Received: 4 October 2007 / Accepted: 12 November 2007 / Published online: 22 November 2007

The effects of SOD contained silk orm po - der on immune regulation and inhibition against Hepatoma 22 tumor cells in vivo ere investigated. The activit of natural killer cell (NK) and the ConA-stimulated spleen proliferation ere measured. The results found that the SOD-contained silk orm po der caused an enhancement on NK cell activit , hich implied this material modulated the immune s stem in mice in vivo. The NK cell activities of Hepatoma 22 tumor modeled mice treated ith silk- orm po der including SOD ere increased signi cantl compared to a modeled control and silk orm po der ithout SOD, reaching 36.18%. In addition, the ConA-stimulated spleen proliferation of SOD treated mice as higher than that of the controls. The treatment of SOD contained silk orm po der presented 40.3% of average inhibition rate to Hepatoma 22 tumor, sho ing stronger inhibition against tumor. There ere no signi cant differ- ence in bod eight bet een modeled control and SOD silk orm po der feeding in Hepatoma 22 tumor modeled mice, suggesting the SOD silk orm po der is safet as an inhibitant to tumor. In conclusion, these ndings demonstrate that administration of silk orm po der containing SOD results in activation of NK cells and immunit , suggesting the silk orm po der containing SOD pla s a positive role in tumor inhibition.

SOD-contained silk orm po der · Natural killer (NK) cells · Hepatoma 22 modeled mice · Immune regulation · Spleen proliferation

Supero ide dismutase (SOD, EC 1.15.1.1) is a metalloen- me, hich catal es the conversion of the supero ide radicals into molecular O_2 and H_2O_2 and thus form a crucial part of the cellular antio idant defense mechanism [1]. The amount of SOD present in cellular and e tracellular environment is crucial for the prevention of disease linked to o idative stress. Mutations in SOD account for appro imatel 20% of familial am otrophic lateral sclerosis (ALS) cases [2].

O idation free radicals have caused more and more interests in medical. Researchers have found that there are close relationship bet een o idation free radicals and tumor development. Antio idants can cancel out the cell-damaging effects of free radicals [3], and people ho eat fruits and vegetables rich in pol phenols and anthoc anins have a lo er risk of cancer, heart disease and some neurological diseases [4]. This observation suggested that these compounds might prevent conditions such as macular degeneration [5], suppressed immunit due to poor

W.-F. Yue · X.-H. Li · B. Ro · G.-L. Li · J.-M. Liu · X.-F. Wu · Y.-G. Miao (✉)
Institute of Sericulture and Apiculture, College of Animal Sciences, Zhejiang Universit , Hang hou 310029, P.R. China
e-mail: miao g@ ju.edu.cn

W. Deng · H.-X. Sun
Institute of Preventive Veterinar Medicine, Zhejiang

nutrition [6], and neurodegeneration, hich are due to o idative stress [7].

While several trials have investigated supplements ith high doses of antio idants, the investigators found there as statisticall signi cant effect of the antio idants on overall survival, cancer, or heart disease, sho ing a 31% reduction in the risk of cancer in men [8].

In other hand, the silk orm bioreactor has sho ed its advantages such as high e pression ef cienc and lo feeding cost, natural activit for its e pressed products and safet for both environment and human [9]. Therefore, it is ver promising to use the silk orm as vector for industrial large-scale mass production [10]. In our previous reports, e used a practical BmNPV bacmid s stem to e press the Mn-SOD en me protein in silk orm larvae b the recombinant bacmid baculoviruses [11]. The availabilit of large quantities of SOD that the silk orm provides should greatl facilitate the future research and testing of this protein for potential application in medicine. We have also investigated the effects of silk orm po der containing SOD on the antio idation and the immune s stem of mouse, focused on hemol sin response, hemagglutination against SRBC, PFC assa and the dose-dependent increase of phagoc tic activit . All treated mice sho ed signi cant promotion in immunit [12, 13].

In this paper, e further investigated the effects of silk orm po der including SOD on NK cell activit and spleen proliferation of mice. The treatment of SOD contained silk orm po der presented 40.3% of average inhibition rate to Hepatoma 22 tumor cell gro th in vivo, sho ing stronger inhibition against tumor. The results sho ed that silk orm po der containing SOD ma be have potential application against tumor cells.

E perimental animals

The e perimental animals ere supplied b Animal E periment Center of Zhejiang Medical Institute, China. Half male and half female ICR mice of 6 8 eeks (eighing 20 2 g) ere used. The animals ere housed in individual stainless steel cages in an air-conditioned room under a 12:12-h light: dark c cle. A commercial pellet diet and ater ere provided throughout the e periment. All procedures ere conducted in accordance ith the P.R. China legislation under No. 8910M047 on the use and care of laborator animals and ith the guidelines established b Institute for E perimental Animals of Zhejiang Universit .

Preparation of silk orm po der including supero ide dismutase (SOD)

The fth instar silk orm larvae e pressed SOD 96 h post-infection ith recombinant virus (rBacmid/BmNPV/SOD) ere collected [11], and dried ith a vacuum dr er (Brocher CHRIST Beta-16, German) under lo temperature of −56 C. The dried larvae ere homogeni ed to po der and stored at −20 C up to use.

Animal e periments

The mice ere randoml divided into groups ith 15 animals in each group: normal health control group (Control), received silk orm po der ithout SOD; three treated groups, administrated through feeding ith silk-orm po der including SOD at the level of 100 mg/kg bod eight/da (Lo dosage group, Group-L), 200 mg/kg bod eight/da (middle dosage group, Group-M) and 400 mg/kg bod eight/da (high dosage group, Group-H), and a positive control as set as oral feeding of *Astragalus membranaceus (Fisch.)* Bunge (supplied b Yang hi River Pharmaceutic Co. LTD, China) at level of 0.3 ml/da (Positive-Control); C clophosphamide group (CPA): 0.2 ml/10 g bod eight as injected through abdominal cavit for successive 2 da s.

The silk orm po der as made to suspension, and fed the mice ith 0.5 ml per da . The immunit inde as assa ed 30 da s after treatment.

Liver cancer Hepatoma 22 cell modeling

Thirt ve male and 35 female ICR mice (5 7 eeks) eighing 18 22 g ere used for liver cancer H22 modeling. The H22 cell line as supplied b Animal E periment Center of Medical College, Zhejiang Universit , China. After three times of passage in abdominal cavit of mouse, 0.2 ml of 5×10^6/ml H22 cells as inoculated through right armpit for successive 10 da s.

Assessment of natural killer (NK) cell activit

Spleen of mice upon e posure to manganese SOD e pressed in silk orm larvae as collected under aseptic conditions, in Hank's balanced salt solution (Sigma), as minced using a pair of scissors and passed through a ne steel mesh to obtain a homogeneous cell suspension, The interface mononuclear cells ere ashed t ice ith Hank's solution, and the er throc tes ere l sed ith NH_4Cl (0.8% (/v)). After centrifugation (1,500*g* at 4 C

for 10 min), the pelleted cells ere ashed three times ith phosphate buffered saline (PBS) and resuspended in RPMI 1640 complete medium (supplemented ith 12 mm HEPES (pH 7.1), 0.05 mm 2-sulfan lethanol, 100 IU/ml penicillin, 100 mg/ml streptom cin, and 10% FCS). Cell numbers ere counted ith a hemoc tometer b the tr pan blue d e e clusion technique. Cell viabilit e ceeded 95%. Splenoc tes ere adjusted to 2 10^5 cell/ml, and seeded into a 96- ell at-bottom microtiter plate ith RPMI 1640 complete medium. NK activit as detected ith the freshl isolated splenic mononuclear cells. Target cells for detection of NK cell c toto icit ere YAC-1 cell line (Shanghai Institute of Biochemistr and Cell Biolog , Chinese Academ of Sciences). The YAC-1 cells ere maintained in continuous suspension culture in the complete culture medium at a concentration of about 8×10^5 cells/ml at 37 C in a humidi ed 5% CO_2 incubator for 24 h. Hundred µl of NK and YAC-1 cells ere added in to a U- bottom microtiter plate hole, and 100 µl of 1% NP40 and YAC-1 cells ere added into another hole for control. The plate as incubated in a humidi ed 5% CO_2 incubator for 4 h. The plate as centrifugated at 1,500*g*, 5 min, and take 100 µl into a 96- ell at-bottom microtiter plate, and add 100 µl of LDH substrate for 5 min of reaction. Thirt µl of 1 M HCl as added to stop the reaction. The absorbance at 492 nm as monitored using a spectrophotometer. The NK cell activit as calculated according to the formula:

$$\text{NK cell activit } (\%) = [(\text{NK} + \text{YAC} - 1)\text{OD} - (\text{Yac} - 1)\text{OD}] / [(\text{Yac} - 1 + \text{NP40})\text{OD} - (\text{Yac} - 1)\text{OD}]$$

In vivo spleen proliferation assa

Spleen of mice e posure to manganese SOD e pressed in silk orm larvae as collected under aseptic conditions, in Hank's balanced salt solution (Sigma), as minced using a pair of scissors and passed through a ne steel mesh to obtain a homogeneous cell suspension, and the er throc tes ere l sed ith NH_4Cl (0.8% (/v)). After centrifugation (1,500*g* at 4 C for 10 min), the pelleted cells ere ashed three times ith phosphate buffer saline (PBS) and resuspended in RPMI 1640 complete medium (supplemented ith 12 mm HEPES (pH 7.1), 0.05 mm 2-sulfan lethanol, 100 IU/ml penicillin, 100 mg/ml streptom cin, and 10% FCS). Cell numbers ere counted ith a hemoc tometer b the tr pan blue d e e clusion technique. Cell viabilit e ceeded 95%. Splenoc te proliferation as assa ed as described b Pan et al. [14]. Brie , splenoc tes ere seeded into a 96- ell at-bottom microtiter plate at 1×10^6 cell/ml in 100 µl of complete medium, and then the Con A (nal concentration 5 µg/ml), RPMI 1640 medium as added to give a nal volume of 200 µl (tetraplicate ells). The plate as incubated at 37 C in a humidi ed atmosphere ith 5% CO_2. After 44 h, 50 µl of MTT solution (5 µg/ml) as added to each ell and incubated for further 4 h. The plates ere centrifuged (1,400*g*, 5 min) and the untransformed MTT as removed carefull b pipetting. T o hundred µl of acidic isopropanol solution (192 µl of isopropanol ith 8 µl of 1 N HCl) as added to each ell, and the absorbance as evaluated in an ELISA reader at 570 nm ith a 630-nm reference after 15 min. The stimulation inde (SI) as calculated based on the follo ing formula: SI = the absorbance value for mitogen-cultures divided b the absorbance value for non-stimulated cultures.

Statistical anal sis

The data ere e pressed as mean S.D, and compared statisticall b *t*- test, $P < 0.05$ being considered signi cant.

Effects of SOD-contained silk orm larvae po der on gro th of organs and tumor in Hepatoma 22 modeled mice

Table 1 sho ed the inoculation of Hepatoma 22 cell caused severe effect on spleen gro th, sho ing 3 5 times increases in splenoc tes/bod eight (mg/g) in modeled

1 Effects of SOD-contained silk orm larvae po der on gro th of organs and tumor in Hepatoma 22 modeled mice

Treatments	Animal No.	Splenoc tes/bod eight (mg/g)		Thoracic gland/bod eight (mg/g)		Tumor eight (g)		Inhibition rate (%)
Normal mice	10	3.77	0.82	2.29	0.38			
Modeled control	10	11.91	1.98**	2.24	0.29	2.86	0.66	
SOD silk orm po der (400 mg/kg)	10	13.30	2.79**	3.09	0.73	2.13	0.31	25.52
SOD silk orm po der (200 mg/kg)	10	16.13	0.85**	1.91	0.18	1.75	0.42*	38.81*
SOD silk orm po der (100 mg/kg)	10	12.27	0.52**	1.94	0.13	2.16	0.30	24.48
Contro (400 mg/kg)	10	12.31	2.47**	2.01	0.38	2.67	0.62	6.62

Values are sho n as the mean SD. * $P < 0.05$, ** $P < 0.01$, $n = 10$

control and SOD silk orm po der treatment compared to normal mice. In contrar , the Thoracic gland/bod eight

Effects of silk orm po der including SOD on splenoc te proliferation (SI) and NK cell activit of Hepatoma 22 tumor modeled mice

Treatments	Splenoc te proliferation (SI)		NK cell activit (%)	
Modeled control	0.973	0.076	23.06	11.24
SOD silk orm po der (400 mg/kg)	1.011	0.096*	36.18	10.64*
Control (400 mg/kg)	0.944	0.058	24.97	7.67
Positive-Control	0.951	0.073	33.42	10.16*
C clophosphamide control (CPA)	0.899	0.049	43.53	14.20**

Values are sho n as the mean SD. * $P < 0.05$, ** $P < 0.01$, $n = 10$

NK cell activit as strong enhanced, but splenoc te proliferation (SI) as reduced compared to controls.

Hepatomas are the most common t pe of cancer originating in the liver. In certain areas of Africa and Southeast Asia, hepatomas are even more common than metastatic liver cancer, and the are a prominent cause of death [15]. Usuall , the survival rate for people ith a hepatoma is poor because the tumor is detected at a late stage. At present, hepatoma as mainl treated ith ph sical therap (i.e. Radiofrequenc ablation (RFA), Intra-arterial iodine-131-lipiodol administration, Combined PEI and TACE, High intensit focused ultrasound) and chemotherap [16].

Chemotherap drugs can be injected into a vein or into the hepatic arter , hich then delivers a high concentration of the drugs directl to the cancer cells in the liver [17]. Although chemotherap drugs can temporaril slo the gro th of the tumor, the do not cure the cancer. Furthermore, almost all of chemical drugs kill both of cancer cells and normal cells at same time ith treatment. This limited the dosage of chemotherap drugs, and reduced the patients' immune function against tumor cells. Therefore, the anti-o idant drugs ere put more and more importance recentl . These drugs are useful to reduce the clinical s mptoms, enhance the life qualit , inhibit the cancer cell transferring, and assist the radio and chemotherap through the cellular antio idant defense and immunit promotion.

In our previous reports, e used a practical BmNPV bacmid s stem to e press the Mn-SOD en me protein in silk orm larvae b the recombinant bacmid baculoviruses. The e pression level as about 20 mg per larval [18]. The time course of the e pressed Mn-SOD activit in larvae infected ith the recombinant virus (rBacmid/BmNPV/SOD) as determined [11]. The availabilit of large quantities of SOD that the silk orm provides should greatl facilitate the future research and testing of this protein for potential application in medicine. We have also investigated the effects of silk orm larvae po der containing SOD on the antio idation and the immune s stem of mouse, focused on hemol sin response, hemagglutination against SRBC, the activit of natural killer (NK) cells, the ConA-stimulated splenoc tes proliferation, PFC assa and the dose-dependent increase of phagoc tic activit . All treated mice sho ed signi cant promotion in immunit [12].

Liu et al. studied the effect of injur of SOD on lipid pero idation of tumor cell, telomerase activities in tumor tissues, e pression of protooncogene. It has been found that content of MDA increased in S180 sarcom group and Le is lung cancer group in situation of SOD injur hile the content of GSH, GPX and activit of T SOD decreased. It has also been found that SOD can reduce the telomerase activities in S180 and H22 and e pression of protooncogene in S180 sarcom, Le is lung cancer tumor tissues. The radiosensitivit of SOD ma have some relationship ith the effect on e pression of protooncogene and telomerase activities, and metabolism of SOD in different kinds of tumor [19].

For silk orm larvae po der containing SOD as potential application in medicine, the safet assessment is ver necessar . Our previous data indicated the feeding treatment as safe ith 360 folds of recommended human dosage in acute to ic test. In long-term test, there ere no effects of silk orm larvae po der containing SOD on treated mice's gro th and inside organs as long as 90 da s. Further the electronic microscope investigation sho ed the intestine, liver, splenoc tes and stomach in mice ere no obvious changes both in organs and sub-organs such as nucleus, endoplasmic reticulum, mitochondrion, Golgi and pero isomes after treated for as long as 90 da s [18].

Natural killer cell is kno n as a major immune s stem in bod through mediating cell death via several possible path a s [20]. NK cells are one of three subpopulations of l mphoc tes functioning as scavenger of tumor, virus infected cells etc. Our present results found the NK cell activities of Hepatoma 22 tumor modeled mice treated ith silk orm po der including SOD ere enhanced signi cantl compared to a modeled control and silk orm po der ithout SOD control, reaching 36.18%, hich implied this material modulated the immune s stem in the Hepatoma tumor modeled mice in vivo. The spleen is an important immunological organ that contains mainl B-l mphoc tes. Hence, the spleen can indirectl re ect humoral immunit . Our data on the effects of continuous treatment ith SOD-containing silk orm po der sho ed the ConA-stimulated spleen proliferation of SOD treated mice as higher than that of the controls. As results in

Table 3, the treatment of SOD contained silk orm po der presented 40.3% of average inhibition rate to Hepatoma 22 tumor, and there ere no signi cant difference in bod eight bet een modeled control and SOD silk orm po der feeding in Hepatoma 22 tumor modeled mice, suggesting the SOD silk orm po der is safet as an inhibitant to tumor.

The c clophosphamide is an effective tumor curing medicine. In CPA, the inhibition rate as highest, but this chemical sho ed strong side-effect, presenting reduced bod eight in treated mice.

In conclusion, these ndings demonstrate that administration of silk orm po der containing SOD results in activation of NK cells and immune s stem, suggesting the silk orm po der containing SOD pla s a positive role in tumor inhibition. The results also suggested the SOD e pressed in silk orm ma be have potential application in medicine.

The orks ere supported b the Hi-Tech Research and Development Program of China (No. 2006AA10A119) and a ke project of Zhejiang Government (No.2005C22042).

1. Weisiger RA, Fridovich I (1973) Supero ide dismutase: organelle speci cit . J Biol Chem 248:3582 3592
2. Louvel E, Hugon J, Doble A (1997) Therapeutic advances in am otrophic lateral sclerosis. Trends Pharmacol Sci 18:196 203
3. Sies H (1997) O idative stress: o idants and antio idants. E p Ph siol 82(2):291 295
4. Stanner SA, Hughes J, Kell CN, Buttriss J (2004) A revie of the epidemiological evidence for the antio idant h pothesis. Public Health Nutr 7(3):407 422
5. Bartlett H, Eperjesi F (2003) Age-related macular degeneration and nutritional supplementation: a revie of randomised controlled trials. Ophthalmic Ph siol Opt 23(5):383 399
6. Wintergerst E, Maggini S, Hornig D (2006) Immune-enhancing role of vitamin C and inc and effect on clinical conditions. Ann Nutr Metab 50(2):85 94
7. Wang J, Wen L, Huang Y, Chen Y, Ku M (2006) Dual effects of antio idants in neurodegeneration: direct neuroprotection against o idative stress and indirect protection via suppression of glia-mediated in ammation. Curr Pharm Des 12(27):3521 3533
8. Hercberg S, Galan P, Pre iosi P, Bertrais S, Mennen L, Malv D, Roussel AM, Favier A, Briancon S (2004) The SU.VI.MAX Stud : a randomi ed, placebo-controlled trial of the health effects of antio idant vitamins and minerals. Arch Intern Med 164(21):2335 2342
9. Maeda S (1994) E pression of foreign genes in insect cells using baculovirus vectors. In: Maramorosch K, McIntosh AH (eds) Insect cell biotechnolog . CRC Press, pp 1 31
10. Choudar PV, Kamita SG, Maeda S (1995) E pression of foreign genes in *Bombyx mori* larvae using baculovirus vectors. In: Richardson CD (ed) Methods in molecular biolog . Baculovirus E pression Protocals, vol 39. Humana Press Inc., Chatper 14
11. Yue WF, Miao YG, Li XH, Wu XF, Zhao AC, Nakagaki M (2006) Cloning and e pression of manganese supero ide dismutase of the silk orm, *Bombyx mori* b Bac-to-Bac/BmNPV Baculovirus e pression s stem. Appl Microbiol Biotechnol 73:181 186
12. Yue W-F, Li G-L, Liu J-M, Sun J-T, Sun H-X, Li X-H, Wu X-F, Miao Y-G (2007) Anti-o idation and immune responses in mice upon e posure to manganese supero ide dismutase e pressed in silk orm larvae, *Bombyx mori* L. Cell Biol Int 31:974 978
13. Yue W-F, Liu J-M, Sun J-T, Li G-L, Li X-H, Wu X-F, Sun H-X, Zhou J-Y, Miao Y-G (2007) Immunit promotion and proteomic identi cation in mice upon e posure to manganese supero ide dismutase e pressed in silk orm larvae. J Proteome Res 6(5):1875 1881
14. Pan HJ, Sun HX, Pan YJ (2005) Adjuvant effect of ethanol e - tractof Semen Cuscutae on the immune responses to ovalbumin inmice. J Ethnopharmacol 99(1):99 103
15. Kumar V, Fausto N, Abbas A (eds) (2003) Robbins & cotran pathologic basis of disease, 7th edn. Saunders, pp 914 917
16. Lau W, Leung T, Ho S, Chan M, Machin D, Lau J, Chan A, Yeo W, Mok T, Yu S, Leung N, Johnson P (1999) Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial. Lancet 353(9155): 797 801
17. Thomas M, Zhu A (2005) Hepatocellular carcinoma: the need for progress. J Clin Oncol 23(13):2892 2899
18. Yue W-F, Liu J-M, Li G-L, Li X-H, Wu X-F, Sun J-T, Sun H-X, Miao Y-G (2007) Effects of silk orm larvae po der containing manganese supero ide dismutase on immune activit of mice. Mol Biol Rep. doi:10.1007/s11033-007-9116-0
19. Liu Y, Zhao J, Liu Y, Hao X, Wang X, Yao S (2004) The studies of effect on tumor cell apoptosis ith SOD and its anti-o idation effect. J Radiat Res Radiat Process 22(4):243 248
20. Sm th MJ, Cretne E, Kell JM, West ood JA, Street SE, Yagita H (2005) Activation of NK cell c toto icit . Mol Immunol 42:501 510