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The antitumor effect of the combination of aconitine and crude monkshood polysaccharide on hepatocellular carcinoma

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Abstract: Aconitine, the main component in *Radix Aconiti Lateralis Preparata*, not only exerts the anti-tumor effect on Hepatocellular Carcinoma (HCC) but also damages on immune system. In the present study, Crude Monkshood Polysaccharide (CMP), another one natural composition component originated from the same herbal with aconitine, combined with aconitine to investigate the effects on HCC and immunity *in vitro* and *in vivo*. The combination of CMP and aconitine enhanced the ability of the immunocyte to kill the tumor cell *in vitro* and had an additive effect on anti-HCC *in vivo*. Aconitine-CMP in combination improved the spleen weights, spleen index, thymus weights, thymus index. Elevated $CD4^+$ T and $CD8^+$ T cells and macrophages in spleen, decreased serum IL-6 level and increased serum IFN-γ and TNF-α levels were observed in mice treated with the combination of aconitine and CMP compare with control group ($P<0.05$). Our results showed that the combination of aconitine and CMP exerts anti-tumor effect by directly killing tumor cells and enhancing the anti-tumor immune responses, which further implies that chemotherapy drugs combined with Chinese medicine immunopotentiator maybe a feasible and effective strategy for HCC.

Keywords: Aconitine, crude monkshood polysaccharide, hepatocellular carcinoma, immunological enhancement.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most frequent malignant cancer around the world (Yu and Keeffe, 2003; PIRCA *et al.*, 2018; Sun *et al.*, 2018). Both new annual incidence and death of HCC patients account for about 54% of the global totality (Xiang *et al.*, 2017). Traditional Chinese medicine (TCM) is an effective-therapeutic tool for HCC with some benefits effects, including the improvement of clinical symptoms, the increment of the quality of life, the prolongment of survival, and retardment of the progression of the disease, especially in advanced HCC patients (Meng *et al.*, 2008; Tian *et al.*, 2010; Meng *et al.*, 2011; Ma *et al.*, 2017).

As the main toxic component in *Radix Aconiti Lateralis Preparata*and other plants, aconitine also is identified to be the main effective component of medicine. Aconitine is reported to have arrhythmia, anti-inflammatory, analgesic and anti-tumor (Zhou *et al.*, 2015; Zhu *et al.*, 2017; Wu *et al.*, 2018). The aconitic injection could relieve pain and prolong life, and even shrank the tumor in some individual cases with an effective rate of 40% ~ 60% in the treatment of tumors (Zhu, 1985). Aconitine displayed obviously anti-tumor effect by inducing differentiation and apoptosis of tumor cells and inhibiting the synthesis of biological macromolecules (DNA or RNA) of tumor cells (Guo *et al.*, 2011; Wada *et al.*, 2011; Du *et al.*, 2013; Chodoeva *et al.*, 2014).

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But recent studies showed that aconitine could impair immune system function by inhibiting the activity of macrophage, the production of TNF-α and the level of CD91 and CD13 (Sun *et al.*, 2012). Therefore, on the basis of guaranty the anti-tumor effect of aconitine, improvement its deleterious effect on immunity becomes be urgent. Some TCMs combined with chemo (radio) - therapy is able to enhance anti-tumor efficacy and reduce side effects of chemo (radio) radiotherapy. Also, they can improve the anti-tumor effect together with delay the generation of drug resistance, which is the main direction of clinical and experimental cancer research (Qi *et al.*, 2015).

Polysaccharides was reported to display a wide range of biological functions, including anticancer and immune regulation (Hou *et al.*, 2014; Li *et al.*, 2015). Monkshood polysaccharide is another component extracted from *Radix Aconiti Lateralis Preparata*. Further research showed that Crude Monkshood polysaccharide (CMP) significantly suppressed tumor growth of H_{22} tumor-bearing mice mainly through enhancing cellular immune function, inducing of tumor cell apoptosis and repressing regulation of tumor gene expression (Liang *et al.*, 2012; Li *et al.*, 2013; Liang *et al.*, 2015). CMP also can augment the anti-tumor effect and decrease the side-effects (such as the cytotoxicity) of adriamycin (ADM) (Dong *et al.*, 2006). However, the combined application of aconitine, another one component of the same plant, has not been studied. So, the combination of aconitine and CMP and the results showed that they both increased anti-hepatoma effect and improved the immunity.

MATERIALS AND METHODS

Drugs

Aconitine ($C_{34}H_{47}NO_{11}$, molecular weight 645.72, 99% pure) was obtained from National Institutes for Food and Drug Control (Beijing, China). Crude Monkshood polysaccharide (CMP) was isolated from *Radix Aconiti Praeparata* through water extraction and alcohol precipitate, and sequentially purified through Sevage method and dialysis (Zhang *et al.*, 2007; Han and Xie, 2013). The content of polysaccharide is 92.03% determined by phenol-sulfuric acid spectro-photometry. It was a brown powder and soluble in water easily. Cisplatin used for injection (10 mg/bottle) was purchased from China Qilu Drug Producing Co. Ltd.

Animals

C57/BL6 male mice (6-8 weeks, 20-22g) obtained from the animal experiment center of Matt Albert Technology Co. Ltd (Suzhou, SCXK (JING) 2014-0004) were treated in accordance with the EU Directive 2010/63/EU for animal experiments. All of the animal protocols were approved by the Local Committee on Ethics of Animal Experiments of Suzhou TCM Hospital Affiliated to Nanjing University of Chinese Medicine.

Hepa1-6 cells culture

The mouse HCC cell line Hepa1-6 was obtained from School of Life Sciences of Sun Yat-sen University. Hepa1-6 cells were cultured in high-glucose (25mM) Dulbecco's modified Eagle's medium (DMEM) containing (Gibco, Grand Island, NY), 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) in a humidified 5% CO_2 at 37°C. Preparation of lymphocytes from the spleen of C57/BL6 mice was performed by cutting the tissue into a 74 mm mesh. Lysis of red blood cells was performed with lysate incubation for 10 minutes and washed twice with PBS.

Cell proliferation assay

The assessment of cell proliferation was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Hepa1-6 cells cells (3×10^3) and for spleen lymphocyte cells (4×10^6) were seeded overnight in 96-well plates and then incubated with different concentrations of aconitine (dissolved in DMEM / PBS with 0.5% dimethyl sulfoxide (DMSO)) separately or CMP separately or aconitine-CMP in combination for the indicated time periods. MTT (Sigma-Aldrich, 0462B32) was dissolved in phosphate buffered saline (PBS) at the concentration of 5mg/mL. The MTT solution was added to a final concentration of 0.5mg/mL. After 4 h, the medium was replaced, and 0.15mL DMSO was added to solubilise the MTT formazan. The optical density (OD) was recorded at 490 nm in a microplate reader (Sepctra Max M2e, Molecular Devices, CA). Cell viability was expressed as percentage of vehicle-treated cells taken as 100% viable.

Spleen lymphocyte killing Heap1-6 cells test

The spleen lymphocyte (Effect cells) and Hepa1-6 cells (Target cells) each of 50μL (Effect: Target =100:1) were planted in 96-well plates and treated with aconitine alone (200μg/mL), CMP alone (1200 and 2400μg/mL) and aconitine with CMP (200+1200, 200+2400μg/mL) for 1h. Calculate the following formula: Killing rate% = $[1-(A_{E+T} - A_E)/A_T]\times100\%$; Killing rate of proliferation % = (Experience group killing rate-Control group killing rate)/Control group killing rate ×100%.

Animal studies

The C57/BL6 mice were used for implanting tumor cells the tumor after adaptive feeding 3 days. After 3 days of acclimatization, C57/BL6 mice were used for establishing tumor-bearing models by implanting tumor cells. Hepa1-6 cells (2×10^6) suspended in 0.2mL PBS were subcutaneously (s.c.) injected into the right flank of C57/BL6 mice. After 10 days, the mice were randomly assigned into four groups (each consisting of eight mice): control group, cisplatin group (positive control), aconitine groups (60, 150, 375μg/kg), CMP groups (900, 1800 μg/kg) and combination groups (aconitine-CMP: 150+900,150+1800μg/kg). Intragastric administration of the corresponding drug at the dose of 0.2ml/20g every day into the mice for 2 weeks was performed, but cisplatin group was intraperitoneally injected 0.2ml/20g once every other day. On the last day of administration, mice were sacrificed. Weigh of tumor tissues, spleens and thymus were determined, and blood samples were collected.

The spleen and thymus were evaluated by the organ index formula: Spleen or thymus weight (mg)/body weight (g) ×10. Tumor inhibition rate (%) = (mean tumor weight of the model control group - mean tumor weight of experiment group) /mean tumor weight of the model control group. q value can be used to determine the type of combined effect of aconitine and CMP. q=EAB / (EA+ EB -EA×EB). EA is the tumor inhibition rate of CMP group, EB is the tumor inhibition rate of aconitine group, EAB is the tumor inhibition rate of CMP-aconitine group. q<0.85 means antagonist, q between 0.85~1.15 means additive, q>1.15 means synergism (Liu *et al.*, 2011).

Detection of lymphocyte subpopulations

Preparation of lymphocytes from the spleen of C57/BL6 mice was performed by cutting the tissue into a 74 mm mesh. Lysis of red blood cells was performed with lysate incubation for 10 minutes and washed twice with PBS.

The spleen lymphocyte was stained with fluorochrome-conjugated Abs against various cell surface markers (Anti-Mouse CD3e PE, eBioscience, E00993-1631; Anti-Mouse CD4 FITC, eBioscience, E00078-133; Anti-Mouse CD8a PerCP-Cy5.5, eBioscience, E08300-1630; Anti-Mouse CD11b FITC, eBioscience, E033742) in the dark for 30 min at 4℃. The cells were subsequently washed twice with FACS buffer. Data were acquired on a FACS

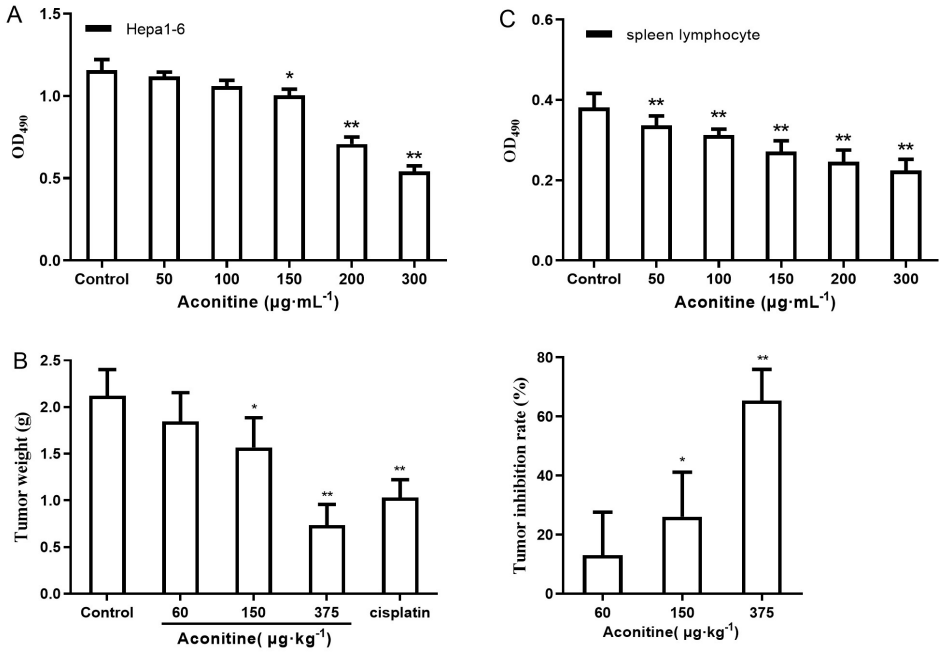

Fig. 1: Effects of aconitine on HCC and immune damage. (A) (C) Viability of Hepa1-6 cells and spleen lymphocyte isolated from C57/BL6 mice treated with aconitine. MTT assay was performed to measure cell growth inhibition at 48 h / 24 h after aconitine treatment. (B) Tumor weights and tumor inhibition rate of the Hepa1-6 tumor cells bearing C57/BL6 mice were treated with aconitine for 14 days. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group. Each data point represented the mean±S.D. of 8 samples.

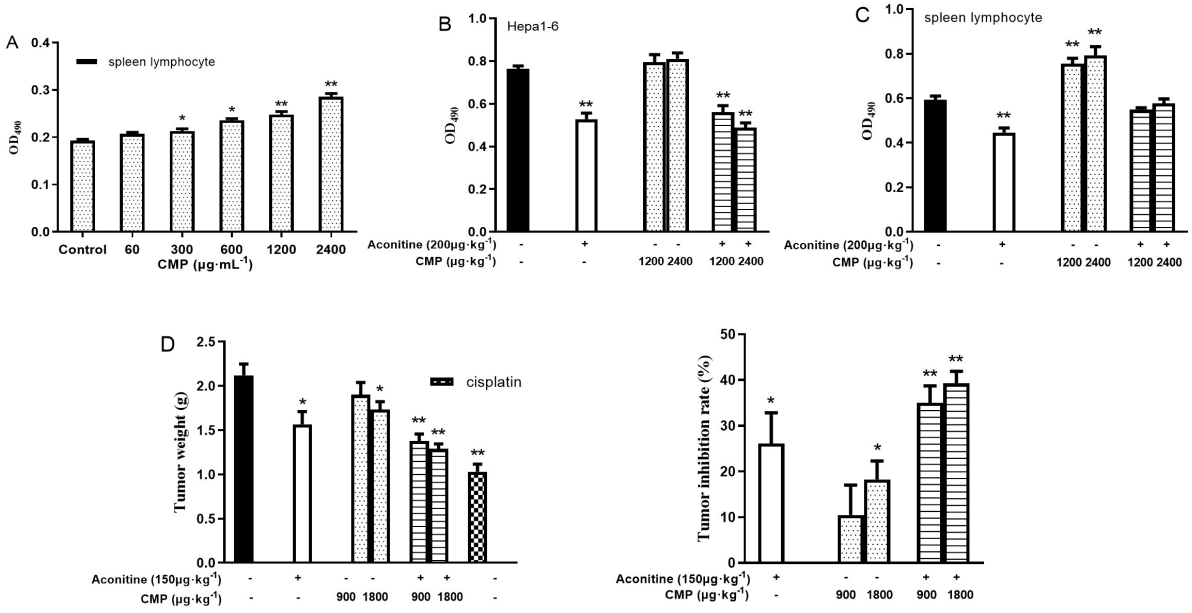

Fig. 2: Effects of CMP on immunity *in vitro* and combined with aconitine *in vivo*. (A) (B) (C) The viability of spleen lymphocyte isolated from C57/BL6 mice /Hepa1-6 cells treated with CMP or combined with aconitine. MTT assay was performed to measure cell growth inhibition rate at 24h / 48h after CMP treatment. (D) Tumor weights of the Hepa1-6 tumor cells bearing C57/BL6 mice were treated with aconitine alone, or CMP alone, or aconitine-CMP in combination for 14 days. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group. Each data point represented the mean±S.D. of 8 samples.

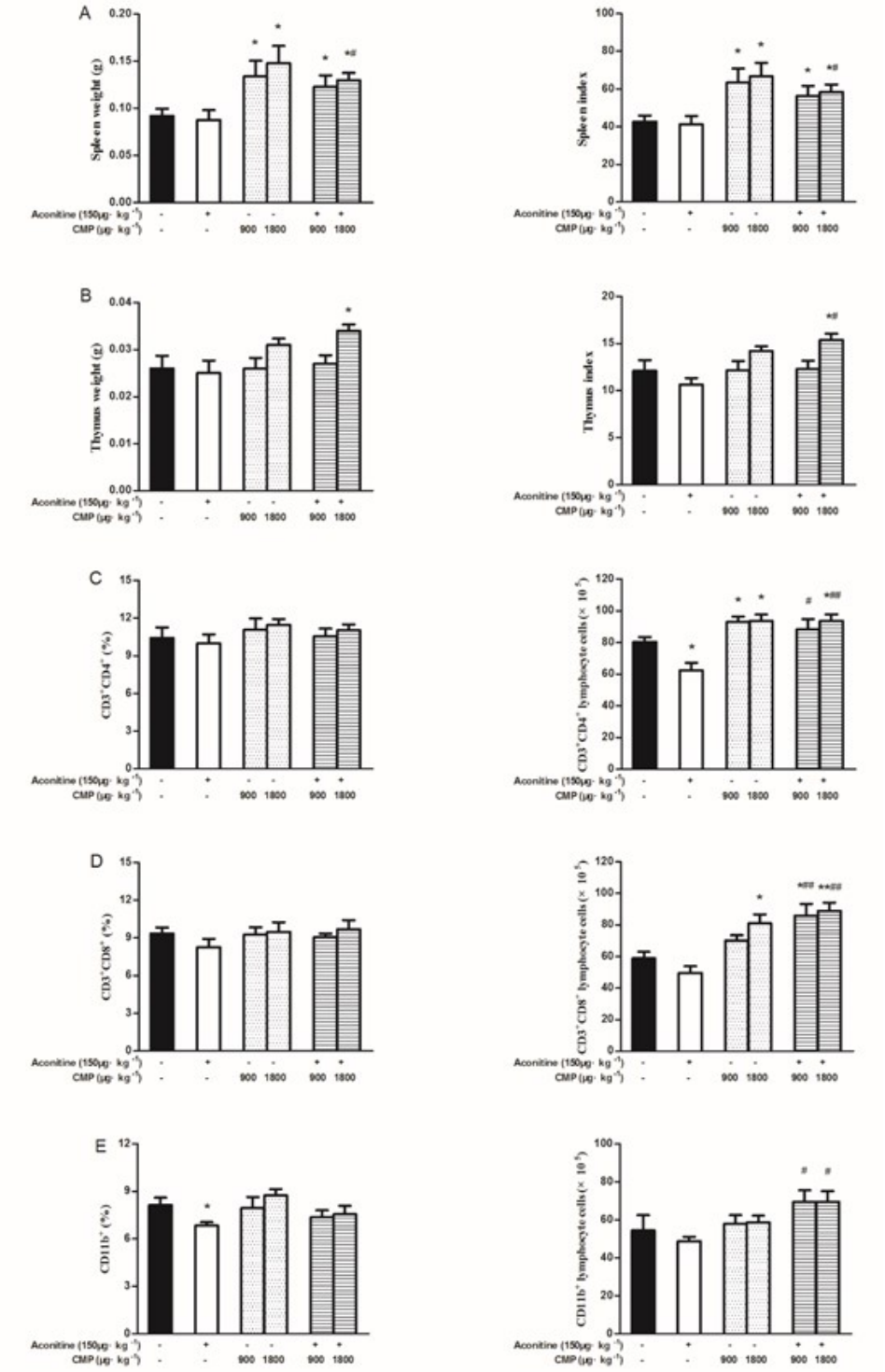

Fig. 3: Effects of CMP combined with aconitine on immunity *in vivo*. The (A) spleen weights and spleen index, (B) thymus weights and thymus index of the Hepa1-6 tumor cells bearing C57/BL6 mice were treated with aconitine alone, or CMP alone, or aconitine-CMP in combination for 14 days. A single cell suspension of mononuclear cells (about $1\times10^{5\text{-}6}$) obtained from spleens of the mice with aconitine alone, or CMP alone, or aconitine-CMP in combination treatment were stained with fluorochrome-conjugated Abs against various cell surface markers and analyzed by flow cytometry. The (C) $CD4^+$ T cell proportion and number, (D) $CD8^+$ T cell proportion and number, (E) $CD11b^+$ macrophages proportion and number were analyzed by Flowjo. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the aconitine group. Each data point represented the mean±S.D. of 8 samples.

Table 1: The effect of aconitine combined with CMP on spleen lymphocyte killing Hepa1-6 cells in different concentration (x±SD)

Groups		OD_{490}	Killing rate%
Control group	Hepa1-6 (A_T)	0.472±0.027	-
	spleen lymphocyte (A_E)	0.801±0.043	-
	Hepa1-6 + spleen lymphocyte (A_{E+T})	1.088±0.047	39.21±9.86
Experiment group	Aconitine 200μg/mL (A_{E+T})	1.142±0.035	27.71±7.43
	CMP 1200μg/mL (A_{E+T})	1.066±0.052	43.88±11.09
	CMP 2400μg/mL (A_{E+T})	1.046±0.048	48.19±10.27
	Aconitine-CMP 200+1200μg/mL (A_{E+T})	1.011±0.043$^{*\#}$	55.62±9.01$^{*\#}$
	Aconitine-CMP 200+2400μg/mL (A_{E+T})	0.955±0.079$^{*\#}$	59.05±16.84$^{*\#}$

Killing rate% = [1－（A_{E+T}－A_E）/A_T]×100%. $^{*}P<0.05$ compared with the A_{E+T} control group (Hepa1-6+spleen lymphocyte), $^{\#}P<0.05$ compared with the Aconitine 200μg/mL group.

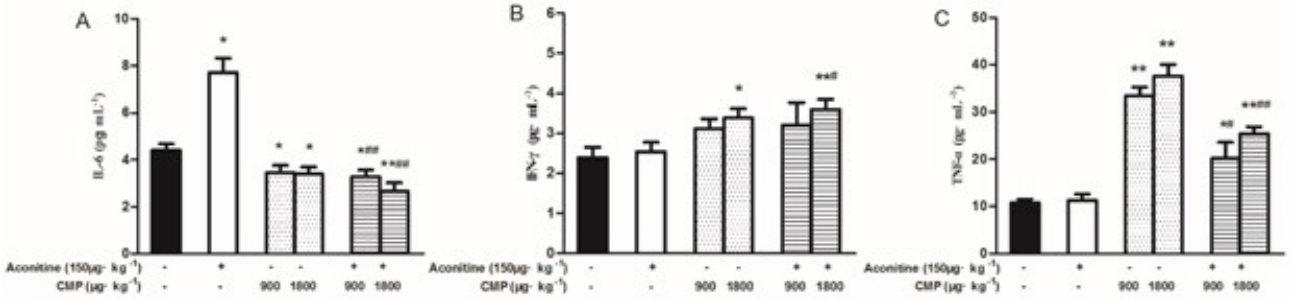

Fig. 4: Effects of CMP combined with aconitine on serum cytokines in murine models of HCC. The (A) IL-6, (B) IFN-γ and (C) TNF-α levels were detected using Cytometric Bead Array (CBA) assays. Data shown were the representatives of three experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the aconitine group. Each data point represented the mean±S.D. of 8 samples.

Calibur (BD Bioscience, San Diego, CA) and analyzed using Flowjo software (FlowJo, Ashland, OR).

Detection of serum IL-6, IFN-γ and TNF-α

The blood samples were collected through the eye socket and centrifuged at 3000 rpm for 20 min to acquire serum. IL-6, IFN-γ and TNF-α were assessed using BD Cytometric Bead Array (CBA, Mouse Th1/Th2/Th17 Cytokine Kit, BD Biosciences, 4234981) assays according to the manufacturer's protocol.

STATISTICAL ANALYSIS

All of the data shown are representative of at least three independent experiments and were expressed as mean ± SD. The statistical significance of differences was assessed by one-way ANOVA with the Bonferroni's multiple comparison post-test (Graph Pad Prism 5.0 Software, San Diego, CA, USA). P values≤ 0.05 were considered as statistically significant.

RESULTS

Effects of aconitine on HCC and immune damage

In vitro, Hepa1-6 cells were treated with aconitine at different concentrations for 48 h. The data showed that aconitine treatment inhibited the proliferation of Hepa1-6 cells in a concentration-dependent manner (IC_{50}: 241.94 μg/mL). *In vivo*, these results showed the tumor weights of aconitine-treated (150 and 375μg/kg) mice were markedly lower than that of control group (control: 2.122±0.281g, 150μg/kg: 1.568±0.319g, 375μg/kg: 0.734±0.223g, P<0.05, P<0.01). It suggested that aconitine could inhibit liver cancer. But spleen lymphocyte isolated from C57/BL6 mice were incubated with different concentrations of aconitine for 24h. Aconitine inhibited the growth of spleen lymphocyte in a dose-dependent manner (IC_{50}: 334.89μg/mL). It suggested that aconitine had an immunosuppressive effect. (fig. 1)

Effects of CMP on immunity in vitro and combined with aconitine in vivo

Spleen lymphocyte was treated with CMP at different doses for 24h. CMP significantly promoted the proliferation of the spleen lymphocytein a concentration-dependent manner. These results suggested that CMP induced the growth of immune cells. In order to observe the effects of CMP combined with aconitine on the relationship between HCC and immunity, Hepa1-6 cells and spleen lymphocyte were treated with aconitine (200

μg/mL) alone, CMP (1200 and 2400 μg/mL) alone, and aconitine-CMP in combination (200+1200 and 200+2400 μg/mL) for 24h or 48h. The data showed that different concentrations of CMP combined with aconitine (200μg/mL) treatment inhibited the proliferation of Hepa1-6 cells (P<0.01) and reduced the inhibitory effect of aconitine on spleen lymphocyte. These results suggested that CMP induces the proliferation of immune cells, has no direct effect on hepatoma cells, and reverses the inhibitory effect of aconitine on splenocytes. (fig. 2A., B. & C.)

In the additional mice experiment, the data showed that the tumor weights of aconitine (150μg/kg) -treated mice (1.568±0.319 g), CMP (900μg/kg and 1800 μg/kg)-treated mice (1.900±0.310g, 1.736±0.194g), CMP-aconitine (150+900μg/kg and 150+1800μg/kg) -treated mice (1.379±0.176 g, 1.289±0.124 g) were obviously lower than that of control group (2.122±0.281g). Compared with the control group, aconitine-CMP in combination groups significantly inhibited tumor growth in tumor-bearing mice (P<0.01) (fig. 2D). The q values of aconitine-CMP two-dose groups were 1.03 and 0.99 respectively calculated by Kim correction formula, which indicated the effects of aconitine combined with CMP on HCC were an additive effect of aconitine alone and CMP alone *in vivo*. These results suggested that the combination of CMP with aconitine enhance the anti-HCC effect, but reduce the immune damage of aconitine.

Effects of CMP combined with aconitine on immunity in vivo

In order to further study the effect of aconitine combined with CMP on immunity in murine models of HCC. We observed the effects of two drugs on immune organs and immune cells after single use and combination. The data showed that CMP could improve the spleen weights and spleen index compare with the control group (P<0.05) and the numbers of $CD4^+$ T cells and $CD8^+$ T cells in spleen were both higher than that of control group (P<0.05). CMP combined with aconitine could improve the spleen weights, spleen index, thymus weights, and thymus index compare with control group (P<0.05). And compared with aconitine alone group, aconitine-CMP group (150 + 1800 μg/kg) had a significant difference (P<0.05). The numbers of $CD4^+$ T and $CD8^+$ T cells in the spleen of aconitine-CMP in combination were both higher than that of control group, which improving the descent of $CD4^+$ T cells and $CD8^+$ T cells caused by aconitine alone (P<0.05, P<0.01). Also, the population of macrophages in the spleen was higher than that of aconitine alone group (P<0.05). There indicated that CMP combined with aconitine could improve the descent of immune caused by aconitine alone (fig. 3).

Effects of CMP combined with aconitine on spleen lymphocyte killing Hepa1-6 cells

In order to further explore the advantage of CMP combined with aconitine, we observed the effects of CMP combined with aconitine on spleen lymphocyte killing Hepa1-6 cells. The data showed that the aconitine-CMP in combination groups could promote the rate of spleen lymphocyte killing Hepa1-6 cells. Compared with control group (A_{E+T} group) or aconitine alone group, the killing rates of aconitine-CMP in combination groups markedly elevated (P<0.05) (table 1). Meanwhile, these results in cell experiments showed that aconitine-CMP in combination could decrease the inhibition of aconitine for spleen lymphocyte (fig. 2C). It indicated that CMP combined with aconitine could protect immunocyte and increase the ability of immunocyte killing tumor cells.

Effects of CMP combined with aconitine on serum cytokines in murine models of HCC

Excessive IL-6 levels in the serum of HCC patients could suppress the function the of Th1 cells and result in the inadequate secretion of TNF-α, IFN-γ and other cytokines, causing the immune function weakens (He *et al.*, 2011; Chew *et al.*, 2012). So, we detected the IL-6, IFN-γ and TNF-α levels of murine serum by CBA assays. Compared with control groups, aconitine alone group significantly increased IL-6 level (P<0.05), CMP alone groups and aconitine-CMP in combination groups could decrease IL-6 level (P<0.05) and increase TNF-α and IFN-γ levels (P<0.05, P<0.01). Compared with aconitine alone group, aconitine-CMP in combination groups could reduce the rasing of the IL-6 causing aconitine, and increase TNF-α and IFN-γ levels (P<0.05, P<0.01) (fig. 4). Therefore, these results further suggested that aconitine can induce the descent of immune, and CMP can activate the anti-tumor immune response by regulating production of IL-6, IFN-γ, TNF-α, and other cytokines.

DISCUSSION

Our results showed that a single administration of aconitine inhibit the proliferation of liver cancer cells and spleen cells *in vitro* and decreases the weights of spleen and thymus of Heap1-6 tumor-bearing mice which there was the occurrence of a certain immune damage. A single administration of CMP promoted the proliferation of splenic lymphocytes *in vitro*, and raised the weight of the spleen and increased the number of $CD4^+$ and $CD8^+$ T cells in tumor-bearing mice which displayed augmented immune responses. Using a combination of CMP and aconitine, the anti-tumor effect of immune cells enhanced *in vitro*, and the weights of spleen and thymus increased, the reduction in the number of $CD4^+$ and $CD8^+$ T cells raised, and the number of macrophages increased in tumor-bearing mice. The current study indicated that the immune damage of aconitine can be improved by using a combination of CMP and aconitine, which may be related to the CMP-mediated regulation of cytokines, like IL-6, IFN-γ and TNF-α.

Radix Aconiti Praeparata, the secondary roots of *Aconitum carmichaeli Debx, (Ranunculaceae)*, has been widely used for anti-tumor therapy in clinical studies with traditional Chinese medicine. Aconitine is not only the effective component for the anti-tumor activity but also the venenosus component of *Radix Aconiti Praeparata*. The dosage of aconitine used in clinical application and animal experiment is strictly controlled due to its greater toxicity (Zhang *et al.*, 2016). 375μg/kg of aconitine which converts from the median lethal dose of human, was chosen as the highest experimental dose in the murine study, and 150μg/kg and 60μg/kg were proportionally setted as the other two doses. The doses of aconitine for cells test was chosen according to the references (Guo *et al.*, 2011; Du *et al.*, 2013). That the present study indicated that aconitine single treatment significantly inhibits the growth of HCC in a concentration-dependent manner, whereas aconitine treatment alone causes immune injury.

The development of tumors is closely correlated with the immune status of the host. The immunolesion and immune disorder occur in mice with progressively growing tumors. In our previous studies, Hepa1-6 tumor-bearing mice displayed the weakly injured thymus, the decreased splenic lymphocytes, and the reduction in the proportion of macrophages, $CD4^+$ T cells, and $CD8^+$ T cells in spleen (Zhang *et al.*, 2012), which suggests that tumor-bearing mice show the immunocompromised state. On the contrary, the immunolesion and immune disorder promoted the development of the tumor. Thus, the ideal anticancer drugs not only have a specific tumor-killing effect but maintain the normal immune function and even enhance the anti-tumor immune responses. The reports showed that aconitine has a direct effect on killing tumor cells (Guo *et al.*, 2011; Wada *et al.*, 2011). The current study also showed that aconitine could restrain the proliferation of both HCC tumor cell and spleen lymphocyte *in vitro*, and suppress the growth of HCC tumor, as well as, decrease the anti-tumor immune responses of HCC tumor-bearing mice *in vivo*. The anti-HCC effect of aconitine is similar to many chemotherapy drugs' effects, including the inhibition of hepatoma cell growth and the damagment of immune function. The kind of anti-tumor drugs for clinical applications often combine with immune-enhancing drugs.

Monkshood Polysaccharide is another one component extracted from *Radix Aconiti Lateralis Preparata*. Previous studies have shown that CMP significantly restrains tumor growth of H_{22} tumor-bearing mice mainly through enhancing cellular immune function (Liang *et al.*, 2012; Li *et al.*, 2013; Liang *et al.*, 2015). Our results also confirmed that CMP had a slight anti-tumor effect (the inhibition rates of dose 900 and 1800μg/kg were 10.48% and 18.22%, respectively) but no effect on the proliferation of tumor cells *in vitro*. These results suggested that CMP didn't directly suppress the proliferation of tumor cells *in vitro*, but had a weakly anti-tumor effect by enhancing immune responses *in vivo*.

Our results also showed that aconitine could restrain the proliferation of tumor cells *in vitro* and *in viv*o, which is related to the induction of apoptosis (Gao *et al.*, 2012; Ji *et al.*, 2016). CMP combined with aconitine could increase the anti-tumor effect, which was the additive effect of aconitine and CMP according to the Kim correction formula (Liu *et al.*, 2011). The combination of CMP and aconitine elevated the decreased numbers of $CD4^+$ T and $CD8^+$ T cells and macrophage caused by aconitine, enhanced the ability of the immunocyte to kill tumor cell, meanwhile inhibited the IL-6 secretion and promoted the secretion of IFN-γ and TNF-α. CMP not only increased the anti-tumor effect but also improved the immune function combined with aconitine.

It has been well known that traditional Chinese medicine exhibits an integral effect via multi-targets and multi-pathways, which is closely associated with "component constitution" (Liu *et al.*, 2012). CMP not only exists in same natural herbal with aconitine but also is an effective component for anti-tumor due to enhancing cellular immune function, which the mechanism was different from aconitine. Our results also supported the anti-HCC efficiency of *Radix Aconiti Praeparata*, which provides ideas to search for ingredient prescription in same natural medicine for a better stagey to the prevention of HCC.

CONCLUSION

Our results revealed that aconitine combined with CMP exerts anti-tumor effect by directly killing tumor cells and enhancing the anti-tumor immune responses, which further implies that chemotherapy drugs combined with Chinese medicine immunopotentiator maybe a feasible and effective strategy for HCC.

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REFERENCES

Chew V, Chen J, Lee D, Loh E, Lee J, Lim KH, Weber A, Slankamenac K, Poon RT, Yang H, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Abastado JP (2012). Chemokine-driven lymphocyte infiltration: An early intratumoural event determining long-term survival in resectable hepatocellular carcinoma. *Gut.,* **61**(3): 427-438.

Chodoeva A, Bosc JJ, Lartigue L, Guillon J, Auzanneau C, Costet P, Zurdinov A, Jarry C and Robert J (2014). Antitumor activity of semisynthetic derivatives of aconitum alkaloids. *Invest. New Drugs,* **32**(1): 60-67.

Dong LF, Zhang YJ, Liu JS, Song SX, Hou J and Lu ZJ (2006). Anti-tumor effect of monkshood polysaccharide with adriamycin long circulating temperature-sensitive liposome and its mechanism. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi.,* **22**(4): 458-462.

Du J, Lu X, Long Z, Zhang Z, Zhu X, Yang Y and Xu J (2013). *In vitro* and *in vivo* anticancer activity of aconitine on melanoma cell line B16. *Molecules.,* **18**(1): 757-767.

Gao F, Li YY, Wang D, Huang X and Liu Q (2012). Diterpenoid alkaloids from the Chinese traditional herbal "Fuzi" and their cytotoxic activity. *Molecules,* **17**(5): 5187-94.

Guo BF, Liu S, Ye YY and Han XH (2011). Inhibitory effects of osthole, psoralen and aconitine on invasive activities of breast cancer MDA-MB-231BO cell line and the mechanisms. *Zhong Xi Yi Jie He Xue Bao.,* **9**(10): 1110-1117.

Han HP and Xie HC (2013). A study on the extraction and purification process of lily polysaccharide and its anti-tumor effect. *Afr. J. Tradit Complement Altern Med*., **10**(6): 485-9.

He X, Li X, Liu B, Xu L, Zhao H and Lu A (2011). Down-regulation of Treg cells and up-regulation of TH1/TH2 cytokine ratio were induced by polysaccharide from radix glycyrrhizae in H22 hepatocarcinoma bearing mice. *Molecules,* **16**(10): 8343-8352.

Hou Y, Ding X, Hou W, Song B, Wang T, Wang F, Li J, Zeng Y, Zhong J, Xu T and Zhu H (2014). Pharmacological evaluation for anticancer and immune activities of a novel polysaccharide isolated from *Boletus speciosus* Frost. *Mol. Med. Rep.,* **9**(4): 1337-44.

Ji BL, Xia LP, Zhou FX, Mao GZ and Xu LX (2016). Aconitine induces cell apoptosis in human pancreatic cancer via NF-κB signaling pathway. *Eur. Rev. Med. Pharmacol. Sci*., **20**(23): 4955-4964.

Li C, Wu X, Zhang H, Yang G, Hao M, Sheng S, Sun Y, Long J, Hu C, Sun X, Li L and Zheng J (2015). A Huaier polysaccharide inhibits hepatocellular carcinoma growth and metastasis. *Tumour Biol.,* **36**(3): 1739-45.

Li H, Sun M, Xu J, Li H, Zang M and Cui Y (2013). Immunological response in H22 transplanted mice undergoing Aconitum coreanum polysaccharide treatment. *Int. J. Biol. Macromol.,* **55**(1): 295-300.

Liang M, Li SC, Shen B, Cai JP, Li C, Wang ZY, Li XG, Gao J, Huang HY, Zhang XY, Li JY (2012). Anti-hepatocarcinoma effects of *Aconitum coreanum* polysaccharides. *Carbohydrate Polymers,* **88**(3): 973-976.

Liang M, Liu J, Ji H, Chen M, Zhao Y, Li S, Zhang X and Li J (2015). A Aconitum coreanum polysaccharide fraction induces apoptosis of hepatocellular carcinoma (HCC) cells via pituitary tumor transforming gene 1 (PTTG1)-mediated suppression of the P13K/Akt and activation of p38 MAPK signaling pathway and displays antitumor activity *in vivo*. *Tumour Biol.,* **36**(9): 7085-91.

Liu D, Jia XB and Yu DH (2012). New idea of traditional Chinese medicine quality control based on "composition structure" theory. *Zhongguo Zhong Yao Za Zhi*., **37**(6): 865-70.

Liu S, Cao M, Li D, Zhang J, Feng S, Nie Y, Yan L, Zhang D, Zhang Y, Yang Z, Zhao J (2011). Purification and anticancer activity investigation of pentacyclic triterpenoids from the leaves of Sinojackia sarcocarpa L.Q. Luo by high-speed counter-current chroma-tography. *Nat Prod. Res.,* **25**(17): 1600-6.

Ma L, Wang B, Long Y, Li H (2017). Effect of traditional Chinese medicine combined with Western therapy on primary hepatic carcinoma: A systematic review with meta-analysis. *Front Med.,* **11**(2): 191-202.

Meng MB, Cui YL, Guan YS, Ying Z, Zheng MH, Yuan CK and Zhang RM (2008). Traditional Chinese medicine plus transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma. *J. Altern Complement Med*., **14**(8): 1027-1042.

Meng MB, Wen QL, Cui YL, She B, Zhang RM (2011). Meta-analysis: traditional Chinese medicine for improving immune response in patients with unresectable hepatocellular carcinoma after transcatheter arterial chemoembolization. *Explore* (NY), **7**(1): 37-43.

Prevention of Infection Related Cancer (PIRCA) Group, Specialized Committee of Cancer Prevention and Control, Chinese Preventive Medicine Association; Non-communicable & Chronic Disease Control and Prevention Society, Chinese Preventive Medicine Association; Health Communication Society, Chinese Preventive Medicine Association (2018). Strategies of primary prevention of liver cancer in China: Expert Consensus (2018). *Zhonghua Zhong Liu Za Zhi.,* **40**(7): 550-557.

Qi F, Zhao L, Zhou A, Zhang B, Li A, Wang Z, Han J (2015). The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer. *Biosci Trends.,* **9**(1): 16-34.

Sun J, Zhou G, Zhang Y, Zhou T, Nie C, Zhu T, Chen S, Wang B, Yu Z, Wang H, Chen X, Hong L, Chen L, Wang W and Zheng S (2018). Comprehensive analysis of common safety profiles and their predictive factors in 520 records of liver cancer patients treated by drug-eluting beads transarterial chemoembolization. *Medicine* (Baltimore), **97**(26): e11131.

Sun T, Du GJ, Zhang YP, Li JH, Liu WJ and Wang YY (2012). Effect of aconiti lateralis radix praeparata and taraxaci herba on Chinese medicine signs and

symptoms of urethane-induced lung cancer in mice. *Zhongguo Zhong Yao Za Zhi.,* **37**(20): 3097-101.

Tian HQ, Li HL, Wang B, Liang GW, Huang XQ, Huang ZQ, Lang JM, Zhang YP, Chen XZ and Chen YS (2010). Treatment of middle/late stage primary hepatic carcinoma by Chinese medicine comprehensive therapy: A prospective randomized controlled study. *Chin J. Integr. Med.,* **16**(2): 102-108.

Wada K, Hazawa M, Takahashi K, Mori T, Kawahara N and Kashiwakura I (2011). Structure-activity relationships and the cytotoxic effects of novel diterpenoid alkaloid derivatives against A549 human lung carcinoma cells. *J. Nat. Med.,* **65**(1): 43-49.

Wu JJ, Zhu YF, Guo ZZ, Lou YM, He SG, Guan Y, Zhu LJ, Liu ZQ, Lu LL and Liu L (2018). Aconitum alkaloids, the major components of Aconitum species, affect expression of multidrug resistance-associated protein 2 and breast cancer resistance protein by activating the Nrf2-mediated signalling pathway. *Phytomedicine.,* **44**(1): 87-97.

Xiang X, Zhong JH, Wang YY, You XM, Ma L, Xiang BD and Li LQ (2017). Distribution of tumor stage and initial treatment modality in patients with primary hepatocellular carcinoma. *Clin. Transl Oncol*., **19**(7): 891-897.

Yu AS, Keeffe EB(2003). Management of hepatocellular carcinoma. *Rev Gastroenterol Disord.,* **3**(1): 8-24.

Zhang DK, Li RS, Han X, Li CY, Zhao ZH, Zhang HZ, Yang M, Wang JB and Xiao XH (2016). Toxic constituents index: A toxicity-calibrated quantitative evaluation approach for the precise toxicity prediction of the hypertoxic phytomedicine-aconite. *Front Pharmacol*., **7**(1): 164.

Zhang LR, Tang Y and Jiang GR (2012). The protection of yupingfeng powder on cisplatin induced oxidative damage of organs in hepatocellular carcinoma mice. *Zhongguo Zhong Xi Yi Jie He Za Zhi.,* **32**(5): 647-651.

Zhang SD, Fang YX, Chen MM, Huang JP and Zhang K (2007). Extraction and anti-tumor activity study of polysaccharide in several traditional Chinese medicine. *Zhong Yao Cai*., **30**(2): 179-82.

Zhou G, Tang L, Zhou X, Wang T, Kou Z, Wang Z (2015). A review on phytochemistry and pharmacological activities of the processed lateral root of *Aconitum carmichaelii* Debeaux. *J Ethnopharmacol.,* **160**(1): 173-93.

Zhu BQ (1985). Clinical efficacy of aconitic injection in asthenia patients suffering from sick sinus syndrome and its mechanism. *Zhong Xi Yi Jie He Za Zhi.,* **5**(4): 219-22, 195.

Zhu L, Wu J, Zhao M, Song W, Qi X, Wang Y, Lu L and Liu Z (2017). Mdr1a plays a crucial role in regulating the analgesic effect and toxicity of aconitine by altering its pharmacokinetic characteristics. *Toxicol. Appl. Pharmacol*., **320**(1): 32-39.