28), and is an antioxidant (24), we considered this to be unlikely. Second, the amount of CrM administered to the G93A mice (2% wt/wt; human equivalent 70–90 g/day accounting for the higher food intake of mice) was much higher than doses used in human trials with neurological disease (17), and the inflammation could be a dose-dependent toxic effect. Finally, we considered that the hepatic changes could be unique to the murine model. To answer these questions, we administered CrM at a lower dose to nontransgenic CD-1 mice and also to a different species of animal, the Sprague-Dawley rat.

CrM was administered to nontransgenic (CD-1) mice at doses that were lower than those used for the rats, and hepatitis changes were found that were similar to future evaluations of CrM using murine models, for it is possible that the inflammatory changes in the liver could alter the pharmacokinetics of any coadministered drug that is metabolized by the hepatic route. In addition, alterations in hepatic function could affect glucose and/or urea/ammonia metabolism, or even animal viability if the changes were extensive. Clearly, these hepatic changes must be considered in the design and interpretation of future studies using CrM supplementation in murine models of human medical disorders.

Table 3. Hepatic pathology in CD-1 mice (study 2)

<table>
<thead>
<tr>
<th>Pathological Category</th>
<th>Creatine, g·kg⁻¹·day⁻¹</th>
<th>Placebo</th>
<th>Normal Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5/6</td>
<td>4/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Grade I</td>
<td>1/6</td>
<td>1/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Grade II</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Grade III</td>
<td>0/6</td>
<td>0/6</td>
<td>1/10</td>
</tr>
<tr>
<td>Total abnormal</td>
<td>1/6</td>
<td>2/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

Pathological Category PL 0.025 0.05 Significance

Normal 9/10 7/10 6/0 5/0 <Placebo*
Grade I 1/10 1/10 1/10 NS between groups
Grade II 0/10 0/10 0/10 NS between groups
Grade III 0/10 0/10 2/10 NS between groups
Total abnormal 1/10 2/10 5/6 >Placebo*

Pathology grades as outlined in text. *P < 0.05.

Table 4. Hepatic pathology in Sprague-Dawley rats (study 3 [50 days treatment])

<table>
<thead>
<tr>
<th>Pathological Category</th>
<th>Creatine + Placebo</th>
<th>Creatine + Prednisolone</th>
<th>Normal Diet + Placebo</th>
<th>Normal Diet + Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>9/10</td>
<td>7/10</td>
<td>6/0</td>
<td>5/10</td>
</tr>
<tr>
<td>Grade I</td>
<td>1/10</td>
<td>1/10</td>
<td>2/10</td>
<td>4/10</td>
</tr>
<tr>
<td>Grade II</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Grade III</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Total abnormal</td>
<td>1/10</td>
<td>3/10</td>
<td>4/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

*Pathological categories are outlined in text. Prednisolone = 7 mg·kg⁻¹·wk⁻¹ methylprednisolone. There were no significant differences between groups.

Table 5. Hepatic pathology in Sprague-Dawley rats [Study 4 (365 days treatment)]

<table>
<thead>
<tr>
<th>Pathological Category</th>
<th>Creatine (2% wt/wt)</th>
<th>Normal Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Grade I</td>
<td>2/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Grade II</td>
<td>2/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Grade III</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Total abnormal</td>
<td>5/6</td>
<td>4/6</td>
</tr>
</tbody>
</table>

*Pathological categories are outlined in text. There were no significant differences between groups.
those seen in the G93A mice. These results suggest that exogenous CrM results in hepatic inflammatory changes in otherwise healthy mice and at a dose (relative to body weight) that is similar to that habitually consumed in food by humans (−1–1.5 g/day). It is likely that the CrM per se and not an impurity accounted for the observations, for the administered CrM was obtained from three separate chemical supply sources (Sigma, Pfannstiel, and Traco) with stringent quality control at the source, including HPLC analysis for impurities. In addition, it would be very improbable that an identical impurity could exist from two separate companies that were used in the mice studies where inflammation was seen from two separate university vivariums. Given that the animals from all groups were caged in the same room, the probability that murine hepatitis virus (MHV) or some other infectious agent selectively affected only those animals on CrM is statistically improbable. A few of the SOD1 G93A mice tested positive for antibodies against the MHV using an ELISA test; however, the numbers of MHV-positive mice were identical to the groups (<2/group). In addition, the detection of anti-MHV antibodies is rarely coincident with liver pathology in wild-type mice (Kwiecien, unpublished observations, 1997–2003). Although MHV was not tested in the CD-1 mice, the statistical probability that identical findings were observed merely by random chance in a different mouse species with the experiments being conducted in a separate vivarium was extremely low (P < 0.01).

Many of the CD-1 mice tested positive for *Helicobacter bili* in the intestines (PCR-based assay), and a few were positive in livers; however, this was not differentially found in those on CrM treatment. Although CrM treatment could have contributed to formation of inflammatory foci, presumably because of periodical bacterial showering via the portal venous system, this potential effect, however, was restricted to mice, both the transgenic and wild type, and was not observed in rats. Without an extensive microbiological evaluation, we cannot exclude the possibility that CrM may render the murine liver more sensitive to microorganisms that would otherwise be nonpathogenic.

It is important to reference the doses of CrM used in the current series of experiments in animals to a human perspective. For longer-term consumption, several studies have employed doses of between 3 and 10 g CrM/day in humans (15, 17, 38, 41). For a 70-kg human, this amounts to between 0.043 and 0.142 g·kg⁻¹·day⁻¹, which is in the range of the dose used in the CD-1 mice (0.05 g·kg⁻¹·day⁻¹), and is less than the estimated intake for the SOD1 G93A studies. Assuming that a 70-kg sedentary human consumed a 2,000-kcal mixed diet of carbohydrate (55%), fat (30%), and protein (15%), and CrM were given at 2% wt/wt, the CrM intake would amount to ~8 g/day or 0.114 g·kg⁻¹·day⁻¹. These results clearly indicate that the amount of CrM provided to the CD1 mice in the current study was not supraphysiological and was well within the dose range that is being used in longer-term human clinical trials with CrM (15, 17, 38, 41); the dose in the rats and G93A mice was also within this range using a human 2% wt/wt dose. If one corrects for the higher food intake in mice and rats relative to their total body mass, a 2% wt/wt diet is 8–12 times higher than the dose used in longer-term human clinical trials with CrM (15, 17, 38, 41). Although we did not measure creatine content in the livers in the current study, a recent study has found that total creatine content in livers of rats and mice ranged from 5 to 8 μmol/g wet wt and showed a large increase (260–500%) in response to oral CrM supplementation (1.4–2.8% wt/wt diet) within 4–8 wk (16).

Given that the CrM supplementation was clearly associated with inflammatory changes in the liver, it was important to determine whether a similar process occurred in a different species of animals. In contrast to the inflammatory changes seen in both strains of mice, there was no evidence of hepatic inflammation in the Sprague-Dawley rat exposed to an identical dose (2% wt/wt) but shorter duration (50 vs. 159 days) of CrM administration. To determine whether duration was a factor, we exposed Sprague-Dawley rats to the same dose (2% wt/wt) for 1 yr (just under 1/2 of the average lifespan) and did not find any evidence of differential hepatitis in the rats. Given the higher relative amount of food eaten by a rat compared with a human, the 2% wt/wt CrM dose for the rats represented ~1.3 g·kg⁻¹·day⁻¹, which was far in excess of that used in any human study; still, there was no evidence of CrM inducing hepatic inflammation. It is difficult to extrapolate the current findings to humans; however, there have been no substantiated reports of alterations in liver function tests or transaminases in intermediate and longer-term human trials with CrM supplementation (5, 17, 22, 36, 37). The lack of any CrM-induced nephritis (renal inflammation) in any of the species studied herein also supports the fact that most human studies have not found evidence for renal dysfunction with CrM (5, 17, 22, 29, 33, 36, 37), and only two unsubstantiated case reports exist (21, 34). Finally, CrM supplementation appears to increase renal disease progression in hypertensive rats (Han:SPRD; see Ref. 8), again showing a species and strain difference.

The above results indicate a species difference in the susceptibility to the hepatotoxic effects from CrM administration. One factor that could explain some of this difference, although by an unknown mechanism, is the fact that mice are predominantly herbivores and rats are more omnivorous (in the wild). As a result, mice may not be accustomed to the delivery of high exogenous creatine concentrations via the portal vein. There does not appear to be any difference in the basal hepatic total or phosphocreatine content between rats and mice, and the magnitude of the increase in response to CrM supplementation is similar for these species (16). As mentioned above, a microorganism that is unique to mice may also be a coinhibitor or corepressor of the hepatic inflammatory changes; however, we cannot support or refute this hypothesis at this time. Similarly, the lack of any renal pathology in the rat or two mice strains, in contrast with the previ-
ous report of renal pathology in the Han:SPRD rats (8), suggests that an unknown factor between and even within species can influence the toxicity of a drug. Irrespective of the mechanism, the mice appeared to have developed hepatitis while on CrM.

The histological findings in the murine CrM-supplemented livers were identical to those of chronic hepatitis caused by persistent viral or bacterial infection, certain hepatotoxins, or autoimmune hepatitis. It is possible that there is some other infectious agent indigenous to mice and not rats that somehow conferred a differential sensitivity to CrM supplementation; however, common agents such as MHV and H. bili did not provide the answer. An autoimmune process was considered unlikely, for there were scattered histopathological findings in the rats that were not suppressed by concomitant methylprednisolone treatment. To rule this out in the mice, further studies with immunosuppressive agents and CrM given separately and in combination would help. A plausible explanation for the inflammatory changes could be the significant swelling that was seen at 56 days of age in the hepatocytes of the CrM-treated CD-1 mice. This swelling may have either represented the osmotic effects of the large increase in total creatine that occurs with CrM supplementation in mice (16), or it was a histopathological feature of a toxic stress. When hepatocytes are first challenged by pathological conditions, they typically exhibit a pale-staining or vacuolated appearance caused by fluid accumulation and swelling of membrane-bound organelles within the cells (6). Similar signs of chronic hepatitis and cirrhosis can be caused by long-term exposure to toxic levels of ethanol; however, the chronic hepatitis observed in the CrM-supplemented mice differed from ethanol-induced hepatitis in several ways. First, the inflammatory infiltrate consists mostly of lymphocytes instead of neutrophils as in alcoholic hepatitis (2). Second, there was no evidence of steatosis in the CrM-treated mice in contrast to that seen with ethanol hepatitis (10). Finally, alcoholic hepatitis and cell swelling are associated with ATP depletion and oxidative stress (6, 10), both of which would be unlikely with CrM supplementation (1, 4, 20).

In summary, CrM supplementation for one-third to one-half of the lifespan of CD-1 mice at doses that are just over those habitually consumed by humans induced hepatitis in most animals. Furthermore, a higher CrM dose given to transgenic mice that exhibit high levels of oxidative stress (SOD1 G93A) also caused hepatitis, yet did not induce any inflammation or other histological abnormality examined in any of the other body tissues and organs. CrM supplementation to rats, in supraphysiological doses for almost one-half their average lifespan, did not differentially induce hepatic inflammatory changes in the many tissues and organs that were sampled, including the liver.

DISCLOSURES

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