Peripheral Blood Mononuclear Cell Cytokine and Proliferative Response to *In Vitro Echinacea* Stimulation in Male College Wrestlers and Soccer Players During Preseason Practice

Nisarg B. Shah[1], Danielle M. Doty[2], David S. Senchina[2]

[1]Pharmacy Program and [2]Biology Department, Drake University, Des Moines, IA, 50311

ABSTRACT:

The effects of dietary botanical supplements on the immune response in athletes are unknown, despite a recent increase in herbal supplement use by both college and professional athletes. We conducted 2 separate studies to examine the effects of in vitro *Echinacea* stimulation on the immune responses of peripheral blood mononuclear cells (PBMCs) taken from athletes during preseason training. College-level male competitive athletes from 2 sports (wrestling and soccer) participated in the studies. PBMCs were isolated from blood sampled either pre- or post-practice, standardized to the same concentration, and then stimulated with extracts from *Echinacea pallida*, *Echinacea simulata*, or solvent vehicle control. Cytokine production (TNF, IL-1β, IL-10, and IFN-g) was measured from supernatants collected between 24-72 hrs contingent on the specific cytokine; proliferation was assessed at 72 hrs. Extracts were phytochemically profiled by high pressure liquid chromatography to quantify known bioactive compounds including alkamides and caffeic acid derivatives. Results differed between the wrestlers and soccer players. In general, *E. simulata* was a more potent immunomodulator than *E. pallida* in both studies. Following exercise, PBMC production of TNF, IL-10, and IFN-g production either decreased or was unaffected. IL-1β levels showed no change in either study. PBMC proliferation increased in the wrestlers as a result of training, but decreased in the soccer players. In conclusion, observed effects were contingent on species chosen, time point within preseason training, and sport (training type).

BACKGROUND & RATIONALE:

- Herbal supplements (such as *Echinacea*) are increasingly used by athletes to offset exercise-induced immunodepression (Petroczi & Naughton 2008).
- Production of cytokines and cytokines tumor necrosis factor (TNF) are known to be influenced by acute exercise and exercise training, though studies report differences in direction and degree specific to each cytokine and TNF and also across studies (Suzuki et al. 2002; Pedersen et al. 1998).
- Different species of *Echinacea* have different immunomodulatory effects *in vitro*; within the genus, *E. pallida* and *E. simulata* have discrepant effects (Senchina et al. 2006).

MATERIALS & METHODS:

Plant Materials: *Echinacea pallida* (PI631275) and *E. simulata* (PI631604A) were harvested from the North Central Regional Plant Introduction Station in Ames, IA and dried for 16 months as intact plants in a climate-controlled facility. Diced roots were used to generate 50% ethanol, 50% water tinctures at a ratio of 1:9 root:solvent. Extracts were stored at -20°C until use. A solvent vehicle control was generated from the same reagents but contained no plant material.

Human Subjects: All procedures for wrestler portion of the study were approved by the Iowa State Institutional Review Board and all procedures for the soccer portion of the study were approved by the Drake University Institutional Review Board. Eight male wrestlers (HT, WT) from the 2006-2007 Iowa State University Wrestling Team and seven male soccer players (HT, WT) from the 2007 Drake University Men's Soccer Team were recruited to the study.

Exercise Protocol: For the wrestlers, practice on Days 1 and 15 was identical and consisted of 2 h intense aerobic drills (target HR 170-180) bookended by 10-minute transition sessions (target HR 130-160). For the soccer players, the single practice session consisted of 1 h aerobic drills followed by 1 h scrimmage.

Phlebotomy: Blood was sampled at rest or post-exercise. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll gradient centrifugation and standardized to 1.0×10^6 cells/mL in AIM-V medium.

Immune Assays: *E. pallida* extract, *E. simulata* extract, and control vehicle preparations were further diluted 1:12.5 in AIM-V medium just prior to use. For cytokine production, 1 mL of cells were cultured with 50 μL of either of the three preparations. ELISA was used to assess TNF production at 24 h, IL-1β or IFN-γ production at 48 h, and IL-10 at 72 h. For proliferation, 100 μL of cells were cultured with 5 μL of either of the three preparations for 72 hours. Each condition was replicated in triplicate and proliferation quantified using a formazine salt assay (CellTiter).

Statistics: Each subject served as their own control. All statistical procedures were conducted in SPSS. Main effects of time, treatment, and treatment × time interactions were determined by ANOVA. When significant effects were discovered, follow-up posthoc tests (LSD) were performed.

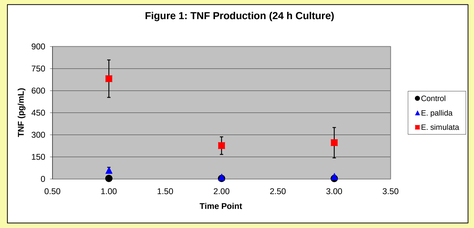

Figure 1: TNF Production in the Wrestling Study: There were time point (p=0.001), treatment (p<0.001), and time point × treatment (p<0.001) interactions. *E. simulata*- induced TNF production was higher than both control and *E. pallida*-induced TNF production at the Pre time point (both p=0.001). There was a significant reduction in TNF production with *E. simulata*-stimulated cultures from the Pre to Day 1 time points (p=0.004).

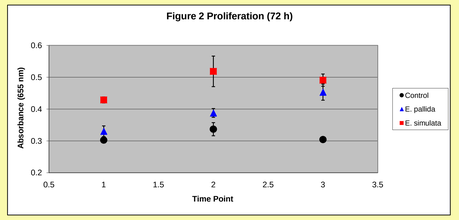

Figure 2: PBMC Proliferation in the Wrestling Study. There were time point (p=0.001), treatment (p<0.001), and time point × treatment (p=0.049) interactions. *E. simulata*-induced proliferation was higher than control at all time points (all $p \leq 0.001$). Within treatment, proliferation due to control or *E. simulata* did not change across time points (all $p \geq 0.094$ and $p \geq 0.012$, respectively); however, *E. pallida* proliferation was higher at Day 15 compared to Pre and Day 1 (both $p \leq 0.001$) and differed from control only at Day 15 (p<0.001).

Cytokine	Time Point	Treatment		
		Control	E. pallida	E. simulata
IL-1β	Pre	9.43 ± 1.2	15.42 ± 3.12	76.22 ± 19.67*
	Day 1	13.15 ± 2.81	17.76 ± 4.19	79.55 ± 21.64*
	Day 15	8.88 ± 1.61	16.89 ± 3.21	73.93 ± 18.19*
IFN-γ	Pre	6.71 ± 0.64	5.72 ± 0.51	7.29 ± 0.65
	Day 1	5.62 ± 0.62	5.91 ± 0.79	7.57 ± 1.71
	Day 15	5.94 ± 0.67	6.4 ± 0.67	6.94 ± 0.86
IL-10	Pre	4.8 ± 0.76	5.41 ± 0.72	23.74 ± 6.5*
	Day 1	4.61 ± 0.35	7.28 ± 0.93	13.97 ± 1.49*
	Day 15	4.19 ± 0.62	6.06 ± 0.53	11.4 ± 2.14*

Table 1: Wrestling study production of IL-1β and IFN-γ after 48 hours culture, and IL-10 after 72 hours culture, expressed as pg/mL. Standard errors are given for each value. Asterisks indicate statistically significant differences as compared to control (α=0.05). There were significant effects of extract but no significant effects of exercise for these cytokines.

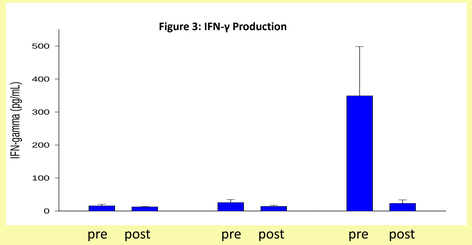

Figure 3: IFN-γ Production in the Soccer Study. There was a main effect of extract such that *E. simulata* stimulated greater IFN-γ production than control or *E. pallida* (p=0.003). There was a main effect of exercise (p=0.025) such that IFN-γ production decreased from Pre to Post acute exercise. There was an exercise × extract interaction (p=0.014); posthoc tests revealed a statistically significant decrease in IFN-γ production with *E. simulata* from Pre to Post (p<0.001).

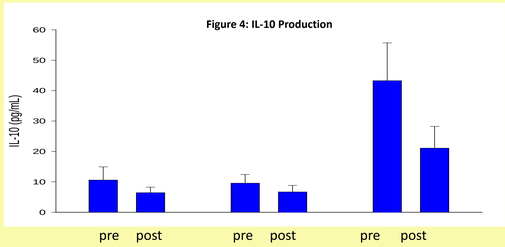

Figure 4: IL-10 Production in the Soccer Study. There was a main effect of extract such that *E. simulata* stimulated greater IL-10 production than *E. pallida* or control (p<0.001). There was a trend for a main effect of exercise (p=0.065) such that IL-10 production decreased from Pre to Post acute exercise.

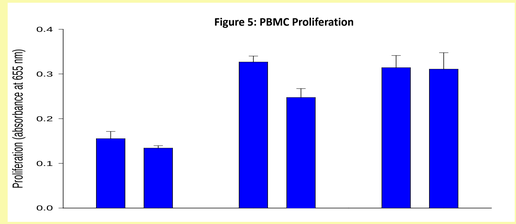

Figure 5: PBMC Proliferation in the Soccer Study. There was a main effect of extract such that *E. simulata* stimulated greater proliferation than control or *E. pallida* (both p≤0.014), and *E. pallida* stimulated greater proliferation than control (p<0.001). There was a trend for a main effect of exercise (p=0.068) such that proliferation decreased from Pre to Post exercise.

Cytokine	Time Point	Treatment		
		Control	E. pallida	E. simulata
TNF	Pre	15.62 ± 1.00	37.61 ± 9.67	1201.80 ± 176.80*
	Post	13.89 ± 1.29	18.11 ± 1.87	860.82 ± 316.62*
IL-1β	Pre	6.58 ± 1.46	13.23 ± 1.76	195.83 ± 43.73*
	Post	10.22 ± 3.01	18.42 ± 5.89	250.14 ± 74.54*

Table 2: Soccer study production of TNF and IL-1β after 24 and 48 h culture, respectively, expressed as pg/mL. Standard errors are given for each value. Asterisks indicate statistically significant differences as compared to control and *E. pallida* (α=0.05). There were significant effects of extract but no significant effect of exercise for these cytokines.

Compound	E. pallida	E. simulata
Amide 2	0.0008	ND
Amide 3	0.0087	ND
Ketone 22	0.0540	0.0148
Ketone 24	0.1668	0.0093
Caftaric Acid	0.0535	0.0359
Chlorogenic Acid	0.0079	0.0069
Cichoric Acid	0.0141	ND
Echinacoside	0.0885	0.0476

Table 3: Phytochemical composition of *E. pallida* and *E. simulata* extracts used in this study, expressed as mg/mL. Though tested for, the following phytochemicals could not be detected: amides 1, 4, 5, 7-14, and cynarin. ND = not detected.

DISCUSSION:

- In the wrestling portion of the study, *E. simulata* consistently stimulated greater levels of PBMC cytokine production (Fig. 1 and Table 1) and proliferation (Fig. 2) relative to *E. pallida* or control. Wrestlers' immune responses to *Echinacea* extracts changed during the course of pre-season training; these changes depended on the training as well as the species.
- In the soccer portion of the study, acute exercise decreased IFN-γ (Fig. 3), IL-10 (Fig. 4), and proliferation (Fig. 5). The *E. simulata* extract was able to consistently enhance cytokine production or PBMC proliferation compared to either solvent vehicle control or *E. pallida* extract (Figs. 3-5). Acute exercise caused a statistically significant decreased response to *E.simulata* extract for IFN-γ (Fig. 3); this was seen for IL-10 (Fig. 4) and TNF (not shown), but was not significant.
- The difference in results between the soccer and wrestler studies could be due to the different training styles and intensities of each sport.
- Extracts were phytochemically profiled to quantify known bioactive compounds (Table 3). The only consistent positive correlation between phytochemical composition and cytokines was chlorogenic acid which significantly increased cytokine production and proliferation (Table 4). Other phytochemicals were consistently negatively correlated with observed immune effects. Given the modest differences in chlorogenic acid between the two extracts, and given that many immunostimulatory effects were observed, our data suggest that other compounds not profiled here may be the mechanism behind the observed effects.

Compound	TNF		IL-1B		IL-10		IFN-γ		Proliferation	
	Wrestler	Soccer	Wrestler	Soccer	Wrestler	Soccer	Wrestler	Soccer	Wrestler	Soccer
Amide 2	-0.294 (0.012*) --	-0.376 (0.014*) --	-0.294 (0.012*) --	-0.361 (0.019*) --	-0.225 (0.058†) -	-0.293 (0.063†) -	-0.133 (0.267) -	-0.192 (0.224) -	-0.031 (0.793) -	0.291 (0.062†) +
Amide 3	-0.294 (0.012*) --	-0.376 (0.014*) --	-0.294 (0.012*) --	-0.361 (0.019*) --	-0.225 (0.058†) -	-0.293 (0.063†) -	-0.133 (0.267) -	-0.192 (0.224) -	-0.031 (0.793) -	0.291 (0.062†) +
Ketone 22	-0.127 (0.288)	-0.185 (0.242)	-0.117 (0.372)	-0.17 (0.280)	-0.07 (0.556)	-0.158 (0.325)	-0.074 (0.537)	-0.088 (0.580)	0.154 (0.196)	0.472 (0.002*) ++
Ketone 24	-0.265 (0.025*) --	-0.342 (0.027*) --	-0.262 (0.026*) --	-0.328 (0.034*) --	-0.197 (0.097†) -	-0.269 (0.089†) -	-0.122 (0.306) -	-0.173 (0.272) -	0.003 (0.979) -	0.326 (0.035*) ++
Caftaric Acid	0.168 (0.158)	0.158 (0.318)	0.191 (0.107)	0.17 (0.283)	0.194 (0.103)	0.093 (0.561)	0.034 (0.778)	0.096 (0.544)	0.434 (0.000*) ++	0.695 (0.000*) ++
Chlorogenic Acid	0.299 (0.011*) ++	0.312 (0.044*) ++	0.327 (0.005*) ++	0.322 (0.038*) ++	0.308 (0.009*) ++	0.210 (0.189)	0.084 (0.485)	0.179 (0.257)	0.539 (0.000*) ++	0.754 (0.000*) ++
Cichoric Acid	-0.294 (0.012*) --	-0.376 (0.014*) --	-0.294 (0.012*) --	-0.361 (0.019*) --	-0.225 (0.058†) -	-0.293 (0.063†) -	-0.133 (0.267) -	-0.192 (0.224) -	-0.031 (0.793) -	0.291 (0.062†) +
Echinacoside	0.070 (0.560)	0.043 (0.788)	0.089 (0.458)	0.056 (0.726)	0.107 (0.373)	0.008 (0.960)	-0.003 (0.982)	0.035 (0.827)	0.347 (0.003*) ++	0.634 (0.000*) ++

Table 4: Correlations between extract phytochemical composition and observed effects. Bivariate correlation Pearson values (p-value) are given for TNF,IL-1B, IL-10, IFN-γ, proliferation as correlated with phytochemical composition of *E. pallida* and *E. simulata* extracts used in both studies. Asterisks indicate statistically significant differences (p ≤0.05) and dagger indicate trends (0.05 <p< 0.1). Plus and minus symbols indicate correlation direction and strength.

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