Original Article

Effects of dexamethasone-contaminated water on mouse growth and intestinal microflora composition

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Abstract: Objective: This study is to investigate the effects of dexamethasone-contaminated water on the mouse growth and intestinal microflora composition. Methods: Totally 20 Balb/c mice were divided into the control group, and the low-, medium-, and high-dose groups. Animal body weight was recorded, and the animal appearance and behavior were observed, after treatment. After animal sacrifice, organ weight analysis was performed. Intestinal microflora composition was analyzed with denaturing gradient gel electrophoresis (DGGE) and 16S rDNA V6 sequencing. Results: Messy fur and hostile behavior were observed in mice in the experimental groups. Compared with the control group (28.88±1.46 g), the mouse body weights in the low-, medium-, and high-dose groups (24.00±1.08, 26.17±1.76, and 25.43±0.95 g, respectively) were significantly declined (P<0.05). Moreover, for the experimental groups, the liver coefficient and spleen coefficient were significantly elevated (P<0.05), compared with the control group. DGGE profiling indicated stable intestinal microflora composition in mice. Principal component analysis (PCA) showed significant differences in the dominant microflora between the control and experimental groups. Microflora diversity analysis showed that, compared with the control group, the intestinal microflora amount (H'=3.74±0.0006368, 3.80±0.01153, and 3.84±0.00831, respectively) and species (S=15.61±0.548, 16.43±1.095, and 16.82±0.548, respectively) were significantly increased in the experimental groups (P<0.05). Moreover, the 16S rDNA V6 sequencing revealed totally 17 species of intestinal microflora, 15 of which were detected in all the groups. For the other 2 species, Lactobacillus bacteria existed only in the control group, while Shigella bacteria were observed only in the medium- and high-dose groups. Conclusion: Dexamethasone-contaminated water reduces the mouse growth, and alters the intestinal microflora composition. Dexamethasone contamination increases the microflora diversity and inhibits the probiotic colonization, facilitating the invasion of pathogenic bacteria.

Keywords: Dexamethasone sodium phosphate, water contamination, intestinal microflora, denaturing gradient gel electrophoresis (DGGE), 16S rDNA V6 region

Introduction

Over the past decade, dexamethasone drugs have been widely used in treating a variety of disorders, including autoimmune diseases, allergies, asthma, as well as skin and eye diseases [1]. Dexamethasone sodium phosphate is a commonly used dexamethasone formulation in clinic, characterized by its high aqueous solubility and easy absorption. Dexamethasone sodium phosphate is comparable in effectiveness with dexamethasone for the prevention and treatment of drug-induced allergy and fever with a cold. Moreover, dexamethasone sodium phosphate is one of the indispensable medicines in rescuing dying patients in emergency.

Clinically, no significant adverse reactions have been reported for the application of dexamethasone drugs at physiological doses. However, long-term or high-dose usage of dexamethasone drugs could lead to iatrogenic Cushing’s syndrome and immune suppression, which would aggravate the epilepticus status and inhibit the children’s growth, probably resulting in glaucoma and cataract [2]. Moreover, phar-
maceutical residues of dexamethasone drugs would become a contamination source, especially in the waste water in hospital. Furthermore, a wider range of water contamination might result from the body fluid secretion and urination from patients using dexamethasone drugs [3, 4]. Currently, the hospital waste water disinfection is limited to physical and biochemical methods, which mainly focus on removing the pathogens, radioactive substances, and heavy metals. However, these traditional disinfection methods are not able to effectively deal with the hormonal contaminants.

In recent years, dexamethasone contamination has been noted in several waters around the world [5-7]. Fortunately, no biohazard cases due to the water contamination have been reported yet. In this study, mouse models were forced to drink water containing dexamethasone sodium phosphate at contamination levels. The mouse growth was detected and observed, and the effects of contaminated water on the intestinal flora composition in these mice were also investigated.

**Materials and methods**

**Study animals and grouping**

Twenty 3-week-old male Balb/c mice (SPF), weighing 18±1.2 g, were purchased from the Experimental Animal Center of Chongqing Medical University. Animals were housed in a room with the humidity of 45%-65% at 22-25°C, in a light/dark cycle of 1:1. Based on the contamination doses previously reported [3, 6, 7] and the animal daily water intake (about 5 mL), these mice were randomly divided into (1) the control group (n=5), in which the mice were fed on normal drinking water and (2-4) the low-, medium-, and high-dose groups (n=5 per group), in which the mice were treated with 0.2 mL drinking water containing 0.035 ng, 0.225 ng, and 2.25 ng dexamethasone sodium phosphate (Haling Biological Technology Co., Ltd., Shanghai, China), respectively, once per day, for 35 consecutive days. The drinking water contaminated with dexamethasone sodium phosphate was freshly prepared each time. After treatment, the animal behavior and appearance were observed every day, and the animal body weight was recorded every week. All animal experiments were conducted according to the ethical guidelines of Chongqing Medical University.

**Organ weight analysis**

After treatment with contaminated water, these mice were sacrificed, and the liver, kidney, spleen, and bilateral femurs were removed under sterile condition. After washing with sterile saline and drying with absorbent paper, these organs were weighted, and the organ coefficient was calculated according to the following formula: the organ coefficient = organ wet weight/body weight × 100. After fixation, dehydration, and paraffin-embedding, the samples were cut into sections and detected with the HE staining.

**16S rDNA amplification**

Under sterile condition, a 2-cm segment of intestine near the ileocecal junction was removed, and the contents were discarded. After washing with saline, the tissue was cut into pieces with a scissor, and then triturated with saline. The bacterial DNA was extracted with the bacterial genomic DNA extraction kit (Tiangen, Beijing, China), according to the manufacturer’s instructions. PCR amplification was performed with the following prokaryote 16S rDNA V6 variable region universal primers containing a GC clamp [5]: V6-GC forward, 5’-CG-CCCGGGCGGCCCCGGGCGGGGCGGGGGCACGAGGCGCAGGCGGGGAAACGCGAAAGA AGAACCTTAC-3’ and V6-GC reverse, 5’-CGGTGTAC-3’. The 25-μL PCR system consisted of 12.5 μL 2 × rTaq Mix (Tiangen), 1 μL DNA template, 1 μL each primer, and 9.5 μL ddH₂O. The amplification program was as follows: denaturation at 95°C for 10 min; 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min, for 30 cycles; and 72°C for 10 min. The products were analyzed with 2% agarose gel electrophoresis.

**Denaturing gradient gel electrophoresis (DGGE)**

DGGE was performed for the analysis of 16S rDNA, according to a previously published protocol [8]. Briefly, the PCR products were loaded to an 8% polyacrylamide gel with 40%-60% urea as denaturing gradient. After electrophoresis, the gel was subjected to silver staining, and the images were acquired and analyzed with the Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA). The band richness (S) was on the DGGE profile was analyzed using the BIO-DAP software [Parks, Canada (PHQ) and National Park, Canada]. The Shannon-Wiener
Results

Mouse behavior, appearance, and growth

Effects of dexamethasone-contaminated water on the mouse behavior, appearance, and growth were first observed and investigated. Our results showed that, all the mice survived after the 35-d treatment with dexamethasone-contaminated water. Compared with the control group, hostile behavior and messy fur were observed in mice from the experimental groups, starting from day 6 of the contaminated water treatment. In the experimental groups, there were 12 mice whose tails had been injured or even bitten off. Repeated injuries and infection induced abnormal hyperblastosis and deformation in these mice (Figure 1).

For the body weight recording, on days 14 and 21 of the contaminated water treatment, the mouse body weights in the experimental groups were significantly declined compared with the control group ($P<0.05$) (Figure 2). For the organ weight analysis, compared with the control group, the liver and spleen coefficients were significantly higher in the experimental groups than the control group ($P<0.05$), while no significant differences were observed in the kidney coefficient ($P>0.05$) (Figure 3). Moreover, HE staining revealed no obvious abnormalities in all the organs and bilateral femurs in the experimental groups (data not shown). These results suggest that, the treatment of dexamethasone-contaminated water could influence the mouse behavior, appearance, and growth.

16S rDNA V6 region amplification

Bacterial genome DNA extracted from the ileocecal region was amplified, and the products were analyzed with 2% agarose gel electrophoresis.
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Figure 3. Organ weight analysis. After treatment with contaminated water, these mice were sacrificed. The liver, kidney, spleen, and bilateral femurs were removed and weighted, and the organ coefficients were calculated accordingly. Compared with the control group, *P<0.05, **P<0.01.

Figure 4. 16SrDNA V6 region amplification (lanes identified by the animal number; M, marker; N, negative control).

resis. As shown in Figure 4, specific 430-bp bands were observed in all the experimental groups, which were not observed for the control group. Based on the detection of the nucleic acid concentration detector, the concentration of amplified nucleic acid was >800 ng/μL, which could be used as template for the 16S rDNA V6 region amplification.

**DGGE profiling**

DGGE profiles of the 16S rDNA V6 region for the control and experimental groups were shown in Figure 5. Profile similarity was assessed with the Quantity one software, and the similarity tree algorithm was obtained with the UPGAM method accordingly (Figure 6). Our results indicated two categories of intestinal microflora composition in these samples, i.e., Category I (the control and low-dose groups) and Category II (the medium- and high-dose groups). In Category I, the similarity between the control and low-dose groups was 0.88, while in Category II, the similarity between the medium- and high-dose groups was 0.86. Moreover, the intestinal microflora similarity among the experimental groups (low-, medium-, and high-dose groups) was higher than 0.90. These results suggest stable intestinal microflora composition in mice from all the control and experimental groups. Moreover, the intestinal microflora composition for the experimental groups (especially the medium- and high-dose groups) is significantly different from the control group.

Figure 5. DGGE profiling of intestinal microflora 16S rDNA (lanes identified by the animal number).
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Principal component analysis (PCA) of the DGGE profile was next performed. The X-axis represents the first principal component PC1 (56.85%), and the Y-axis represents the second principal component PC2 (13.71%). The percentage indicates the main component contribution rate. As shown in Figure 7, the data points of the control and experimental groups were distributed in different regions, suggesting the differences in the dominant microflora species between these groups.

In the DGGE profiles, the dominant bands in each lane reflect the dominant intestinal microflora for each animal, and the band pattern reflects the specific intestinal microflora composition. Accordingly, the analysis of microflora species showed that, the control and experimental groups share majority of the intestinal microflora composition, with a few different microflora species as indicated by the differential DGGE pattern. Microflora diversity was also analyzed. The Shannon-Wiener diversity index (H') reflects the changes in the microflora amount and species, and the Pielou index (E) reflects the dominant species and its relative content. As shown in Table 1, compared with the control group, the H’ indexes were significantly increased in the experimental groups (P<0.05), especially for the medium- and high-dose groups. On the other hand, no significant differences were observed in the E index between the control and experimental groups (P>0.05). These results suggest that, there are significant differences in the amount and species of the intestinal microflora between the control group and experimental groups, while these mice might share similar dominant microflora species and relative contents.

16S rDNA V6 cloning and sequencing

Dominant bands in the DGGE profiles were extracted, amplified, cloned, and sequenced. Then the DNA sequences were blasted against the NCBI Genbank database. As shown in Table 2, the sequence alignment revealed totally 17 species, 15 of which were detected in all the control and experimental groups. For the other 2 species, Lactobacillus bacteria existed only in the control group, while Shigella bacteria were observed only in the medium- and high-dose groups.
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Moreover, as shown in Table 2, the inherent ileocecal microflora mainly included *Lachnospira*, *Blautia*, *Helicobacter*, and *Mucispirillum* bacteria. In the control and low-dose groups, *Lachnospira* bacteria were the most common species, accounting for 23.5% and 14.5% in the microflora community, respectively, followed by *Blautia*, *Helicobacter*, and *Mucispirillum* bacteria. On the other hand, in the medium- and high-dose groups, *Mucispirillum* bacteria ranked the first in the microflora community (accounting for 33% and 35.1%, respectively), followed by *Lachnospira*, *Blautia*, and *Helicobacter* bacteria. The specific *Lactobacillus* bacteria accounted for 1.2% in the microflora community in the control group, while *Shigella* bacteria accounted for 0.5% and 0.7%, respectively, in the medium- and high-dose groups. Taken together, these results suggest that, the drinking water contaminated with dexamethasone at indicated doses could alter the dominant bacteria species and the ileocecal microflora composition in mice.

**Table 1. Microflora diversity analysis of DGGE profile**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low-dose</th>
<th>Medium-dose</th>
<th>High-dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shannon-Wiener index (H’)</td>
<td>3.65±0.01096</td>
<td>3.74±0.0006368</td>
<td>3.80±0.01153</td>
<td>3.84±0.00831</td>
</tr>
<tr>
<td>Richness (S)</td>
<td>14.41±1.517</td>
<td>15.61±0.548</td>
<td>16.43±1.095</td>
<td>16.82±0.548</td>
</tr>
<tr>
<td>Pielou index (E)</td>
<td>0.98±0.004</td>
<td>0.98±0.003</td>
<td>0.99±0.003</td>
<td>0.99±0.002</td>
</tr>
</tbody>
</table>

Compared with the control group, *P*<0.05, **P**<0.01.

**Table 2. Intestinal microflora sequence alignment and composition analysis**

<table>
<thead>
<tr>
<th>Similarity</th>
<th>Control</th>
<th>Low-dose</th>
<th>Medium-dose</th>
<th>High-dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lachnospira</em></td>
<td>99%</td>
<td>28.7%</td>
<td>23.5%</td>
<td>15.7%</td>
</tr>
<tr>
<td><em>Blautia</em></td>
<td>97%</td>
<td>21.8%</td>
<td>19.5%</td>
<td>12.2%</td>
</tr>
<tr>
<td><em>Helicobacter</em></td>
<td>99%</td>
<td>14.5%</td>
<td>19.1%</td>
<td>17.4%</td>
</tr>
<tr>
<td><em>Mucispirillum</em></td>
<td>98%</td>
<td>11.3%</td>
<td>17.1%</td>
<td>33%</td>
</tr>
<tr>
<td><em>Alistipes</em></td>
<td>97%</td>
<td>4.9%</td>
<td>5.1%</td>
<td>5.3%</td>
</tr>
<tr>
<td><em>Ruminococcus</em></td>
<td>97%</td>
<td>4.5%</td>
<td>3.6%</td>
<td>2.8%</td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td>98%</td>
<td>2.2%</td>
<td>2.5%</td>
<td>3.2%</td>
</tr>
<tr>
<td><em>Roseburia</em></td>
<td>97%</td>
<td>1.8%</td>
<td>0.8%</td>
<td>1.1%</td>
</tr>
<tr>
<td><em>Desulfobulbus</em></td>
<td>99%</td>
<td>1.7%</td>
<td>2%</td>
<td>0.9%</td>
</tr>
<tr>
<td><em>Defluviicoccus</em></td>
<td>98%</td>
<td>1.6%</td>
<td>1.2%</td>
<td>1.1%</td>
</tr>
<tr>
<td><em>Rikenella</em></td>
<td>97%</td>
<td>1.5%</td>
<td>1.5%</td>
<td>1.7%</td>
</tr>
<tr>
<td><em>Oscillobacter</em></td>
<td>99%</td>
<td>1.4%</td>
<td>0.9%</td>
<td>0.7%</td>
</tr>
<tr>
<td><em>Odoribacter</em></td>
<td>97%</td>
<td>1.2%</td>
<td>1.4%</td>
<td>1.7%</td>
</tr>
<tr>
<td><em>Bacteroides</em></td>
<td>99%</td>
<td>1.1%</td>
<td>1.3%</td>
<td>1.5%</td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td>98%</td>
<td>0.6%</td>
<td>0.5%</td>
<td>1.2%</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>97%</td>
<td>0</td>
<td>0</td>
<td>0.5%</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>99%</td>
<td>1.2%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Moreover, as shown in Table 2, the inherent ileocecal microflora mainly included *Lachnospira*, *Blautia*, *Helicobacter*, and *Mucispirillum* bacteria. In the control and low-dose groups, *Lachnospira* bacteria were the most common species, accounting for 23.5% and 28.7% in the microflora community, respectively, followed by *Blautia*, *Helicobacter*, and *Mucispirillum* bacteria. On the other hand, in the medium- and high-dose groups, *Mucispirillum* bacteria ranked the first in the microflora community (accounting for 33% and 35.1%, respectively), followed by *Lachnospira*, *Blautia*, and *Helicobacter* bacteria. The specific *Lactobacillus* bacteria accounted for 1.2% in the microflora community in the control group, while *Shigella* bacteria accounted for 0.5% and 0.7%, respectively, in the medium- and high-dose groups. Taken together, these results suggest that, the drinking water contaminated with dexamethasone at indicated doses could alter the dominant bacteria species and the ileocecal microflora composition in mice.

**Discussion**

Dexamethasone is a kind of long-term glucocorticoid hormone, which could enter the water through a variety of ways. In recent years, glucocorticoid hormones have been found in the waste water at farms, hospitals, and factories, in which the contaminant concentrations are mainly between 7-450 ng/L [3, 6, 7]. Based on the daily water intake of each mouse (about 5 mL), the doses of dexamethasone sodium phosphate were set at 0.035 ng, 0.225 ng, and 2.25 ng in 0.2 mL drinking water, respectively, for the low-, medium-, and high-dose contamination groups. The effects of dexamethasone-contaminated water on the mouse growth and intestinal microflora composition in these mice were investigated.

Balb/c mice have been known for being docile and timid, which rarely fight with each other. In our study, from day 6 of the contaminated water treatment, messy fur and hostile behavior were observed in mice from the experimental groups. There were 12 mice whose tails had been injured or even bitten off. Repeated injuries and infection induced abnormal hyperplasia and deformation. These results suggest that, drinking water contaminated with different doses of dexamethasone could affect the mouse temperament (from docile to annoying anxious), which might be induced by the changes in the nervous system development. These findings were in line with the fact that, the long-term or high-dose usage of dexamethasone...
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would aggravate the epilepticus status in clinic. On day 35 of the contaminated water treatment, compared with the control group, the mouse body weight in the experimental groups were significantly reduced. Moreover, the enlargement of spleen and liver was observed in these mice, which was in line with previous findings [11].

Our results showed that dexamethasone-contaminated water could reduce the mouse growth, which was consistent with the fact that the long-term or high-dose usage of dexamethasone would inhibit the children's growth in clinic. Zhao et al. [12] have found that, the oral administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) together with dexamethasone (25 mg/kg) can induce cleft palate in mouse embryos. Moreover, Wei et al. [13] have shown that, water containing 2.5 μmol/L, 10 μmol/L, and 25 μmol/L dexamethasone could cause osteoporosis in zebrafish. In this study, histopathological examination showed no abnormalities in the liver, kidney, spleen, and bilateral femurs (data not shown). Further studies are still needed to investigate whether the water contaminated with great doses of dexamethasone and/or the long-term usage of dexamethasone would induce histopathological changes in mice.

From the perspective of probiotics, in humans and animals, the normal microflora could be divided into the abundant inherent microflora (>1%) and minor temporary microflora (<1%). In this study, the effects of dexamethasone-contaminated water on the intestinal microflora composition in mice were analyzed. Intestinal region was suitable for the investigation of inherent microflora [14]. DGGE profiles indicated stable microflora composition in the ileocecal region in mice, and water containing different doses of dexamethasone sodium phosphate would change the species and amount of ileocecal microflora, altering the proportion of dominant species, and increasing the microfloradiversity. Our results indicated that water contaminated with medium- and high-dose of dexamethasone significantly changed the intestinal microflora composition, while negligible changes were noted for the low-dose of dexamethasone contamination. Lactobacillus bacteria only existed in the control group, while Shigella bacteria were only seen in the medium- and high-dose groups. Lactobacillus bacteria comprise the key part of the intestinal microflora, which are nowadays widely used as probiotics in health foods, while Shigella bacteria are mostly recognized as the pathogenic bacteria in the intestinal region. The effects induced by dexamethasone-contaminated water on the intestinal microflora composition might be due to the fact that some bacteria could grow and metabolize on dexamethasone [15].

In conclusion, our results showed that, the mouse body weight would be significantly declined by the dexamethasone-contaminated water treatment, compared with the control mice. Moreover, the liver and spleen coefficients were significantly higher in the experimental groups treated with dexamethasone-contaminated water than the control group. DGGE profiling indicated significant differences in the intestinal microflora amount and species between the control and experimental groups. 16S rDNA V6 sequencing revealed totally 17 species of intestinal microflora, 15 of which were detected in all the control and experimental groups. For the other 2 species, Lactobacillus bacteria existed only in the control group, while Shigella bacteria were observed only in the medium- and high-dose groups. These results suggest that dexamethasone-contaminated water could reduce the mouse growth and alter the intestinal microflora composition. Dexamethasone contamination increased the microflora diversity and inhibited the probiotics colonization, which facilitated the invasion of pathogenic bacteria. These findings provide evidence that dexamethasone contamination in natural waters might be a potential threat for human health.

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Disclosure of conflict of interest

None.

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References


