Original article

Immuno-modulatory and cellular antioxidant activities of \( \kappa \)-selenocarrageenan in combination with Epirubicin in H22 hepatoma-bearing mice

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, being the sixth most prevalent tumor type as well as the third leading cause of cancer-related mortality worldwide [1]. Therefore, as a major public health problem, researches in reducing mortality and therapeutic on liver cancer should be a priority in the coming years [2]. Epirubicin (EPI), a member of the anthracycline family, is widely used in liver cancer therapeutic regimens for its effective anticancer activity. Nevertheless, it has serious adverse effects, such as cardiotoxicity, hepatotoxicity, bone marrow suppression and gastrointestinal reactions, which may be linked to the formation of reactive oxygen species (ROS) derived from redox activation of anthracycline [3,4]. Therefore, some natural compounds with better effectiveness and lower toxicity are receiving significant attention as potential new therapeutic drug replacements [5].

\( \kappa \)-selenocarrageenan (KSC), made from natural red algae, is a kind of polysaccharide selenate, in which the partial substitution of Sulfur (S) by Se forms a selenate [6]. KSC has been demonstrated to have double function of polysaccharide and selenium with lower toxicity and higher biological availability, especially in inhibiting the proliferation of cancer cells, and the possible antitumor molecular mechanisms were through antioxidation, induction of tumor cell apoptosis, blockade of cell cycle, and enhancement of immunity [7]. In addition, KSC can alleviate adriamycin-induced cardiotoxicity and exert the protective effects in cardiovascular and liver diseases, likely through ameliorating the blood
hemorrhology and reducing oxidative injury [8]. Furthermore, the combination of KSC treatment with other chemotherapeutic drugs can better improve antitumor efficacy, enhance immune functions and decrease the toxic effects of chemotherapeutic drugs [9,10].

Cancer cells are usually subjected to high levels of reactive oxygen species (ROS) [11]. ROS may be the cause of cell damage and the progression of normal cells to cancer cells. Therefore, tissues must be protected from oxidative injury through intracellular (SOD, GSH-Px and CAT) as well as extracellular (vitamins, micronutrients) antioxidants [12]. It has been demonstrated that different natural compounds induce the production of GSH as one of the principal anticarcinogenic mechanisms [13].

In recent years, combination therapy with multiple drugs or patterns is commonly used for treatment, achieves better therapeutic efficacy, and minimizes side effects in the cancer therapy [14,15]. However, the mechanism of the effect on KSC-EPI combination was seldom reported. The objectives of this study were to examine the antitumor, antioxidant, immune function of EPI in combination with KSC against hepatocarcinoma in H22 mice, and to explore the effect of KSC in reducing toxicity and enhancing efficacy on chemotherapy epirubicin in H22 tumor-bearing mice.

2. Materials and methods

2.1. Drugs and reagents

*k*-selenocarrageena (organic selenium containing 1.68% selenium and polysaccharide, solid powder, made by TianCifu Biological Engineering Co., Ltd); Epirubicin, purchased from Sigma Chemical Co., Ltd.; RPMI1640 medium, bought from GibCO BRL, Life Technologies Inc. (New York, USA); Fetal bovine serum (FBS), obtained from Hangzhou Sijiqing Life Technologies; 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) purchased from Sigma (St. Louis, MO, USA). In addition, detecting kits for tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Biotechnology Co. (Jiangsu, China).

2.2. Experimental animals and culture of H22 hepatoma cells

Mice with hepatoma H22 were obtained from Chinese Academy of Medical Sciences, and Kunming mice (females and males weighing18 g–22 g) from Changchun Yisi experimental animal technology Co., Ltd. They were raised in a temperature and humidity controlled environment under a 12 h light/12 h dark cycle, and given food and water ad libitum. Animal experiments were performed in accordance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals and approved by the local Ethics Committee.

Under aseptic conditions, H22 mice were sacrificed by cervical dislocation following intra peritoneal inoculation with H22 tumor cells for 7 days. The thick and white ascites including tumor cells were collected aseptically and stained by Trypan Blue to determine their viability, which exceeded 95%. H22 cells were then diluted with saline and cell concentration was adjusted to 1 × 10⁷/mL.

2.3. In vivo antitumor experiment

Forty Kunming mice, half male and half female, were randomly divided into four groups, including the control group, the EPI group (10 mg/kg/d), the KSC group (40 mg/kg/d), and the EPI + KSC group (using the same doses of EPI and KSC). The concentration design of KSC and EPI were based on some studies [16,17], and the selected dose is within epirubicin dose range for human [18]. Mice in each group were inoculated with 0.2 mL of 1 × 10⁷/mL H22 cell suspension in the right armpit subcutaneously. After 24 h inoculation, the mice in the EPI group and the combined group were injected of EPI intravenously once, and the KSC group administered intragastrically, once daily doses consecutively for 10 days, while those in the control group were given the same volume of saline. On the next day after the last administration, the body weights of the mice were measured, and blood samples were taken by removing eyeball. The tumor was removed and weighed, and tumor inhibition rates were calculated. At the same time, the thymus and spleen were also removed and weighed for the calculation of organ indexes. The inhibition rate was calculated using the following formula:

\[
\text{Inhibition rate (\%)} = \left(1 - \frac{\text{tumor weight of drug group}}{\text{tumor weight of control group}}\right) \times 100\%.
\]

2.4. Evaluation of drug interaction

The combined effect was assessed via the Q value using the following Jin’s formula [19]:

\[
Q = \frac{\text{E(A+B)/EA} \times (1-\text{EA})}{\text{EB}},
\]

where EA, EB, and E(A+B) indicate the inhibition rates of A, B, as well as their combination, and the combination effect can be classified as antagonistic (Q < 0.85), additive (0.85 < Q < 1.15), or synergistic (Q > 1.15).

2.5. The life-prolonging rate of mice

After continuous administration for 10 days, the conditions of mice with ascetic tumors were observed and survival time was recorded. The life-prolonging rate was calculated according to the following formula: The life-prolonging rate (\%) = \left(\frac{\text{survival time of drug group – survival time of the control group}}{\text{survival time of the control group}}\right) \times 100\%.

2.6. Splenocyte proliferation assay

Under aseptic conditions, spleen from sacrificed mice were immediately minced into small pieces and squeezed to obtain a homogeneous cell suspension. Spleen cells were lysed with lysis buffer (0.15 M NH₄Cl, pH 7.4) for 5 min to remove erythrocytes. After centrifugation (1500 rpm at 4 °C for 10 min), the spleen cells were washed three times in PBS and re-suspended in a RPMI-1640 medium. Cell numbers and viability (over 95%) were estimated using Trypan blue dye. The Splenocyte suspension was a mixture of cells containing T lymphocytes, B lymphocytes and monocytes. After 24 h incubation, only lymphocytes proliferated.

Assay of lymphocyte proliferation was performed by using the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method [20]. Briefly, the splenocyte suspension in RPMI-1640 (5 × 10⁵ cells/mL, 100 μL aliquot/well) was pre-incubated in 96-well plate for 24 h before the addition of concanavalin A (Con-A, final concentration of 1 μg/mL), or lipopolysaccharide (LPS, final concentration of 5 μg/mL) alone or in combination with KSC or EPI. The control had added the same volume of RPMI-1640 medium. The treated cells were then incubated at 37 °C in a humidified 5% CO₂ atmosphere for a further 44 h. Thereafter, 20 μL MTT (5 mg/m) was added to each well, and the plates were incubated for another 4 h at 37 °C. Following which, the plates were centrifuged (1500 rpm, 5 min), and the supernatants were carefully removed by pipetting. Then 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve purple formazan. After incubation at 37 °C for 15 min, the optical density (OD) values were measured at 570 nm in an ELISA reader (Bio-Rad, USA).
Table 1
Effects of KSC and EPI on tumor weight, inhibition rate, survival time and life-prolonging rate in the H22 tumor-bearing mice (n = 10).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg/d)</th>
<th>Tumor weight (g)</th>
<th>Inhibition rate (%)</th>
<th>Q value</th>
<th>Survival time (d)</th>
<th>Life-prolonging rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>4.25 ± 0.74</td>
<td>–</td>
<td>–</td>
<td>18.68 ± 1.67</td>
<td>–</td>
</tr>
<tr>
<td>KSC</td>
<td>40</td>
<td>2.45 ± 0.62</td>
<td>42.25</td>
<td>–</td>
<td>26.44 ± 2.54$^*$</td>
<td>41.54%</td>
</tr>
<tr>
<td>EPI</td>
<td>10</td>
<td>1.22 ± 0.67</td>
<td>71.27</td>
<td>–</td>
<td>23.26 ± 1.64$^*$</td>
<td>24.52%</td>
</tr>
<tr>
<td>KSC + EPI</td>
<td>40 + 10</td>
<td>0.80 ± 0.44$^*$</td>
<td>81.23</td>
<td>0.97</td>
<td>28.88 ± 2.25$^*$w</td>
<td>54.60%</td>
</tr>
</tbody>
</table>

$^*$P < 0.01 vs control group, $^*$P < 0.05 vs EPI group.

2.7. Natural killer (NK) cell activity

Natural killer (NK) cell activity was measured as described by Sarangi et al. [21]. Briefly, the spleens prepared as described above were used as the source of effector cells, and isolated splenocytes were seeded into 96-well plates at 5 × 10⁵ cells/mL. The cells were then stimulated at 37°C by KSC or EPI for 24 h. To eliminate the direct effects of KSC or EPI on target cells, spleens were washed once with RPMI-1640, then target YAC-1 cells (5 × 10⁴ cells/mL, yielding a 100:1 expected effector-target ratio) were added to each well in 100 μL aliquot. The plates were then incubated for 4 h at 37°C in 5% CO₂ atmosphere. An aliquot (40 μL) of MTT solution (5 mg/mL) was then added to each well, and the plates were incubated for a further 2 h. After that, the plates were centrifuged, the MTT solution in each well removed and 100 μL of DMSO added to each well. After incubation at 37°C for 15 min, OD value in each well was measured at 570 nm in a microplate reader. Three kinds of control measurements were performed: target cell control, blank control, and effector cell control. NK cell activity was calculated as follows: NK activity (%) = 100 × [ODT – (ODS – ODE)]/ODT, where ODT = optical density value of target cell control, ODS = optical density value of test samples, and ODE = optical density value of effector cell control.

2.8. Count of WBC and detection of serum biochemical indicators

A unit of 20 μL blood was taken from each mouse and 380 μL of white blood cell count solution was added to it. This was mixed evenly and used to measure the white blood cell (WBC) count. After the blood sample was centrifuged and the serum obtained, the kit instructions were followed to detect the serum TNF-α and IL-2 levels. The livers of the mice were used to prepare 10% liver homogenates, and the kit instructions were followed to detect GSH-Px, CAT and SOD activities, and GSH, MDA contents in the homogenates.

2.9. Statistical analysis

SPSS 16.0 software was used for the statistical analysis. All data were expressed as mean ± SD from triplicate experiments. The data were analyzed using GraphPad Prism 5.0 to calculate two-sided Student’s t-test. P < 0.05 or P < 0.01 was considered statistically significant.

3. Results

3.1. General condition of H22-bearing mice

Within 5 days of inoculation, there were no significant changes in the appearance of the mice. One week later, subcutaneous masses on the right axilla in the four groups were visible, the tumor formation rate being 100%. The tumors in the control group grew rapidly, while the tumors in the drug-treated groups grew slowly. Mice in the EPI group showed visible drug toxicity, such as emaciation, diarrhea, anorexia and superficial skin peeling. On the other hand, mice in the KSC + EPI group did not show obvious signs of drug toxicity, and they had no significant weight loss or decreased activity and their feeding behavior appeared normal.

3.2. Tumor growth inhibition and survival time of mice

There was a significant inhibition of tumor growth in the treated groups when compared to the control group (P < 0.01). The inhibition rates of KSC, EPI and KSC + EPI were 42.25%, 71.27% and 81.23%, respectively. The inhibition rate of KSC + EPI group was significantly higher than that of the EPI group (P < 0.05). Q value was less than 1.15 but more than 0.85, which indicated an additive effect between KSC and EPI (Table 1).

In addition, compared to the control group, the mean survival time of mice bearing H22 was significantly extended in all the treated groups (P < 0.01). The life span of mice in the KSC + EPI group was longer than that of EPI group (P < 0.05) (Table 1).

3.3. Thymus and spleen index in H22 tumor-bearing mice

The results showed that after the administration of KSC and EPI for 10 d, compared with the control group, the thymus and spleen indexes of H22 tumor-bearing mice were increased in the KSC group while decreased in the EPI group (P < 0.01) (Fig. 1). Meanwhile, the decreases in thymus and spleen indexes caused by EPI could be improved by KSC (P < 0.05), which suggested that KSC could enhance the immunity of H22 mice.

3.4. Splenocyte proliferation

Splenocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses [22]. To assess whether KSC and EPI can modulate cell immunity, splenocyte proliferation of H22 mice were cultured with KSC or EPI and Con-A (T lymphocyte mitogen) or LPS (B lymphocyte mitogen). The effect of KSC or EPI on splenocyte proliferation in H22-bearing mice was shown in Fig. 2. KSC significantly promoted Con-A- and LPS-stimulated splenocyte proliferation in H22-bearing mice compared with the control group (P < 0.01), whereas EPI has...
no significant effect on splenocyte proliferation ($P>0.05$). Compared with the EPI group, the splenocyte proliferation in combination group (KSC + EPI) was obviously elevated ($P<0.05$).

### 3.5. NK cell activity

NK cells are a major component of the innate immune system and play an important role in tissue inflammation or liver disease. They can represent a first line of defense against pathogens [23]. Measures of the cytotoxic activity of splenic NK cells against NK sensitive tumor cells (i.e., YAC-1 cell line) revealed that, as compared with the control cells, KSC significantly enhanced NK cell activity and reversed EPI-induced inhibition of NK activity ($P<0.01$) (Fig. 3).

#### 3.6. Changes of WBC in peripheral blood

Compared with the control group, the number of WBC in the EPI and KSC + EPI mice was significantly lower ($P<0.01$ or $P<0.05$). There was no difference between KSC and the control group, but the WBC number of KSC + EPI group was higher than that of the EPI group ($P<0.05$) (Fig. 4), which suggested that KSC could lower the side effects induced by EPI.

#### 3.7. TNF-α and IL-2 levels in H22 tumor-bearing mice

Many studies have shown that polysaccharides may exert their indirect anti-tumor effect through improving the immune response, and then regulating certain immune-related factors such as TNF-α and IL-2 levels [24,25]. Effects of KSC and EPI on serum TNF-α and IL-2 levels were investigated in H22 tumor-bearing mice. The results showed that KSC could significantly elevate the levels of TNF-α and IL-2 ($P<0.01$), and EPI could increase the TNF-α level but decrease the IL-2 level ($P<0.05$) in H22 tumor-bearing mice. Moreover, compared to the EPI group, the TNF-α and IL-2 levels of KSC + EPI group were obviously improved ($P<0.01$ or $P<0.05$), which suggests that the anti-tumor effect of KSC may be related to regulating the level of serum related factors to enhance immunity in H22 tumor-bearing mice (Fig. 5).

### 3.8. Cellular antioxidant activity

The activities of liver antioxidant enzymes GSH-Px, SOD and CAT were evaluated in the mice. As shown in Table 2, the GSH-Px, SOD and CAT activities and GSH level were obviously higher and MDA level was lower in the KSC group than those in the control group ($P<0.01$ or $P<0.05$), whereas EPI had the opposite effect. In addition, KSC could antagonize the decrease in the GSH-Px, SOD, CAT activities and GSH level and increase in MDA level caused by EPI ($P<0.01$ or $P<0.05$). The results of GSH-Px, SOD and CAT activities clearly showed that KSC exhibited a free radical scavenging activity.

### 4. Discussion

The development of HCC is a highly complex process involving numerous pathological and molecular mechanisms at the various stages of growth and metastasis, which makes the use of a single drug a poor choice [26]. Chemotherapy is one of the important treatment methods; however, much evidence show that the anti-tumor activities of many chemotherapeutic agents result in severe side effects [27]. Therefore, identification and development of a
novel agent with higher effectiveness and lower toxicity for patients fighting against HCC are urgently needed. Studies have shown that KSC has a variety of physiological activities such as anti-tumor, anti-oxidation, and immunity enhancement in addition to decreasing the toxic effects of chemotherapeutic drugs [7].

The in vivo study had found that KSC could improve the general condition of tumor-bearing mice, significantly inhibit the growth of H22 tumor, and extend the survival time of mice. More importantly, KSC had an additive anti-tumor effect with EPI and attenuated the indicators of EPI-induced toxicity such as diarrhea, anorexia, weight loss and the shrinkage of the thymus and spleen. Specifically speaking, KSC not only increased the inhibition rate of EPI induced by 9.96%, reaching 81.23%, but also extended the life span by 30.08%, reaching a life span for KSC+ EPI of 54.60%.

Lowered immune function can favor the formation and development of tumors, and moreover, tumors can often cause a decrease in immune function and atrophy immune organ [28]. Thymus and spleen are important immune organs, thymus and spleen indexes can directly reflect the level of immune function in the body, and effects of drugs on the thymus index and spleen index can be used as the preliminary indicators for the study on immuno-pharmacological mechanisms in animals [29,30]. In this study, it was demonstrated that KSC could improve thymus index and spleen index in H22 tumor-bearing mice and reverse the decrease of thymus index and spleen index induced by EPI. This suggested that KSC could alleviate the damage of organs and protect the thymus and spleen shrinkage caused by EPI, and enhance the immune function in H22 tumor-bearing mice while exerting its inhibitory effect on the tumor growth.

The inhibitory effect of polysaccharides on tumor cells is produced primarily by its effects on immune functions and direct cytotoxic effects on tumor cells, and most of them exert an indirect effect on tumors by enhancing immune function of the body [31]. TNF-α and IL-2 are cytokines with immuno-modulatory effects in the body, closely related to the formation, development and metastasis of tumors, and can both indirectly reflect the growth of tumors to some extent and be involved in the immuno-modulatory activities associated with tumors [32]. The results showed that KSC could increase TNF-α and IL-2 levels in H22 tumor-bearing mice and antagonize the effects induced by EPI, suggesting that KSC may enhance the immunity by regulating the serum TNF-α and IL-2 and other cytokines to exert its anti-tumor effect.

The disorder of free radical metabolism is closely related to the occurrence and development of tumors [33]. Normally, there is a complete set of antioxidant defense systems in the body, including GSH-Px, CAT, SOD, etc. [34]. Under physiological conditions, antioxidant enzymes (GSH-Px, CAT and SOD) are maintained in a dynamic equilibrium in the body, but when the antioxidant systems are damaged, lipid peroxides, like MDA, are produced excessively, leading to the damage of cells and tumorigenesis [35,36]. The results also showed that KSC could significantly improve the activities of GSH-Px, SOD and CAT and GSH level, and reduce the content of MDA in the liver tissue of H22 tumor-bearing cells, indicating that KSC can exert its synergistic anti-tumor effect with EPI by improving the activity of antioxidant enzymes and reducing the content of MDA in H22 tumor-bearing mice.

5. Conclusions

This study indicated that in mice implanted with hepatocellular carcinoma cells, KSC could significantly inhibit the growth of tumors, and prolong the survival time and enhance the immune function. Moreover, the study also proved the potential of κ-selenocarrageenan as combinational agent with epirubicin in improving the antioxidant and immuno-modulatory capacity to scavenge the excessive free radicals and reducing the drug side effects of epirubicin caused by generation of oxidative stress, making it more effective aided-drug in tumor therapy.

Conflicts of interest

All authors declare they have no conflicts of interest.

Compliance with ethical standards

All the animal studies were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC).

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