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Food Chemistry 91 (2005) 139–145
Food Chemistry
www.elsevier.com/locate/foodchem

The in vivo effects of cytokine modulation for Balb/C mice given *Canavalia ensiformis* (L.) seeds with different heat treatment

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Received 17 July 2003; revised 6 June 2004; accepted 6 June 2004

Abstract

An experiment was conducted to investigate the effects of *Canavalia ensiformis* seeds, roasted at 180 °C for 25 min (group A), 190 °C for 15 min (group B) and 200 °C for 5 min (group C), respectively, on the cytokine modulation in Balb/C mice. Six groups of mice, each divided into two parts, were administered a diet containing either 50% dried-ground seed powder (groups A1, B1, C1) or 75% dried-ground seed powder (groups A2, B2, C2) for 6 months. Another group (group D) was administered the normal diet during the same time. The physiological characteristics of the mice were observed during the study period. The results revealed neither weight difference nor pathological changes among the seven groups. However, serum-cytokine assays indicated that increased secretion, of both TH1- and TH2-pattern cytokines in mice serum can be achieved in group A1 and the most obvious increased secretion of TH2-pattern cytokines in mice serum was demonstrated in group C1. We conclude that increased secretion of both TH1- and TH2-pattern cytokines in mouse serum can be achieved with a diet of *C. ensiformis* seeds and the effect on cytokine modulation in vivo is related to its dosage and the process of heat treatment.

Keywords: Jack bean; Con A; TH1; TH2

1. Introduction

Canavalia ensiformis is a good source of protein (23–34%), carbohydrate (55%) and minerals Na, K, Ca, Mg, P, Fe, Zn, Cu, Mn and Ni (Akpapunam & Sefa-Dedeh, 1997; Rajaram & Janardhanan, 1992). *C. ensiformis* is a tropical legume, which is used for animal feeding. In China, the planting of *C. ensiformis* was recorded in the local Taiwan archives as early as the Ming Dynasty (AD 1368–1644). In India, the seeds of *C. ensiformis* (jack bean) have traditionally been eaten by indigenous peoples. However, certain antinutritional and toxic factors, such as lectins, hemagglutinins, cyanogen glucosides, oligosaccharides, trypsin inhibitors (TI) and other compounds present in the *C. ensiformis*, seed have hindered its use by modern more civilized people (Akpapunam & Sefa-Dedeh, 1997; Rajaram & Janardhanan, 1992).

The lectin, concanavalin A (Con A) comprises up to 15% of the protein in the cotyledons of jack bean

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doi:10.1016/j.foodchem.2004.06.008

(*C. ensiformis*) seeds (Carrington, Auffret, & Hanke, 1985). It is a well known polyclonal mitogen inducing human and mouse T-cell mitosis (Barral-Netto et al., 1992; Rodriguez, Cavada, Abreu-de-Oliveira, de-Azevedo-Moreira, & Russo, 1992), although it is harmful to animals (Larue-Achagiotis, Picard, & Louis-Sylvestre, 1992). The use of Con A in vitro has demonstrated that activation of T cells leads to the production of cytokines and cytokine receptors, which together drive the selected clones through their life cycle (proliferation) and ultimately to maturation and the production of effetor, or memory cells. Our research of the in vivo effects of *C. ensiformis* seeds-extract solution (5 mg/ml) on cytokine modulation demonstrated that levels of both TH1- and TH2-pattern cytokines in mice serum could be elevated by feeding with jack bean seeds (Tsai et al., 2000).

Since jack bean seeds have a long and varied history as a food source for different animals (Marfo, Wallace, Timpo, & Simpson, 1990; Mendez, Vargas, & Michelangeli, 1998) and have an effect on immunoregulation (Mendez et al., 1998), the use of the raw seeds for nourishment must be safe and practical. In order to develop more nutritious and less toxic food products by *C. ensiformis* seeds, choosing a proper and economical process for preparing them is very important. This study has been conducted to investigate the effects on the T-cell immune response in Balb/C mice fed a meal basal diet to which either 50%, or 75% of dried-ground *C. ensiformis* seeds, roasted at different temperatures, were added.

2. Materials and methods

2.1. Mice and serum samples

One hundred and twenty female Balb/C mice, purchased from the National Cheng Kung University (Tainan, Taiwan) at four weeks of age and weighing between 14 and 16 g, were used in this animal study. The mice were randomly divided into seven groups. *C. ensiformis* seeds were roasted at 180 °C for 25 min, 190 °C for 15 min and 200 °C for 5 min, and a basal diet for mice was prepared to which either 50%, or 75% of dried-ground *C. ensiformis* seeds were added, respectively. Mice, in three groups, were fed a 50% diet (180 °C: group A1; 190 °C: group B1; 200 °C: group C1), respectively, and the mice in the other three groups were fed with a 75% diet (180 °C: group A2; 190 °C: group B2; 200 °C: group C2), respectively. Another group was fed with a diet without *C. ensiformis* seeds and served as the normal control (group D). There were 15 mice in each group. A minimum of two mice were sacrificed at the end of each month for 6 months after the regimen had commenced. Sera were separated from the blood samples of sacrificed mice by centrifugation at 3500*g* for 15 min, and aliquoted and stored at −70 °C until required for cytokine assay. The brain, heart, liver, spleen and kidneys were sectioned for hematoxylin-eosin (H&E) staining.

2.2. Hematoxylin-eosin staining

All specimens were examined by routine fixation with H&E staining. All brains, hearts livers, spleens and kidneys were fixed immediately in a 10% formalin solution overnight at 4 °C and embedded in paraffin. Serial sagittal sections, 5 μm thick, were cut, and stained with H&E for histological study.

2.3. Cytokines determinations by ELISA

Cytokines (IL-2, IL-4, IL-10 and IFN-γ) were assayed using commercial ELISA kits (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions as described previously (Lin et al., 2004). Using 96-well plates, 100 μl of 1:250 diluted anti-mouse capture antibodies were added and incubated overnight at 25. Volumes of 100 μl of sera or standard were added in duplicate and plates were incubated at room temperature for 2 h. After incubation, plates were washed five times with ELISA washing solution. Then, 100 μl of working detector (biotinylated anti-mouse monoclonal antibody conjugated to horseradish peroxidase at 1:250 dilution in assay diluents) were added. Plates were incubated at 25 °C for 1 h. After incubation, the wells were washed seven times with ELISA washing solution, using an ELISA washer (BIO-RAD, Hercules, CA, USA). Substrate solution (Tetramethylbenzidine) was added and plates were incubated at 37. After incubation for 15 min, the reaction was terminated by the stop solution (2N H_2SO_4) and the absorbance was read at 450 nm (Spectrophotometer Model 550, BIO-RAD). For every test, a standard curve was also derived for IL-2, IL-4, IL-10 and IFN-γ. The range of the standard curve covered 0.0000–1000 pg for IL-2, 0.0000–1000 pg for IL-4, 0.0000–4000 pg for IL-10, and 0.0000–8000 pg for IFN-γ.

2.4. Statistical analyses

The Kruskal–Wallis test was used to test the differences in weight among different groups and P values <0.05 were taken as significant. The Wilcoxson rank sum test was used to test the weight difference and cytokine ratios between each two different groups and P values <0.05 were taken as significant.

3. Results

At the end of each month during the study period, at least two mice from each group were sacrificed. The

Table 1
The mean weight (g) ± SD of mice fed with 50% (groups A1, B1, C1), 75% (groups A2, B2, C2) or 0% (group D) *Canavalia ensiformis* seed roasted at different temperatures ($P > 0.05$)

Month	Mean weight (g) ± SD						
	180 °C Group A1	180 °C Group A2	190 °C Group B1	190 °C Group B2	200 °C Group C1	200 °C Group C2	Control Group D
1	29.00 ± 1.00	32.50 ± 0.50	28.50 ± 2.50	29.00 ± 1.00	31.25 ± 1.75	30.00 ± 2.00	33.00 ± 0.00
2	29.00 ± 1.00	32.50 ± 0.50	28.50 ± 2.50	29.00 ± 1.00	31.25 ± 1.75	33.00 ± 5.00	32.00 ± 0.00
3	30.50 ± 0.50	29.50 ± 0.50	30.00 ± 2.00	27.00 ± 1.00	35.00 ± 1.00	30.00 ± 2.00	29.65 ± 0.55
4	29.65 ± 0.55	27.30 ± 1.10	30.10 ± 1.50	30.15 ± 1.25	27.15 ± 8.55	29.30 ± 1.80	30.70 ± 0.70
5	35.50 ± 3.10	35.85 ± 1.05	37.10 ± 2.60	37.55 ± 1.95	35.75 ± 2.55	35.50 ± 1.40	34.70 ± 1.00
6	35.32 ± 0.84	31.32 ± 2.28	27.54 ± 1.60	31.30 ± 2.65	33.48 ± 4.39	34.14 ± 2.95	37.80 ± 1.38

Twenty mice were included in each group.

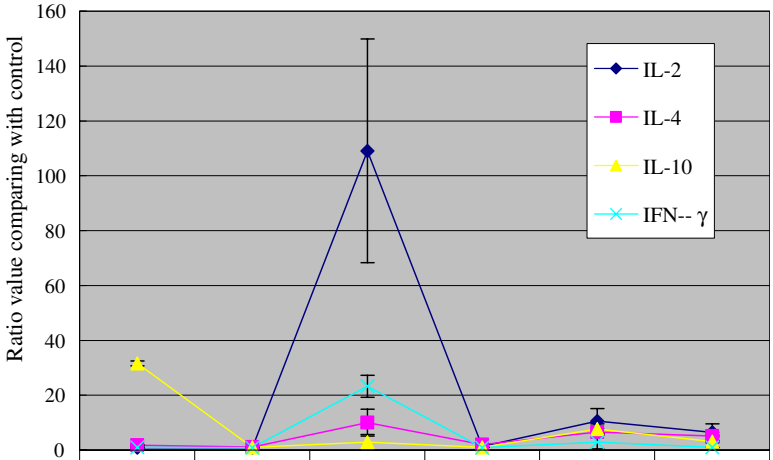
weights (Table 1) and physiological characteristics of the mice were recorded for each group. The results indicate no weight differences among different groups ($P > 0.05$). Furthermore, there were no pathological changes in the brain, the heart, the liver, the spleen or the kidneys of the mice given *C. ensiformis* seeds (Histology data not shown).

No mice in any group died during the experimental period. The serum samples from each mouse were collected separately for IL-2, IL-4, IL-10 and IFN-γ ELISA assays. Standard curves for the cytokines were also derived and covered a wide range. The cytokine concentration for the control group ranged from 0.346 to 0.853 pg/ml for IL-2, 0.0114 to 0.704 pg/ml for IL-4, 0.0105 to 0.193 pg/ml for IL-10, and 0.165 to 2.66 pg/ml for IFN-γ. The mean cytokine concentrations for mice fed with 50% (groups A1, B1, C1), 75% (groups A2, B2, C2), or 0% (group D) *C. ensiformis* seed rosted at different temperatures were identified. Compared with the control group, the cytokine concentrations for the mice fed with the diet containing *C. ensiformis* seed show greater variation. When the mice fed with a diet with 50% *C. ensiformis* seed roasted at 180 °C (group A1), IL-2 varied from 0.337 to 13.8 pg/ml, IL-4 from 0.0155 to 2.42 pg/ml, IL-10 from 0.0110 to 0.903 pg/ml and IFN-γ from 0.143 to 25.5 pg/ml; at 190 °C (group B1), IL-2 varied from 0.355 to 4.90 pg/ml, IL-4 from 0.0138 to 5.94 pg/ml, IL-10 from 0.0148 to 0.753 pg/ml and IFN-γ from 0.163 to 10.8 pg/ml; and at 200 °C (group C1), IL-2 varied from 0.439 to 12.5 pg/ml, IL-4 from 0.0150 to 355 pg/ml, IL-10 from 0.0141 to 8.11 pg/ml and IFN-γ from 0.176 to 38.5 pg/ml. When the mice were fed with the diet with 75% *C. ensiformis* seed roasted at 180 °C (group A2), IL-2 varied from 0.463 to 5.73 pg/ml, IL-4 from 0.0153 to 2.27 pg/ml, IL-10 from 0.0110 to 5.91 pg/ml and IFN-γ from 0.176 to 3.58 pg/ml; at 190 °C (group B2), IL-2 varied from 0.407 to 12.2 pg/ml, IL-4 from 0.0168 to 6.03 pg/ml, IL-10 from 0.0112 to 4.01 pg/ml and IFN-γ from 0.172 to 5.36 pg/ml; and at 200 °C (group C2), IL-2 varied from 0.311 to 1.09 pg/ml, IL-4 from 0.0152 to 2.42 pg/ml, IL-10 from 0.0098 to 20 pg/ml and IFN-γ from 0.167 to 1.04 pg/ml.

Figs. 1–6 compare the varied cytokine ratio for mice given 50% or 75% *C. ensiformis* seed roasted at 180, 190 and 200 °C with those in control groups ($P < 0.05$). In group A1, serum IL-2 and IFN-γ increased significantly in month 3, IL-10 increased significantly in the first month and then declined rapidly in the following months ($P < 0.05$). IL-4 increased less markedly than IL-10 in month 3. In group A2, only serum IL-10 varied significantly and reached a maximum in the first month and a lower maximum in month 5. In group B1, four tested cytokines, namely, IL-2, IL-4, IL-10 and IFN-γ, increased in month 3, and IL-10 reached a much higher maximum in month 5. In group B2, cytokines IL-4 and IL-10 increased much more significantly ($P < 0.05$) than cytokines IL-2 and IFN-γ. IL-4 increased significantly in months 3 and 5, and IL-10 increased significantly in months 1 and 5 ($P < 0.05$). In group C1, compared with group D, four tested cytokines, i.e., IL-2, IL-4, IL-10 and IFN-γ, showed significant variation, but the concentration ratio for IL-4 increased to 31144.73 in month 6, and for IL-10 increased to 559 in month 5. In group C2, cytokines IL-2 and IFN-γ were not significantly increased ($P > 0.05$) but cytokines IL-4 and IL-10 increased significantly ($P < 0.05$). IL-10 increased in months 1, 3 and 5, respectively, and its level was much higher than that of IL-4, which increased most significantly ($P < 0.05$) in month 3.

4. Discussion

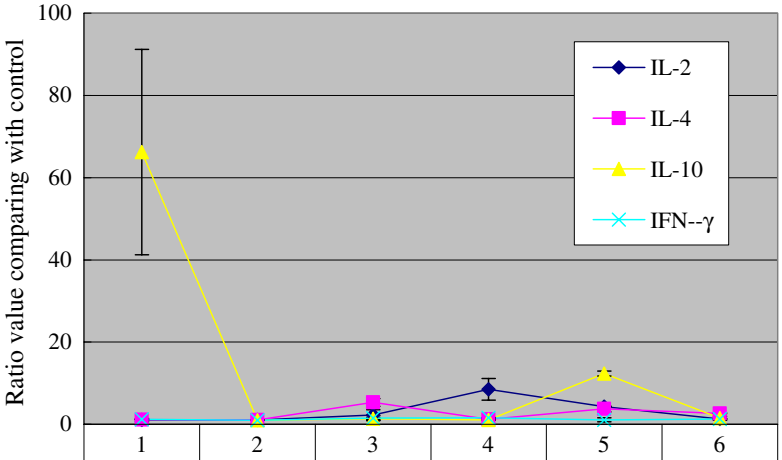
Studies were performed to investigate the effect of processing on the nutritive value of *C. ensiformis* or the activity of antinutritional and toxic factors in Balb/C mice. Bressani and Sosa (1990) reported that pressure-cooking alone and roasting at 170 °C for 15 min destroyed the antiphysiological factors in *Canavalia* but possibly also damaged its protein quality. Melo and D'Souza (2000) mixed *C. ensiformis* seed meal extract with crude cell-free extract to precipitate all the invertase. Babar, Chavan, and Kadam (1988) found that treating the seeds by soaking for 24 h, followed by



	1	2	3	4	5	6
IL-2	0.92	0.91	109	1.34	10.54	6.42
IL-4	1.81	1.14	10	1.96	6.56	5.15
IL-10	31.6	1.02	2.89	1.04	7.71	3.12
IFN-- γ	0.95	0.92	23.3	0.94	2.92	0.95

Month

Fig. 1. The ratio of cytokines in mice ($n=20$) given 50% *Canavalia ensiformis* seeds roasted at 180 °C (group A1) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).

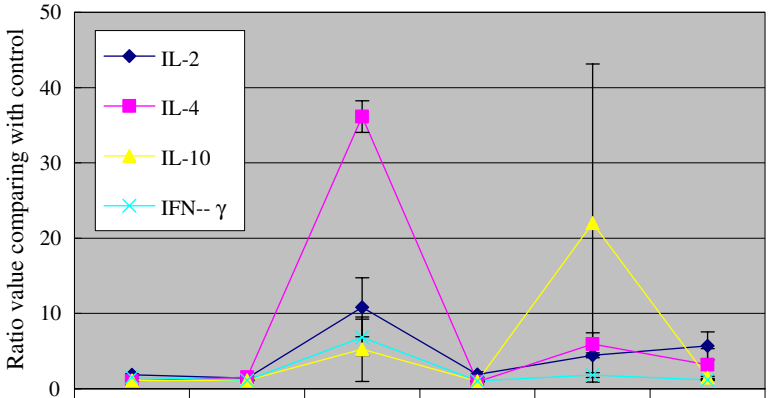


	1	2	3	4	5	6
IL-2	1.03	1.04	2.22	8.51	4.32	1.28
IL-4	1.15	1.04	5.31	1.21	3.75	2.66
IL-10	66.2	1.01	1.38	1.02	12.3	1.49
IFN--γ	1.19	1.01	1.48	1.57	1.09	1.31

Month

Fig. 2. The ratio of cytokines in mice ($n=20$) given 75% *Canavalia ensiformis* seeds roasted at 180 °C (group A2) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).

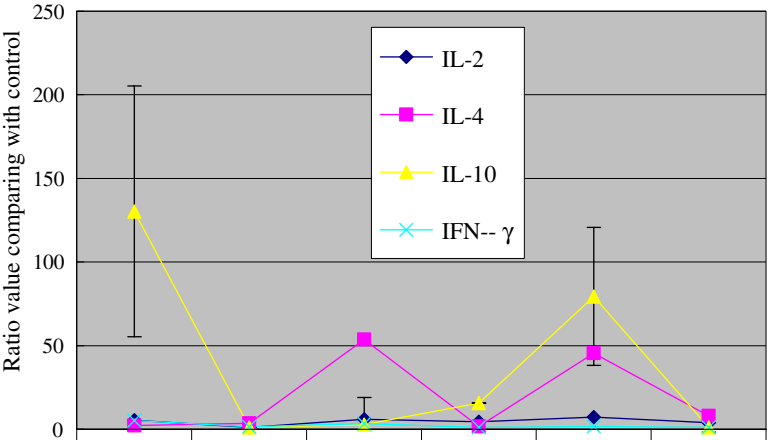
cooking for 20 min, not only reduced the polyphenol content, but was also equally effective in destroying the TI activity. Risso and Montilla (1992) showed that the autoclaved (121 °C/15 psi/90 min) *Canavalia* flour substantially improved porcine growth, but it was not effective in eliminating or minimizing the toxic effects of the raw *Canavalia*. Although animals may reject food containing Con A (Larue-Achagiotis et al., 1992; Vargas, Castillo, & Michelangeli, 1996), the mice in this study demonstrated no obvious rejection of food, suggesting that the amount of Con A in the six different diets is tolerable for the Balb/C mouse. This is consistent



	1	2	3	4	5	6
IL-2	1.87	1.41	10.8	1.88	4.45	5.68
IL-4	1.06	1.55	36.2	0.91	5.94	3.21
IL-10	1.11	1.13	5.26	1.01	22.0	1.51
IFN-- γ	1.54	1.14	6.82	1.03	1.81	1.19

Month

Fig. 3. The ratio of cytokines in mice ($n=20$) given 50% *Canavalia ensiformis* seeds roasted at 190 °C (group B1) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).



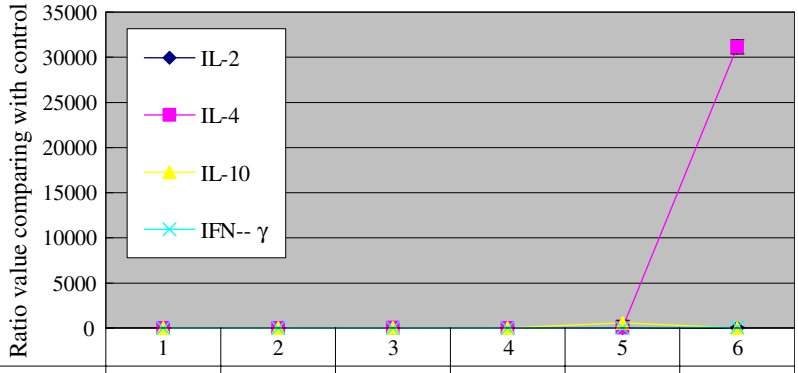
	1	2	3	4	5	6
IL-2	5.32	1.13	5.97	4.54	7.18	3.99
IL-4	2.31	3.54	53.6	1.73	45.4	8.04
IL-10	130	1.05	2.74	15.7	79.4	1.17
IFN-- γ	5.25	1.53	3.37	1.19	1.71	1.04

Month

Fig. 4. The ratio of cytokines in mice ($n=20$) given 75% *Canavalia ensiformis* seeds roasted at 190 °C (group B2) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).

with the results reported by Mendez et al. (1998), who have demonstrated that broiler chicks can tolerate a daily intake of 100 mg of Con A, a constituent of *C. ensiformis* seeds, over a 6-week period without growth being affected. The toxicity of the jack bean seed was also noted. Since neither significant death rate nor pathological change in the mice was observed in this study, it is unlikely that the jack bean seed treated by the indicated process was toxic to the mice at the set percentage of the meal.

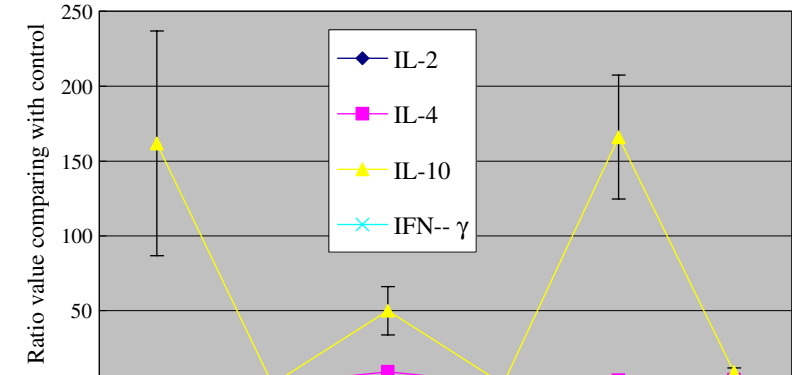
Mendez et al. (1998) have studied the immune response stimulated by the jack bean seed. They found that Con A binds to the cells of the gastrointestinal tract, is absorbed into the general circulation and, eventually, elicits an immunological response without affecting the production of antibodies to *Brucella abortus*.



	1	2	3	4	5	6
IL-2	2.35	1.23	19.3	2.93	13.01	4.06
IL-4	2.19	1.03	20.8	1.66	34.07	31145
IL-10	1.68	0.91	20.4	1.02	559	13
IFN-- γ	1.49	1.01	1.17	0.91	1.01	89.0

Month

Fig. 5. The ratio of cytokines in mice ($n=20$) given 50% *Canavalia ensiformis* seeds roasted at 200 °C (group C1) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).



	1	2	3	4	5	6
IL-2	1.08	1.09	1.01	1.18	1.59	1.31
IL-4	1.27	1.25	9.24	1.43	3.68	3.91
IL-10	161.8	1.02	50.01	1.01	166	9.04
IFN-- γ	1.68	1.03	1.23	1.02	1.51	1.65

Month

Fig. 6. The ratio of cytokines in mice ($n=20$) given 75% *Canavalia ensiformis* seeds roasted at 200 °C (group C2) compared with those in control group (group D) from months 1 to 6 ($P<0.05$).

Our in vivo studies also indicated that *C. ensiformis* can activate T-cell immune response by stimulating the secretion of TH1/TH2 – pattern cytokines (Tsai et al., 2000). The CD4+ lymphocytes can be grouped as TH1 and TH2. When stimulated by polyclonal mitogens such as Con A, they rapidly enter the G1 phase and progress through the cell cycle. If an organism triggers the release of IL-2 and IFN-γ, the TH subset that develops will bias it toward TH1, whereas release of IL-4 and IL-10 will bias it towards TH2. Thus TH1 cells release IL-2 and IFN-γ, while TH2 cells release IL-4 and IL-10. Once the TH subset has been established, either of these patterns of response is able to promote itself while suppressing the other.

In this study, when mice were fed with *C. ensiformis* seeds roasted at 180 °C, both TH1- and TH2-pattern cytokines varied significantly ($P<0.05$). In group A1, serum TH1-pattern cytokines, IL-2 and IFN-γ, increased significantly in month 3; IL-10 increased significantly in the first month and declined rapidly in month 2, and IL-4 increased less markedly than IL-2 and IFN-γ in month 3. In group A2, only serum IL-10 varied signif-

icantly and reached a higher maximum in the first month and a lower maximum in month 5. When fed with seeds roasted at 190 °C, in group B1, both TH1- (IL-2 and IFN-γ) and TH2-pattern (IL-4 and IL-10) cytokines in month 3 were significantly ($P<0.05$) increased, and IL-10 in month 5 reached a much higher maximum. In group B2, TH2-pattern cytokines increased much more significantly than TH1-pattern cytokines. IL-4 increased significantly in months 3 and 5, and IL-10 increased significantly in months 1 and 5 ($P<0.05$). When *C. ensiformis* seeds were roasted at 200 °C, in group C1, compared with group D, both TH1- and TH2-pattern cytokines showed significant variation in months 3 and 6 ($P<0.05$), especially the concentration ratio for IL-4, which increased to 31145 in month 6. In group C2, only TH2-pattern cytokines increased significantly. IL-10 increased in months 1, 3 and 5, respectively, and its level was much higher than that of IL-4, which increased in month 3.

These data reveal that increased secretion of both TH1- and TH2-pattern cytokines in mice serum can be achieved with a diet of 50% *C. ensiformis* seeds roasted at 180 °C for 25 min (group A1), and TH2-pattern cytokines increased earlier and more than TH1- pattern cytokines, but when *C. ensiformis* seeds were increased to 75% in the diet, only TH2-pattern cytokine-IL10 increased rapidly with significance ($P<0.05$). When *C. ensiformis* seeds were roasted at 190 °C for 15 min, only TH2-pattern cytokines increased significantly ($P<0.05$) in mice serum, especially when mice were fed with the diet with 75% *C. ensiformis* seeds. A similar effect was identified for each cytokine when the mice were fed with *C. ensiformis* seeds that were roasted at 200 °C for 5 min, but the TH2-pattern cytokines in mice serum increased significantly in the 50% group ($P<0.05$), and TH1-pattern cytokines had a lower up-regulation in this group. The most obvious increased secretion of TH2-pattern cytokines in mice serum was demonstrated in group C1.

Comparing these results with other published reports (Babar et al., 1988; Bressani & Sosa, 1990; Risso & Montilla, 1992), the results suggest that dry heat may be the choice for treating *C. ensiformis* seeds without damaging the cytokine modulation effects and it is non-toxic to the mice. Different ways of treatment may have different effects on *C. ensiformis* seed toxicity and TH1/TH2 balance. In the present study, in vivo modulation effects of TH2-pattern cytokines (IL-4 and IL-10) were more obviously increased than TH1-pattern cytokines (IL-2 and IFN-γ) with higher heat treatment (200 °C). The factors that might be the active components stimulating the responses recorded need further study. We conclude that the effect of *C. ensiformis* seed on cytokine modulation in vivo is related to its dosage and the process of heat treatment.

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