Protein and mRNA expression of Shh, Smo and Gli1 and inhibition by cyclopamine in hepatocytes of rats with chronic fluorosis

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HIGHLIGHTS

- Excess fluoride may be able to participate in the pathway of Shh signaling.
- The Shh signaling pathway in hepatocytes of fluorosis rats can be blocked by cyclopamine.
- Interaction between Shh and other cell signaling pathways may be due to liver injury by fluorosis.

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ABSTRACT

In order to investigate the Sonic hedgehog (Shh) signaling pathway and the effect of cyclopamine in rat hepatocytes with chronic fluorosis, 48 Wistar rats were randomly divided into 4 groups. The control group was provided with tap water in which the fluoride concentration was <1 mg/L, while the remaining three groups were provided with water containing sodium fluoride (NaF) at a concentration of 50 mg/L. After 6 months, the blocking and blocking control groups were injected intraperitoneally once every 2 days for 6 days with 10 mg/kg cyclopamine or dimethyl sulfoxide, respectively. The urinary and skeletal fluoride contents were determined by the ion selective electrode method. Levels of aspartate transaminase (AST), alanine transaminase (ALT), total protein (TP) and albumin (Alb) in the serum were determined by using auto biochemical machine. Histological changes in liver tissue were evaluated with Hematoxylin & Eosin (H&E) staining using light microscopy. The protein and mRNA expression of Shh, Smo and Gli1 in hepatocytes of experimental animals was determined by immunohistochemistry (IHC), Western blotting (Wb) and Real-time quantitative PCR (RT-qPCR). Fluoride content of the urine and bone was increased in the fluorosis and blocking groups compared to those in the control group (P < 0.05), while fluoride content in the blocking group was decreased compared to the fluorosis and blocking control groups (P < 0.05). The expression of Shh, Smo and Gli1 at the mRNA and protein levels was significantly increased in hepatocytes from the fluorosis and blocking control groups compared with the control group, and expression in the blocking group was lower than that of the fluorosis and blocking control groups. The difference between any two groups was considered to be statistically significant (P < 0.05). Taken together, our study indicates that the expression of Shh, Smo and Gli1 at the protein and mRNA level in hepatocytes of rats with chronic fluorosis can be increased by fluoride and may be inhibited by cyclopamine and that the Shh signaling pathway plays an important role in the liver pathogenesis caused by fluorosis.

1. Introduction

Endemic fluorosis is a widespread public problem that negatively impacts human health in many parts of the world as a result of high fluoride content in groundwater and airborne fluoride released from the burning of fluoride-containing coal. Fluorosis is prevalent throughout the world, and Guizhou, China is the main endemic fluorosis region. Fluorosis causes damage not only to skeletal tissue and teeth but also to soft tissues such as liver, kidney and brain. Epidemiological investigations and in vivo studies have shown that fluorosis may interfere with the liver, particularly hepatocyte function and morphology (Yu et al., 1990; Sun et al., 2001; Guo et al., 2003; Li, 2004).

The Hedgehog (Hh) signaling pathway is crucial for proper development and differentiation of the liver during embryogenesis. The progenitor cells of mature liver survival depends on Hh signaling pathway (Sicklick et al., 2006). Additional studies have found that aberrant activation of Hh signaling, especially the Shh gene plays an important role in the development and occurrence
of liver diseases. However little is known about Hh signaling in endemic fluorosis. This study aimed to examine the role of the Hh signaling pathway in liver damage due to fluorosis in order to further understand the pathogenesis of endemic fluorosis as well as to reduce its occurrence in the future.

2. Materials and methods

2.1. Animals and treatments

Forty-eight Wistar rats approximately 4–5 weeks of age were fed for six months with standard solid feed containing less than 1 mg/kg fluoride (The Experimental Animal Center of the Third Military Medical University, SCXK (YU) 2007–0005). After one week the animals were randomly divided into 4 groups (N = 12 per group): Groups 1, 2, 3, 4 represented the control group, fluorosis group, blocking group and blocking control group, respectively. The numbers of males and females were equivalent within each group and sub-group. Except for the control group, which was given tap water, all animals were given water containing sodium fluoride (NaF) at a concentration of 50 mg/L. After 6 months, the fluorosis and blocking control groups were injected intraperitoneally with either cyclophamine or dimethyl sulfoxide (10 mg/kg) every once 2 days for 6 days, respectively. All the animals were killed by phlebotomy of the femoral artery, a portion of liver tissue was fixed in 4% neutral formalin, others were stored at −80 °C.

2.2. Chemicals and equipment

Total RNA extraction reagent Trizol (Invitrogen, USA), real-time quantitative PCR iQ™ SYBR® Green Supermix (Bio-Rad, USA), Real Time–PCR amplification ABI7300 cycler (ABI USA), Cycloamine (Selleck, USA), rabbit anti-mouse Shh, Gli1, Smo, β-actin polyclonal antibody (Santa Cruz, CA), GTVisionTM II immunohistochecmical detection kit (Gene Technology, China), diethyl pyrocarbonate (DEPC) (Promega, USA), PBS buffer (Beijing Zhongshan Golden Bridge Biotechnology, China), −80 °C ultra-low temperature freezer (Forma, USA), SB10 R-refrigerated centrifuge (Eppendorf, Germany), adjustable micro-pipettes (Eppendorf, Germany), Olympus CH20 microscope (Leica, Germany), Immun-Blot PVDF membrane (Bio-Rad, USA), Auto-biochemical machine AUS400 (OLYMPUS, Japan), chemiluminescent detection (Bio-Rad, USA), BIOMIAs-2001 high-resolution image analysis system (Institute of Image and Graphics Science University, China), Millipore ultrapure water meter (Millipore, USA), ELX800UV microplate reader (Bio-Rad, USA).

2.3. Experimental methods

2.3.1. Dental fluorosis analysis

After 6 months, dental fluorosis in rats was examined and evaluated according to the dental fluorosis indexing criteria of Dean (Wang, 2007): Degree I: tooth surface was yellow and white, with clear chalk-stripes; Degree II: tooth surface was dull with chalky white spots; Degree III: minor grooves and cracks in the surface of the tooth or partial loss, serration, or serious tooth defects.

2.3.2. Urine and skeletal fluorine

Urine fluorine was measured using an ion-selective electrode and skeletal fluorine was measured with a cineration-electrode.

2.3.3. Liver function

Liver function was investigated by analysis of aspartate transaminase (AST), alanine transaminase (ALT), total protein (TP) and albumin (Alb) in serum of rats with chronic fluorosis using an autobiochemical machine analyzer according to the manufacturer’s instructions.

2.3.4. Hematoxylin and eosin and immunohistochemical analysis

Rat liver samples were fixed in 10% neutral buffered formalin, processed, and paraffin-embedded sections were stained with Hematoxylin & Eosin (H&E). Morphological changes in H&E-stained liver tissue were examined using optical microscopy. The Envision two-step immunohistochemical method was utilized for detection of Shh, Smo and Gli1 expression using rabbit anti-mouse Shh, Smo and Gli1 polyclonal antibodies at a concentration of 1:100. Negative controls were incubated with PBS instead of primary antibody.

Positive staining for Shh, Smo and Gli1 was defined as the appearance of yellow or yellow-brown granules in the cytoplasm and nucleus. After routine observation, the immunohistochemical results were analyzed with a BIOMIAs-2001 high-resolution image analysis system. Five non-overlapping fields (400×) were randomly selected in each section for the detection of IOD (Integrated Optical Density) of Shh, Smo and Gli1. The IOD value was utilized to represent expression intensity, where larger IOD values reflect increased expression.

2.3.5. Western blot analysis

Expression of Shh, Smo and Gli1 was measured by Western blot using Shh, Smo and Gli1 antibodies. Equivalent amounts of protein (40 μg per lane) were loaded onto a 10% Tris–glycine gel, separated by electrophoresis, and transferred to an

### Table 1

Real-time PCR primer sequences.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5′-3′)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FCAAGTGACGCCAAGCACTCAAGG</td>
<td>123</td>
</tr>
<tr>
<td>Shh</td>
<td>FATTGCTAGCCAGGCAAGGG</td>
<td>131</td>
</tr>
<tr>
<td>Gli1</td>
<td>FGAGCTGAGCTGACATCCCA</td>
<td>195</td>
</tr>
<tr>
<td>Smo</td>
<td>FGGCGTCTTCTTGGTGGCC</td>
<td>133</td>
</tr>
</tbody>
</table>

Immun-Blot PVDF membrane. Membranes were incubated in blocking buffer (5% Bovine serum) for 1 h and then probed with Shh, Smo, Gli1 (1:200) or β-actin (1:500) antibody overnight at 4 °C. Membranes were rinsed with wash buffer (TBST) and incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h at room temperature, followed by chemiluminescent detection.

2.3.6. Real-time quantitative PCR analysis

Rat liver samples were assayed for Shh, Smo and Gli1 mRNA expression using quantitative real-time PCR. Total RNA was extracted with Trizol reagent according to the manufacturer’s instructions. RNA purity was tested by gel electrophoresis, and indicated by an optical density ratio of 1.8~2.0. Isolated RNA was stored at −80 °C until further analysis.

cDNA was prepared according to manufacturer’s protocol. Reverse transcription reactions were assembled as follows: 1 μl total RNA (1 ng), 1 μl Oligo (dt) 18 primer, 4 μl 5× reaction buffer, 1 μl Ribohock RNase inhibitor (20 U/μl), 2 μl 10 mM dNTP Mix, 1 μl Revert-aid M-MUL Reverse Transcriptase (200 U/l), 10 μl ddH₂O (total volume of 20 μl). According to Genbank retrieve SD rats housekeeping gene sequences of Shh, Gli1 from primers of Shanghai Generay Biological Engineering were designed and synthesized. Primer sequences are listed in Table 1. qPCR reactions were assembled as follows: 1 μl primer (2 μM), 1 μl cDNA, 10 μl FastStart Universal SYBR Green Mastermix with ROX, 8 μl PCR grade H₂O (total reaction volume of 20 μl). Sterile DEPC water was used as a negative control instead of 1 μl cDNA template. PCR cycling parameters were as follows: 95 °C for 10 min; 95 °C for 15 s, 60 °C for 30 s (40 cycles); 60 °C for 10 min to terminate the reaction, and a 4 °C hold. With SYBR green I was a fluorescent marker to internal reference as an internal reference, according to each specimen measured in the number of cycles (Ct) value, calculate to 2−ΔΔCt relative quantification of the target gene that test results (Livak and Meserschmitt, 2001).

2.4. Statistical analysis

In statistical analysis of all experimental data, results were expressed as x ± s, and statistical significance was assessed by T test or F test. Statistical significance among groups was determined using analysis of variance (ANOVA) with subsequent Least Significant Difference (LSD) test using SPSS (19.0) software. The level of significance was set at P < 0.05.

3. Results

3.1. The situation of rat feeding and dental fluorosis

During the treatment period, control rats exhibited smooth fur, healthy appetites, normal activity level, and showed no significant tooth lesions. With increased time of exposure to fluoride, experimental rats appeared listless, and exhibited loss of appetite, decreased activity level, and dull–appearing fur.

While dental fluorosis was not observed in control rats over the six-month course of study, the occurrence of dental fluorosis in the fluorosis group was 88.89% (32/36). The incidences of fluorosis in the fluorosis, blocking and blocking control group were 91.67%, 91.76% and 83.33%, respectively (Table 2).

3.2. Urinary and skeletal fluorine contents

The urinary and skeletal fluorine contents of rats in the fluorosis and blocking control groups were significantly increased compared to the control group, while the fluorine contents in the blocking group were lower than those in the fluorosis and blocking control
Table 2
The incidence of dental fluorosis in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Animal no.</th>
<th>Case of dental fluorosis</th>
<th>Happen</th>
<th>Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fluorosis</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blocking</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blocking control</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3
Alterations in hepatocyte function in rat livers.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TP (g/L)</th>
<th>ALB (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.85 ± 7.79</td>
<td>128.37 ± 16.61</td>
<td>75.60 ± 8.54</td>
<td>39.05 ± 5.53</td>
</tr>
<tr>
<td>Fluorosis</td>
<td>65.15 ± 12.78</td>
<td>68.00 ± 69.48</td>
<td>67.20 ± 4.64</td>
<td>33.10 ± 2.61</td>
</tr>
<tr>
<td>Blocking</td>
<td>47.00 ± 10.05</td>
<td>220.07 ± 36.79</td>
<td>79.70 ± 5.53</td>
<td>41.45 ± 3.09</td>
</tr>
<tr>
<td>Blocking control</td>
<td>54.78 ± 9.47</td>
<td>149.25 ± 17.61</td>
<td>71.50 ± 5.95</td>
<td>36.62 ± 2.92</td>
</tr>
</tbody>
</table>

3.5. Real-time qPCR analysis of Shh, Smo and Gli1 mRNA expression in hepatocytes

Real-time qPCR analysis indicated that expression of Shh, Smo and Gli1 mRNA in rat hepatocytes were increased in the fluorosis group. In particular, expression of Shh, Smo and Gli1 in the fluorosis and blocking control groups showed a greater increase compared to their corresponding control group. The decrease in expression of Shh, Smo and Gli1 in the blocking group was lower than that of the fluorosis and blocking control groups. Except for the fluorosis and blocking control group (P > 0.05), the difference between any two groups was statistically significant (P < 0.05) (Fig. 4).

4. Discussion

This study expands upon our previously described in vivo model of chronic fluorosis (Chen et al., 2013), in which it was demonstrated that rats fed water containing 50 mg/L fluoride for 6 months exhibited dental fluorosis as well as increased urinary and bone fluoride content, consistent with chronic fluorosis.

The liver, one of the important detoxification organs, is mainly involved in the regulation of metabolism, detoxification and other functions of the body. Liver damage in rats with chronic fluorosis is characterized by hepatocyte degeneration, necrosis, fibrosis and apoptosis following fluoride exposure, and has been previously associated with oxidative stress, apoptosis, and activation of signaling pathways (Wang et al., 2004; Jose et al., 2010). In the current study we found that fluoride exposure can result in liver injury, which is consistent with other studies (Guo et al., 2003; Sun et al., 2001). Additionally, in experiments examining biochemical serum markers of liver function, we observed that, compared with the control group, the fluorosis groups showed increased activity of AST and ALT in contrast to decreased activity of TP. Based on our experimental data and previous studies (Li et al., 2012; Sun et al., 2001; Mei et al., 2007), we hypothesize that this may be due to the low fluoride concentration in the body and generation of free radicals caused by excessive fluoride intake, as well as fluoride metabolism, leading to liver damage.
The Hh signaling pathway was first discovered in Drosophila (Nüsslein-Volhard and Wieschaus, 1980) and three homologous Hh genes have been identified in mammals: Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog, which encode Shh, Ihh and Dhh proteins, respectively. Shh, one of the largest potential and most widely expressed Hh homologues, is involved in asymmetrical development of the embryonic limb buds and development of the central nervous system (Ehlen et al., 2006). Hh proteins interact with the membrane-bound receptor Patched (Ptc), which is not only a tumor suppressor gene, but also represses downstream target genes of the Hh signaling pathway thus contributing to the formation of a self-regulating mechanism of negative feedback. Smo (Smo), another key molecule in the Hh signaling pathway, belongs to the G protein-coupled receptor superfamily. Smo plays a key role in transduction of the extracellular-to-intracellular Hh signal, and is responsible for transduction of the intracellular signal to the nucleus and activity of downstream Hh target genes (Deneff et al., 2000). The zinc finger transcription factor Gli family downstream of Hh includes Gli1, Gli2, and Gli3; Gli1 is the only Gli family member that can bind to Ptc, which activates Smo; subsequently the Hh signal is transmitted from Smo to Gli1 however, the mechanism is not well understood in vertebrates. Here, we describe the expression of Shh, Smo and Gli1 in all fluorosis treatment groups as well as the relationship of fluorosis and Hh signal transduction. In the present study we found stable and comparatively lower expression of Shh, Smo and Gli1 in livers of normal rats; moreover, there was upregulation of Shh, Smo and Gli1 in livers of rats with chronic fluorosis. This is consistent with the findings of others that when compared with normal liver tissue, Shh, Smo and Gli1 were over-expressed in some liver diseases, including non-alcoholic fatty liver, alcoholic liver disease, liver cancer, liver fibrosis, regenerating liver after partial hepatectomy and that liver tissue damage (Jung et al., 2008; Choi et al., 2011; Jin Hai et al., 2012; Hirsova et al., 2013; Hyun et al., 2013; Jeng et al., 2013; Sviderska-Syn et al., 2013). We hypothesized that a closely association exists between the Hh signaling pathway and liver damage associated with fluorosis, and that excess fluoride could activate and upregulate the Shh signaling pathway, which may have long-lasting consequences for normal liver function via effects on of stem/progenitor cell subsets, which normally function to maintain homeostasis under various pathological conditions ( Sicklick et al., 2006), thus leading to liver injury.

The Hh signaling pathway can be specifically suppressed by small molecule inhibitors. The Shh neutralizing antibody 5E1 acts as a Shh antagonist to inhibit the Hh signaling pathway. Rab23 acts as a negative regulator of gene expression, and can reduce the expression of Smo and enhance the expression of Gli1 to impact subcellular localization of Shh signaling pathway members (Evans et al., 2003; Eegenschwiler et al., 2006). Cyclopamine is a steroidal alkaloid obtained from separation and extraction of Veratum lily plants, and it can have direct effects on Smo in the Hh signaling pathway, leading to inhibition of the Hh signaling pathway and is considered to be a Hh signaling pathway-specific inhibitor (Vanden Heuvelton, 1996; Karreni et al., 2004; Feldmann et al., 2007; Liao et al., 2009). In our experiments, it was demonstrated that the expression of Shh, Smo and Gli1 could be partially inhibited by cyclopamine which interfered with the Hh signaling pathway. In addition, the Shh/Pi3K/ Bcl-2 signaling pathway can be inhibited by cyclopamine, showing not only that Shh signaling can be blocked, but that cyclopamine can also reduce the activate of Pi3K/Akt, NF-κB, and MAPK signaling pathways, resulting in apoptosis (Dormoy et al., 2009); and when oxidative stress induced secretion of internal and external sources of Shh can play a protective role in astrocytes (Xia et al., 2012; Dai et al., 2011). There was a reason speculated: the induction of Hh was partly accelerated at the transcriptional level, leading to and increase in expression at the level of translation and transcription. In addition, recent studies (Bijlsma et al., 2006) have shown vitamin D can inhibit the Hh signaling pathway. While cyclopamine can directly bind to Smo leading to inhibition of Hh signaling, VitD not only bound with high affinity to Smo, but also possibly acted via other mechanisms, such as oxidative stress. Many studies of fluorosis have shown that vitamins, minerals, proteins and other natural products have the potential to reduce the symptoms of fluorosis, thereby reducing its occurrence (Asimuguli–Kelimu et al., 2008).

In addition, there may also be interactions between OPN, Wnt, MAPK, NF-κB and other cell signaling pathways, including the Shh signaling pathway (Fig. 5) in the pathogenesis of fluorosis-induced liver damage. Das et al. (2009) and Syn et al. (2011) found that Hh signaling pathway could regulate osteopontin (OPN) at the level of transcription, as OPN is primarily regulated by the transcription factor Gli1, a key downstream effector of Hh. At the same time, OPN can promote liver fibrosis via Hh signaling in cases of non-alcoholic fatty liver. Zhou et al. (2011) also found that fluoride exposure can promote the OPN expression in rat liver tissue. It was speculated that the Hh signaling pathway is closely related to liver damage in rats with chronic fluorosis; however, other researchers have shown (White et al., 2003; Kessaris et al., 2004; Qu et al., 2013) that Wnt can inhibit and induced expression by the Hh signaling pathway. Gli1 can regulate the interaction between Wnt, Shh and MAPK, but Shh itself does not seem to activate MAPK, and while Hh and NF-κB signaling were positively correlated, Shh can not only activate NF-κB, but Shh and CyclinD1 also synergize to regulate the NF-κB signaling pathway. Other studies (Chen et al., 2013; Guan Zhizhong et al., 2011) have also shown that excessive fluoride can activate the Shh signaling pathway and promote the expression of Shh target genes in livers of rats with chronic fluorosis; meanwhile fluoride can stimulate the over-expression of Wnt3a and β-catenin in the Wnt signal transduction pathway, enhance osteogenesis and cause skeletal fluorosis in chronic fluorosis, thus there is a synergistic effect between MAPK and the Hh signaling pathway. Moreover, fluoride can affect hepatocyte apoptosis, leading to liver injury through the NF-κB signaling pathway. It was speculated that the Wnt, MAPK, NF-κB and Shh signaling pathways may be cooperating to induce liver damage as a result of fluorosis, leading to

Fig. 2. Histological changes in liver tissue were observed with Hematoxylin and Eosin (H&E) staining. Control group (a), liver injury in fluorosis group (b), blocking group (c) and blocking control group (d).
Fig. 3. The expression of Shh, Smo and Gli1 protein in the hepatocytes of the different groups. (A) IHC staining of rat livers (fluorosis group) showing expression of Shh, Smo and Gli1. (B) Protein expression of Shh, Smo and Gli1 in livers of rats with fluorosis were increased in the fluorosis and blocking control groups compared to the control group; however, expression in the blocking group was increased compared to the fluorosis and blocking control groups. (a) P < 0.05, relative to the control group; (b) P < 0.05 relative to the blocking group. (C and D) Western blot analysis of hepatocyte extracts from the control group not exposed to fluoride (lane 1), the fluorosis group exposed to 50 mg/L fluoride (lane 2), the blocking and blocking control groups exposed to 50 mg/L fluoride followed by injection of cyclopamine or DMSO (lanes 3 and 4, respectively).
changes in gene expression, cell proliferation and apoptosis. But thus far the precise mechanisms of liver injury due to fluorosis and the interaction of multiple genes and gene regulation in distinct signaling pathways are not known.

Combined with all related knowledge above, it was of great significance that the Shh signaling pathway could be a potential therapeutic target for endemic fluorosis as well as for prevention, diagnosis and treatment of fluorosis. However, the Shh signaling pathway in the liver was affected by fluoride and intervened by cyclopamine, but the detailed underlying mechanisms of fluorosis in liver is unknown. Further research will be performed to determine how excessive fluoride influences Hh signaling and other signal pathways, as well as if cyclopamine, vitamins and other natural products could effectively inhibit liver injury in chronic fluorosis and whether there are some connections among that.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References


