Study the therapeutic effect of capsule CTHepaB on nude mice carrying human hepatocellular carcinoma cells infected with hepatitis B virus

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ABSTRACT

Objective: To assess the effect the therapeutic effect of CTHepaB capsules on nude mice carrying human hepatocellular carcinoma cells (HCC) infected with hepatitis B virus (HBV). Method: Nude mice after HCC transplant Hep3B infected with HBV on the right thigh, appeared a tumor on the thigh on the 14th day of transplantation, had an average size of 74.6 ± 14.3 mm³), randomly divided into 2 groups of 15 rats each and randomly divided into 3 lots (5 mice / lot): in the control group, drink 0.9% NaCl solution; Treatment, oral dose of CTHepaB 0.96g / kg / 24h; Reference batch, oral dose of 5FU 10mg / kg / 24h. Give the rats the drug according to the above division for 4 weeks. Group 1 used to evaluate the effect of research drugs on tumor size, survival / death rate; Average lifetime of the mouse. Group 2, used for splenectomy to evaluate the densit of some immune cells and the HBV-DNA quantification of tumor cells. Results: CTHepaB capsules reduced the average tumor size compared to the control group, equivalent to that of the group using 5FU at dose of 10mg/kg/24h; Limiting the death rate of mice at the evaluation points 70, 76, 85, 90 days after taking the drug compared to the control group and on 85, 90 days, compared with the reference group; increases average survival time of mice; Increased number of NK cells, Macrophage cells and DC cells in mouse spleen; Reduces the amount of HBV-DNA in mouse tumor cells. Conclusion: CTHepaB capsule with dose of 0.96g/kg/24h has good therapeutic effect, in nude mice carrying HCC infected with HBV.

Keywords: CTHepaB, hepatocellular carcinoma, hepatitis B virus, hepatitis B virus-infected hepatocellular carcinoma cell transplant model
1. INTRODUCTION

Cancer is a dangerous disease with a high mortality rate. According to the International Agency for Research on Cancer (IARC), in 2008, there were about 12.7 million new cases around the world with 7.6 million deaths from cancer. Forecast by 2030, each year, 21.4 million new people will get cancer and 13.3 million people will die from this disease. The main risk factor for liver cancer is chronic hepatitis B virus (HBV) infection. People who are chronically infected with the hepatitis B virus are 100 times more likely to develop liver cancer than non-infected people, because the virus attacks it directly and repeatedly, causing cirrhosis and leading to cancer. Phan Thi Phi Phi examining 1,251 patients with liver disease detected 193 patients with HCC (15.4%), screened 4,677 people in the population, and found 2 patients with HCC (Phan, 1993).

One of the achievements in cancer research, since the late 20th century, scientists have created mice that carry human cancer (Nguyen, 2018; Ho, 2012). Faced with the challenge of the level of malignancy and resistance to treatment of liver cancer, today people are developing new therapies and pharmaceuticals, especially active plant-derived active ingredients must have a pathological model to apply to preclinical testing. Human cancer cells are tolerated and mice will carry human cancer. On this mouse model of human cancer, tumor formation and development were assessed and applied to detect and treat cancer patients (Teng et al., 2016; Jilkova et al., 2019). CTHepaB capsules are made from the remedy CTHepaB, which include a number of medicinal herbs such as Solanum hainanense Hance, Ophiocordyceps sinensis, Ganoderma lucidum, Rheum palmatum Bail (Trinh, 2014; Nguyen, 2014; Nguyen, 2014; Gong et al., 2000; Ha et al., 2004) has been shown to have a protective effect on liver cells, inhibition of HBV replication, inhibit the development of cirrhosis, liver cancer etc., creating a premise for the development of herbal products for liver cancer prevention and treatment.

Therefore, the thesis was conducted with the aim: to evaluate the effects of CTHepaB capsules on immunocompromised mice carrying HCC cells infected with HBV.

2. MATERIALS AND METHODS

Research subjects and materials

Research drug: CTHepaB capsules are formulated from CTHepaB remedy with ingredients: Solanum procumbens 15g, Euphorbia thymifolia 10g, Gardenia jasminoides 5g, Rheum palmatum 2.5g, Polyscias fruticosa 5g. Ophiocordyceps sinensis 2.5g, Ganoderma lucidum 5g, Fallopia multiflora 5g. The medicinal herbs meet the Vietnamese Pharmacopoeia V standards. CTHepaB capsules are prepared at Military Medical Academy, meeting the basic standards.

Reference drug: 5-Fluorouracil (5FU), anticancer drug, anti-metabolic type.

Experimental animals: mice BALB/c are immune deficient, do not have T lymphocytes (nude mouse, Foxn1nu) 8-10 weeks old, weigh 160-180g. These mice will be transplanted Hep3B human HCC cells infected with HBV to the right thigh of mice to create liver cancer cell mass of people infected with hepatitis B virus. 30 nude mice after transplantation were tumor in the right thigh (with Hep3B liver carcinoma cells infected with B virus)

Chemicals used in research: Hep3B liver carcinoma strain Hep3B infected with HBV; Cell culture and preservation medium: EMEM (Eagle’s Minimum Essential Medium) medium, Catalog No.30-2003, ATCC supplemented with 10% FBS and 1% antibiotic penicillin and streptomycin and some other chemicals.

Research equipment: Laboratory system for cell culture: clean room, CO2 incubator, reverse microscope, centrifuge, 40°C cooler, -20°C, -80°C, liquid nitrogen storage tank, Cellular flow counter planing; Mouse cage system with independent ventilation and air filtration; Clean room for mice with immune deficiency; Temperature and humidity control system; Biochemical automated testing machine; Small animal slaughter kits and other laboratory tools.

Research Methods

Study duration
from April 2020 to April 2021

Hep3B liver carcinoma cell transplant with HBV infection in the thigh of a nude mouse (immune deficient mouse)

Hep3B liver carcinoma cells infected with HBV cultured in EMEM medium, supplemented with 10% FBS and 1% antibiotic penicillin and streptomycin. Each 75 cm2 culture bottle inoculated contains 0.3-0.4 x 10^6 cells. Cells were cultured to proliferate and change the medium 3 times / week at temperatures of 37°C, CO2 5%. When cells grow to 75-80% area, transfer to new bottles. Fixed nude mouse and inject 0.1 ml of prepared cancer cell solution (10^6 cell/ rat) under the skin of the right thigh.
Evaluate the therapeutic effect of CTHepaB capsules in mice with hepatocellular carcinoma infected with HBV

After the appearance of tumor in mice (14 days since transplantation, when the tumor has formed and developed, with size of 70-75 mm³; (average 74.6 ± 14.3 mm³). Rats were randomly divided into 2 groups of 15 mice each: Group 1, used to evaluate the effect of research drugs on tumor size, survival / death rate; Average lifetime of the mouse; Group 2, was used to evaluate the effect of research drugs on the density of some immune cells in mouse spleen and to quantify HBV-DNA of tumor cells. Each group of mice 15, was randomly divided into 3 lots (5 mice / lot): in the control group, drink 0.9% NaCl solution; Treatment, oral dose of CTHepaB 0.96g / kg / 24h; Reference batch, oral dose of 5FU 10mg / kg / 24h. The 14th day of transplantation was counted as day 0 of the cancer-carrying mice. Give the rats the drug according to the above group for 4 weeks. CTHepaB oral administration stopped when all mice in the control group died.

Evaluation indicators

* Tumor size, survival / death ratios; Average survival time of rat groups
  + Tumor size in mice: by observing, palpating and measuring tumor length and width by NSK exact size, 1 time / week.
  + Tumor volume (mm³) = D (length) x R² (width) x 0.5
  + Tumor sizing was stopped when rats died in any of the groups; others were still monitored to determine their survival.
  + Survival time of groups: rat life was calculated from cancer cell transplantation. The monitoring of the lifetime of each group of mice stopped when all mice in the control or reference group died.

Density of some immune cells in the spleen of mouse groups

After finish taking the drug, dissecting the rats and taking the spleen to assess the density of some immune cells in the spleen groups of mice: rate of NK cell (natural killer cell-NK), macrophage (macrophages DC cells (Dendritic cells) by antibody-specific staining and running flowcytometry.

Quantitative HBV-DNA of tumor cells

Quantitative HBV-DNA of tumor cells after treatment in the study groups.

Data processing

The data were processed according to the biomedical statistical methods, compared with anova, after the Turkey test, using SPSS 16.0 software. The data are presented as X ± SD.

3. RESULTS

Results of liver carcinoma transplantation infected with hepatitis B virus in the thighs of nude mice

Results on day 14 after transplantation, 100% mice formed and developed tumors, size reached 70-75 mm³ (average 74.6 ± 14.3 mm³) (Figure 1). At this time it was counted as day 0 of the cancer-carrying mice while undergoing treatment.

Figure 1 Image of tumor formation and development in the right thigh of mouse

Results of evaluation of the therapeutic effect of CTHepaB capsules in mice with hepatocellular carcinoma infected with HBV

Table 1 shows that, during the initial phase (≤ 35 days) of treatment, there was no difference in the mean cancer tumor size of the rat groups, p > 0.05. The mean tumor sizes in both the treatment and reference groups were smaller than in the control group starting day 42, and the differences were significant, p < 0.05. Comparing between the group using CTHepaB and the group using
5FU, the group using 5FU tended to reduce tumor size better than the group using CTHepaB, but the difference was not statistically significant, \( p > 0.05 \).

**Table 1** Tumor size of mice studied

<table>
<thead>
<tr>
<th>Time</th>
<th>Control lot (1)</th>
<th>Treatment lot (2)</th>
<th>Reference lot (3)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>74.35 ± 15.26</td>
<td>74.69 ± 16.52</td>
<td>74.59 ± 18.21</td>
<td>0.16</td>
</tr>
<tr>
<td>Day 7</td>
<td>221.62 ± 19.64</td>
<td>218.65 ± 21.06</td>
<td>212.32 ± 20.68</td>
<td>0.11</td>
</tr>
<tr>
<td>Day 14</td>
<td>591.45 ± 62.24</td>
<td>586.91 ± 61.33</td>
<td>580.92 ± 71.02</td>
<td>0.11</td>
</tr>
<tr>
<td>Day 21</td>
<td>792.47 ± 96.93</td>
<td>789.64 ± 102.33</td>
<td>779.96 ± 101.54</td>
<td>0.06</td>
</tr>
<tr>
<td>Day 35</td>
<td>986.36 ± 102.62</td>
<td>968.46 ± 116.48</td>
<td>960.95 ± 121.09</td>
<td>0.06</td>
</tr>
<tr>
<td>Day 42</td>
<td>1194.84 ± 106.56</td>
<td>1126.19 ± 112.44</td>
<td>1118.95 ± 116.36</td>
<td>0.05</td>
</tr>
<tr>
<td>Day 49</td>
<td>1356.49 ± 121.43</td>
<td>1268.42 ± 132.46</td>
<td>1259.28 ± 139.54</td>
<td>0.05</td>
</tr>
<tr>
<td>Day 56</td>
<td>1562.96 ± 216.83</td>
<td>1432.68 ± 236.71</td>
<td>1425.89 ± 241.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Day 63</td>
<td>1792.64 ± 261.85</td>
<td>1596.67 ± 283.64</td>
<td>1588.24 ± 279.44</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**Table 2** Number of mice that survived in experimental plots

<table>
<thead>
<tr>
<th>Lot mouse</th>
<th>Number of days from the start of taking the drug</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td>Control lot (1)</td>
<td>n</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>TB ± SD</td>
<td>68.60 ± 5.77</td>
</tr>
<tr>
<td>Treatment lot (2)</td>
<td>n</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>TB ± SD</td>
<td>81.80 ± 8.61</td>
</tr>
<tr>
<td>Reference lot (3)</td>
<td>n</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>TB ± SD</td>
<td>79.20 ± 8.11</td>
</tr>
</tbody>
</table>

\( p \)        \( p_{2,1} > 0.05 \) \( p_{2,1} < 0.05 \) \( p_{2,2} > 0.05 \) \( p_{2,2} < 0.05 \)

Table 2 shows that control lot (without using drugs): at 63 days (from the time the drug started), the mice died, to the day 85 all rats died. In the group using 5FU, the number of mice died was faster than that of the group using CTHepaB. At the end of the experiment (90 days), the number of rats in the reference lot had died. The group used CTHepaB at the end of the experiment (90 days) to have 1 live mouse (rate 20.0%). The difference is statistically significant with \( p <0.05 \). The mean survival time of rats in the treatment and reference lots were both significantly longer than in the control lot (81.80 and 79.20 versus 68.6 days), with \( p < 0.05 \). Compared to the reference lot, the treatment lot had a longer study period, but the difference was not statistically significant (\( p > 0.05 \)).

**Table 3** Density of some immune cells in mouse spleen (average ± SD)

<table>
<thead>
<tr>
<th>Density of some immune cells in mouse spleen</th>
<th>Control lot (1)</th>
<th>Treatment lot (2)</th>
<th>Reference lot (3)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of NK cells (x 10^6)</td>
<td>1.26±0.24</td>
<td>1.43±0.19</td>
<td>1.16±0.21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Percent cell Macrophage</td>
<td>5.86±0.35</td>
<td>6.97±0.82</td>
<td>5.24±0.71</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Number of DC cells</td>
<td>2.35±0.29</td>
<td>3.51±0.43</td>
<td>2.08±0.53</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Table 3 and Figure 2 show that, in the lot treatment: the number of NK cells, the percentage of Macrophage cells and DC cells in the mouse spleen increased compared to the control group, as well as compared to the reference lot, respectively (1.43 vs 1.26 and 16 x 10^6 cells); (6.97 vs. 5.86 and 5.24) and (3.51 vs. 2.35 and 2.08), Meaningful difference is p < 0.05.

Figure 2 Density of some immune cells in mouse spleen

Table 4 Quantitative results of HBV-DNA in mouse tumor cells

<table>
<thead>
<tr>
<th>HBV-DNA (x 10^5 UI)</th>
<th>Control lot (1)</th>
<th>Treatment lot (2)</th>
<th>Reference lot (3)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average ± SD</td>
<td>12.68±2.93</td>
<td>10.84±2.61</td>
<td>12.36±2.84</td>
<td>p2,3&lt; 0.05; p2,&gt; 0.05; p1,&gt; 0.05</td>
</tr>
</tbody>
</table>

Table 4 shows that, in the 5FU lot, the amount of HBV-DNA in mouse tumor cells was smaller than in the control lot, but the difference was not statistically significant, p > 0.05. In the lot using CTHepaB, the amount of HBV-DNA in tumor cells decreased compared to the control lot, as well as compared with the lot using 5FU, p < 0.05.

4. DISCUSSION

About the mouse tumor size change

Hepatitis B virus-infected hepatocellular carcinoma cell transplant studies in nude mice have been successful, as well as progression of cancer formation has provided an implantable model in animals that can treatment trials (Teng et al., 2016), and effective screening of new anti-HCC drugs (Jilkova et al., 2019). In our study, when tumors formed, after just 1 week (from day 0 to 7) the size of tumors in all lots increased by 3 times. Our research results are also consistent with those of the authors (Nguyen, 2018; Nguyen, 2013; Kubota et al., 1994).

Rats were given the drug for 4 weeks, the tumor size in the lots of mice using CTHepaB and 5FU at the following times, decreased compared to the lot group. A statistically significant difference was achieved, starting at day 42 from the initiation of the drug. Thus, the effects of the drug may have begun to work from the very beginning when taking CTHepaB and 5FU, then tumor cells were inhibited, inhibiting growth, and gradually becoming apparent at later times, as tumor size grew slowly, while tumor cells in the control lot were still growing with the fast speed, the tumor size is much larger than the drug lot.

Some medicinal herbs in CTHepaB capsules have anti-inflammatory, antioxidant and antibacterial effects such as Solanum hainanense Hance, Euphorbia thymifolia, Ganoderma lucidum, Gardeniae jasminoidis Ellis, Rheum palmatum Bail. Some herbs have the effect of nourishing the body, enhancing immunity such as Ophiocordyceps sinensis, Polyscias fruticosa (L.) Harms, Fallopia multiflora Thunb, Solanum hainanense Hance, Rheum palmatum Bail. It is also proven to contain ingredients that inhibit the growth of hepatitis B virus, cancer cells, anti-oxidants, prevent cirrhosis (Nguyen, 2014); Thus, the tumor growth inhibitory effect of CTHepaB capsules may have many different supportive mechanisms: immune enhancement mechanism, tumor cell suppression mechanism, anti-inflammatory mechanism, antioxidant.

Effect of reducing mortality, increasing survival time of mice

Rats in the group receiving CTHepaB had a higher survival rate than the control lot, at the time of assessment 70, 76, 85, 90 days after taking the drug, and even higher than the reference lot using 5FU dose of 10mg / kg / 24h at the times of 85 and 90. The mean survival time of mice in the lot using CTHepaB was also higher than that in the control lot (p <0.05), and also higher than the reference lot using 5FU, but not statistically significant (p > 0.05). The reduction in tumor size is one of the reasons that helped the
On the results on NK cells, Macrophage and DC cells in mouse spleen

In cancer treatment, in addition to directly inhibiting the growth of cancer cells, the effect of enhancing the body’s immune response will limit the growth of cancer cells also very important. NK immune cells contain granzymes and perforin which are two essential components to create toxic activity, destroy foreign agents, infected cells and cancer cells. In addition, NK cells are capable of producing inflammatory agents (IFN-γ) and tumor necrosis agents (TNF-α), which play a large role in their ability to fight off bacteria and perform immune surveillance. Translation of tumors. NK cells increase, helping the body to protect itself against pathogens, especially the ability to eliminate cancer cells.

Macrophage cells, which act as phagocytes, are the constituents of the cell’s residue and pathogens. Dendritic cells (DC) are cells that present antigens to T lymphocytes in the immune response. The demonstration with modern medical evidence, to determine the number of immune cells in the mouse spleen carrying human hepatocellular carcinoma, has high scientific and practical significance. The results of our study showed that: NK, Macrophage and DC cells in the nude mouse spleen were statistically significant higher in the group using CTHepaB with p <0.05, compared to the control and reference lots. Possibly an indirect result through the capsule’s immunostimulating effect.

The result was a decrease in the amount of HBV-DNA in mouse tumor cells

Compared with the control group and the reference group using 5FU, CTHepaB capsules reduced HBV-DNA. This may be the result of the direct effect of CTHepaB capsules on the hepatitis B virus, possibly an indirect result through the capsule’s immunostimulating effect. Solanum hainanense Hance, a medicinal ingredient in CTHepaB capsules that has been shown to be effective in the treatment of HBV disease, reduces the number of hepatitis B viruses (Nguyen, 2014; Nguyen, 2014). Research by Trinh Thi Xuan Hoa (2014) using HAINA preparation of Solanum hainanense Hance extract 250 mg. After 60 days of treatment, the markers of hepatitis B virus had 23.3% of patients with HBsAg loss, 44% of patients with anti-HBe, compared to the control group 0% và 15%.

5. CONCLUSION

CTHepaB capsules at the dose of 0.96g g / kg / 24h have good therapeutic effects, in nude mice carrying HCC cells infected with HBV through indicators that, CTHepaB capsules reduced the average tumor size compared to the control group, equivalent to that of the group using 5FU at dose of 10mg / kg / 24h. Limiting the death rate of mice at the evaluation points 70, 76, 85, 90 days after taking the drug compared to the control group and on 85, 90 days, compared with the reference group; Increases average survival time of mice. Increased number of NK cells, Macrophage cells and DC cells in mouse spleen. Reduces the amount of HBV-DNA in mouse tumor cells.

Author contribution

All authors have contributed equally to this work. All authors have read and approved the final manuscript. All authors have agreed to publish this manuscript.

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Conflict of Interest

The authors declare that there are no conflicts of interests.
Ethical approval
The study was approved by the Medical Ethics Committee of National Hospital of Traditional Medicine (ethical approval code: 34/IBR-NHTM).

Data and materials availability
All data associated with this study are present in the paper.

REFERENCES AND NOTES


