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Alcohol extract of *Echinacea pallida* reverses stress-delayed wound healing in mice

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Abstract

Healing of open skin wounds begins with an inflammatory response. Restraint stress has been well documented to delay wound closure, partially via glucocorticoid (GC)-mediated immunosuppression of inflammation. *Echinacea*, a popular herbal immunomodulator, is purported to be beneficial for wound healing. To test the hypothesis, an alcohol extract of *E. pallida* was administered orally to mice for 3 days prior to, and 4 days post wounding with a dermal biopsy on the dorsum. Concomitantly, mice were exposed to 3 cycles of daily restraint stress prior to, and 4 cycles post wounding. *Echinacea* accelerated wound closure in the stressed mice, but had no apparent wound healing effect for the non-stressed mice when compared to their respective controls. To test if the positive healing effect is through modulation of GC release, plasma corticosterone concentrations were measured in unwounded mice treated with restraint stress and the herbal extract for 4 days. Plasma GC in restraint stressed mice gavaged with *Echinacea* was not different from mice treated with restraint only, but was increased compared to the vehicle control. This data suggests that the improved wound healing effect of *Echinacea* in stressed mice is not mediated through modulation of GC signaling.

Keywords

Alcohol extract; *Echinacea pallida*; Glucocorticoid; Restraint stress; Wound healing

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Introduction

The wound repair process has three orderly but temporally overlaid stages: inflammation, cell proliferation and tissue regeneration (Hubner, Brauchle et al. 1996; Park and Barbul 2004). The inflammatory response is a double-edged sword; it has important physiological significance in the normal healing process, but excessive inflammation is detrimental to wound repair (Midwood, Williams et al. 2004). Homeostatic regulation of the early inflammatory event is beneficial for faster wound healing.

The genus *Echinacea*, a popular herbal medicine, is a promising anti-inflammatory agent. Historically, *Echinacea angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP), have been empirically used in phytotherapy for wound healing, pain relief and alleviation of cold symptoms (Borchers, Keen et al. 2000). Recent laboratory studies indicate that alkamides and alkamide-rich alcohol extracts of *Echinacea* inhibit production of inflammatory mediators (LaLone, Hammer et al. 2007; Zhai, Haney et al. 2007).

Macrophages are important players in the inflammatory response and the early stage of wound healing. They can undergo classical activation by lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF- α) to activate inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO) that causes inflammation, or undergo alternative activation by interleukin (IL)-4 and IL-10 to activate arginase leading to production of L-ornithine (Modolell, Corraliza et al. 1995), an important precursor for cell growth and collagen synthesis in wound recovery (Meurs, Maarsingh et al. 2003). We recently investigated the differential effects of *Echinacea* on these two competitive metabolic pathways; alcohol extracts of *Echinacea* reduced TNF- α production, but increased IL-4 and IL-10 production in an *in vivo* gavage model (Zhai, Liu et al. 2007). Moreover, *Echinacea* inhibited NO production, but stimulated arginase activity in activated macrophage cell line (unpublished). These data indicate that *Echinacea* presents a clear anti-inflammatory activity that may promote wound tissue recovery.

Alcohol extracts of *Echinacea* are typically composed of two classes of natural chemicals, lipophilic alkamides and water-soluble caffeic acid derivatives. Caffeic acid derivatives are effective antioxidants in free radical generation systems (Dalby-Brown, Barsett et al. 2005), and have an antihyaluronidase activity (Facino, Carini et al. 1993). A suppression of hyaluronidase will allow accumulation of enough hyaluronan in the extracellular matrix for scarless wound repair and has been functionally linked to the healing effect of topical application of *Echinacea* on vocal fold wounds (Rousseau, Tateya et al. 2006). In rats, excision wounds treated topically with EPA extract or its constituent echinacoside, a caffeic acid derivative, showed a positive healing process characterized by reduced inflammatory response and higher hyaluronan content (Speroni, Govoni et al. 2002). However, there are no studies that have tested the efficacy of oral administration of *Echinacea* or its chemical constituents in wound healing.

Another advantage of oral *Echinacea* treatment of wounded animals is the herb's multiple immunomodulatory properties. *Echinacea* is known for strengthening the immune system against pathogenic infections partially through the activation of neutrophils and macrophages to produce inflammatory mediators (Awang 1999; Goel, Chang et al. 2002). Although chronic inflammation can disrupt normal wound repair, acute inflammation occurring at the very early stages of a wound healing process has critical physiological implications, i.e. removal of cellular debris and pathogens from wound tissue (Kondo and Ohshima 1996; Weller 2003; Midwood, Williams et al. 2004). Extrinsic factors that disturb this physiological inflammatory process might adversely affect wound healing. One notable example is stress. In a model of cutaneous wound healing, a decreased inflammatory response induced by repeated restraint stress resulted in increased corticosterone and delayed wound healing (Padgett, Marucha et al.

1998; Rojas, Padgett et al. 2002). The dual biological activities (immunomodulation and alternative macrophage activation) of *Echinacea* could be beneficial to wound healing under unfavorable conditions like chronic stress, however, direct evidence is needed.

We evaluated for the first time the wound healing effect of oral administration of EPA extract on the wound healing process in mice exposed to repeated restraint stress and non-restrained controls. We chose EPA as the *Echinacea* treatment as it decreased NO production to the greatest degree and enhanced arginase activity equivalently to two other medicinally used *Echinacea* species, EA and EP (unpublished).

Materials and methods

Preparation of EPA extract

Plants of *Echinacea pallida* (Nutt.) Nutt. (Asteraceae), accession PI 631293, were harvested in 2006 at the USDA North Central Regional Plant Introduction Station (Ames, IA). Identity to species for this accession was verified by Dr. M.P. Widrlechner, and its voucher (Rapp et al. 80) is deposited at ISC. Alcohol extract from the dried roots was prepared by Soxhlet extraction followed by evaporation to dryness (Zhai, Liu et al. 2007). The dry residue was subsequently dissolved and diluted to 16 mg/ml in 5% ethanol. Aliquots of this dilution were stored at -20°C and thawed for gavage with each aliquot used once. The phytochemicals in the dilution were analyzed using high performance liquid chromatography (HPLC) as described previously (Wu, Bae et al. 2004; LaLone, Hammer et al. 2007). *N*-isobutylundeca-2-ene-8,10-diynamide and 3,5-dimethoxy-4-hydroxy-cinnamic acid were used as internal standards for quantification of lipophilic compounds and water soluble compounds, respectively. To exclude bacterial or endotoxin contamination, the endotoxin levels were evaluated in aliquots using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD, USA) and were below the limit of detection (< 0.1 EU/ml).

Animals

Male SKH-1 mice, 5 weeks of age, were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA), and acclimated to the new environment for 2 weeks. This outbred strain of mice was used to evaluate wound healing, because they are hairless which permits better viewing of the wounds (Padgett, Marucha et al. 1998), yet immune competent. The mice were housed 2–4/cage under a reverse 12-h light/dark regimen with lights off at 8:30 AM. Unless indicated, the mice had access to water and Harlan Teklad standard rodent chow (#2018; 18% protein rodent diet) *ad lib*. All experimental manipulations were approved by the Iowa State University Institutional Animal Care and Use Committee.

Animal study 1: Wound healing effect of EPA

This study consisted of two independent replicate experiments with an identical study design (total $n = 6-7$ mice/group).

Restraint stress treatment

Mice were stressed in a manner described previously (Padgett, Marucha et al. 1998). After the mice were randomly assigned to groups, the restraint stressed groups of mice were placed individually into 50-ml conical centrifuge tubes drilled with holes of 0.4 cm diameter for air flow, heat exchange, and some urine and fecal drainage. The restraint was administered for 12 h per day (one cycle) during the light phase, with a total of 7 cycles applied: 3 cycles prior to and 4 cycles post wounding. The first stress cycle post wounding began 30–32 h after wounding. When the stressed mice were subjected to the restraint process, the non-stressed animals were simultaneously food and water deprived (FWD), but remained in their home

cages. To determine the potential effect of FWD on wound healing, an extra group was tested with continued free access to food and water (Food *ad lib*) throughout the experimental procedure.

Echinacea administration

An alcohol extract of EPA (130 mg/kg body weight) was administered by gavage to the animals once daily for a total of 7 doses; 3 doses were given immediately after each stress cycle prior to wounding, and 4 doses were given before each stress cycle post wounding. This dosage and regimen was generally selected based on an extrapolation of the dose recommended for humans and calculated as previously described (Zhai, Haney et al. 2007). Control groups of non-restrained and restrained mice received gavage with vehicle or no-gavage (FWD + vehicle, FWD or RST + vehicle, RST).

Wound biopsy

Mice were anesthetized with an intraperitoneal injection of rompum (20 mg/ml) and ketamine (100 mg/ml) mixture at 8.8 µl/g body weight. The dorsal area was disinfected and a sterile 3.5 mm biopsy punch (Miltex, Inc., York, PA, USA) was used to make two full-thickness wounds on the back (Padgett, Marucha et al. 1998). To prevent dehydration and hypothermia, mice were injected intraperitoneally with sterile 0.9% saline solution (1 ml) and placed under a warm lamp until conscious.

Wound healing evaluation

Wounds and a standard dot (3.5 mm) were photographed immediately after wounding and daily until the end of the experiment. In order to obtain high quality pictures, mice were briefly anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL, USA) prior to photography. The area of each wound and standard dot were analyzed by measuring the horizontal and vertical length using Canvas X software (ACD Systems of America, Miami, FL, USA). Beginning 7 days after wounding, healing was also assessed by the absence of an enzymatic reaction during application of 3% hydrogen peroxide (Padgett, Marucha et al. 1998) which has no negative influence on wound healing (Drosou A 2003).

Animal study 2: Immune and corticosterone modulation of EPA

Study 2 was an extension of study 1, and the animals from study 1 were reused in study 2. To rule out the potential effect of wounding history on the observational parameters, the animals were given one month to recover after experimental manipulations in study 1. Half the animals from study 1 were sampled for plasma corticosterone without further manipulation, and corticosterone was found to return to the baseline levels after one month. The remaining animals were assigned to *Echinacea* and restraint treatments without wounding in a partial crossover design (i.e. they did not receive the same stress or gavage treatment that they were assigned in study 1). Study 2 includes the same 7 groups as study 1, 3–4 mice per group. Mice were stressed or FWD as described above but with a total of 4 consecutive cycles. They were gavaged with *Echinacea* extract (130 mg/kg) or vehicle once daily before each stress cycle or assigned as no-gavage.

Two hours after the last stress cycle, the stressed mice and the non-restraint stressed mice were euthanized. Body weight and spleen weight were recorded. Blood was collected, separated, and plasma frozen at -80°C until assayed. The spleens were dissociated into a single cell suspension as previously described (Zhai, Liu et al. 2007)

Corticosterone sensitivity assay

Corticosterone is known to suppress splenocyte proliferation *in vitro* (Avitsur et al., 2001) while splenocytes from animals with high levels of plasma corticosterone are not affected as strongly by corticosterone in culture. To test the sensitivity of splenocytes to inhibition by corticosterone, cells were seeded at 5×10^5 cells per well in 96-well tissue culture plates with or without 1 $\mu\text{g/ml}$ of LPS (*E. coli* 055:B5, L6529; Sigma, St. Louis, MO, USA) and 0.1–1 μM corticosterone (Sigma) in ethanol [0.1% (v/v)]. Following 45 h of incubation, MTS (15 $\mu\text{l/well}$)(CellTiter 96 Aqueous One Solution ; Promega Corporation, Madison, WI, USA) was added and the extent of formazan formation was determined at 3 h using a plate reader (Abs, 490 nm) (Bio-Tek Instruments, Winooski, VT, USA).

Plasma corticosterone and IL-6

Corticosterone was determined using an enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA).

Plasma IL-6 was determined by an OptEIA ELISA mouse kit (BD Biosciences, San Diego, CA, USA).

Statistical analysis

A repeated measures analysis of variance was used to analyze the data from study 1. The first step was to find a reasonable model for the correlation between the repeated observations (days 1–10) on individual animals. This was done by computing Akaike Information Criterion (AIC) values for various correlation models. An autoregressive model with heterogeneous variances (ARH) had the smallest AIC; inspection of the variances for each day and the correlations among pairs of days supported this choice of model. The main effects of group (7 groups) and the group by day interaction were tested using ANOVA with the ARH correlation model and the Kenward-Rogers adjustment for degrees of freedom. The Kenward-Rogers adjustment accounted for the correlation between observations on the same animal. If the group by day interaction term was significant the data was sliced by day and each day was tested by a one-way ANOVA using *a priori* contrasts to determine effects of EPA compared to non-stressed and stressed controls. Briefly, data for the Food *ad lib* and FWD groups were considered non-stressed groups. If there were no significant differences between these groups on any day of the study, the data for these groups was pooled for subsequent analyses to increase the statistical power. Similarly, the RST and RST + vehicle groups were considered the stressed groups and if there were no significant differences between these groups on any day of the study, the data for these groups was pooled for subsequent analyses to increase the statistical power. For analysis of study 2, differences between treatment groups were tested by one-way ANOVA with subsequent *a priori* contrasts. A value of $p < 0.05$ was considered significant.

SAS PROC MIXED (version 9.1, SAS Institute, Cary, NC, USA) was used to fit the repeated measures ANOVA; Statistix software (version 8.0, Analytical Software, Tallahassee, FL, USA) was used for the separate analyses of each day and for study 2.

Results

Phytochemical analysis

The HPLC profiles of EPA extract are shown in Fig. 1 with the amounts of phytochemicals identified and quantified shown in Table 1. The alcohol extract of EPA contains two basic groups of natural chemicals, lipophilic alkamides and ketones and water-soluble caffeic acid derivatives. Alkamide 2 and 8 are the main forms of alkamides, while echinacoside is the main caffeic acid derivative, followed by cichoric acid. One novel aspect of the EPA extract is that

it contains high levels of ketones (mainly ketone 20), which are not detectable in EA or EP extracts (LaLone, Hammer et al. 2007).

Study 1. Wound healing effect of EPA

Effect of stress and EPA on body weight gain—Restraint stress can cause many physical and psychological changes. Decreased body weight gain and increased plasma corticosterone levels are two typical indicators of chronic stress (Munhoz, Lepsch et al. 2006). The restraint stress paradigm resulted in loss of body weight relative to the non-restraint stressed animals whose body weight increased gradually (Fig. 2). Upon discontinuation of stress exposure, the body weight of the stressed mice returned rapidly, but was still lower than the non-stressed groups on day 12 ($p < 0.001$). EPA, (FWD + EPA) and (RST + EPA), has no effect on body weight gain when compared to their respective controls FWD + vehicle or RST + vehicle

Effect of stress and EPA on wound closure—Fig. 3 shows daily changes in wound area compared to the original wound size. The results of the repeated measures ANOVA demonstrated a significant main effect of group ($p = 0.0027$), as well as a significant group by day interaction ($p < 0.001$). The results of the ANOVA for wound size differences between the treatment groups for each day are shown below Fig. 3. Although data for all groups was collected at the same time, we have split the data between two graphs in order to better illustrate the results across time. Among the non-stressed groups (Fig. 3A), there was no difference in wound closure between the Food *ad lib* and FWD controls. Thus, these no gavage, no restraint groups were combined. Wound healing in the FWD + vehicle group was significantly delayed compared to the combined Food *ad lib* and FWD control groups on days 1–2 and 5–6 post biopsy (p values ≤ 0.031). Interestingly, the FWD + EPA group was not different from the combined Food *ad lib* and FWD control groups except for day 6 ($p = 0.026$), indicating that gavage was stressful and that EPA administration could partially compensate for effects on wound healing.

Fig. 3B displays the data for wound closure for the RST stressed mice along with the FWD control. The RST and the (RST + vehicle) stress groups were not significantly different from each other on any day of the analysis. Thus, these two stressed groups were combined for further analyses. The combined stress groups (RST and the RST + vehicle) are significantly delayed in wound healing from day 1 through day 12 compared to the Food *ad lib* and FWD controls (the Food *ad lib* group is not shown on this graph in order to simplify the graph) (p values < 0.05). More interestingly, the wound closure for the RST + EPA group was equivalent to the Food *ad lib* and FWD controls for days 1–5 post wounding and significantly different from the RST and RST + vehicle groups on these days (p values < 0.05). On day 6 post biopsy the positive effect of EPA is no longer seen and the RST + EPA group demonstrated wound healing that is significantly behind than the Food *ad lib* and FWD controls ($p = 0.01$), and not significantly different from the RST and RST + vehicle groups.

Effect of stress and EPA on healing time—Wound healing was also assessed by determining the total time to complete wound closure (Fig. 4). Compared to the Food *ad lib* and FWD groups only the RST and RST + vehicle treatments demonstrated a significant delay in complete re-epithelization (p values < 0.001). The time to complete wound healing for the FWD + EPA and RST + EPA groups was not significantly different from the combined Food *ad lib* and FWD controls. The EPA treatment of stressed mice (RST + EPA) shortened healing time compared to the stressed groups (RST and RST + vehicle; $p < 0.001$).

Study 2. Immune and corticosterone modulation of EPA

Effect of stress and EPA on spleen/body weight ratio—In order to determine if stress reduces spleen mass, spleen/body weight ratio was calculated. Restraint stress showed a trend to reduce spleen/body weight ratio, but there was no significant difference between the stressed (all RST groups) and the non-stressed (non-restrained) mice. EPA did not significantly affect the spleen/body weight ratio (Fig. 5A).

Effect of stress and EPA on corticosterone sensitivity—The effect of oral EPA on splenocyte proliferation was assessed. Proliferation increased in the presence of LPS alone, with no significant differences among the seven groups. Addition of corticosterone decreased LPS-stimulated cell proliferation. To better evaluate the corticosterone sensitivity of splenocytes, a “corticosterone resistance” index (Avitsur, Stark et al. 2001) was calculated: $[(\text{proliferation with LPS+corticosterone})/(\text{proliferation with LPS alone})] \times 100$. Splenocytes of the stressed mice lose the sensitivity to inhibition by corticosterone, when compared to the non-stressed mice (Fig. 5B). When compared to the RST + vehicle group, the RST + EPA group showed increased corticosterone resistance, indicating the RST + EPA group might have higher corticosterone levels *in vivo*.

Effect of stress and EPA on plasma corticosterone and IL-6—Plasma collected 2 h after the last restraint cycle confirmed restraint stress without gavage significantly increased plasma corticosterone (RST vs FWD group, $P = 0.007$) (Fig. 5C). For the stressed mice, gavage with vehicle (RST + vehicle), but not EPA (RST + EPA) significantly decreased the stress-induced corticosterone levels ($P = 0.013$). Although the corticosterone concentration at the 2 h time point was lower for the RST + vehicle group compared to the RST group, the loss of corticosterone sensitivity was similar for the RST + vehicle and the RST groups. Taken together these data suggests that corticosterone is elevated in the RST + vehicle group, but has a more rapid return to baseline.

Restraint stress was also found to increase plasma IL-6 levels (the RST group vs the FWD group, $P = 0.002$) (Fig. 5D). The RST + vehicle group, rather than the RST + EPA group significantly decreased the IL-6 levels when compared to the RST control ($P = 0.031$).

As the statistical analysis of results of study 2 did not indicate that EPA was able to modulate stress induced GC, we did not repeat this experiment as increased animal numbers would result in no increased statistical significance.

Discussion

Wound healing is a multifactorial process that involves neutrophils, macrophages, fibroblasts and wound remodeling (Diegelmann and Evans 2004; Park and Barbul 2004). Neutrophils and macrophages are two important immune cell types responsible for the inflammatory stage of wound healing that takes place from the time of injury to about 5–6 days after injury. Although the inflammatory response reflects host immune defense, it must be tightly regulated in order for the later healing stages to be initiated. Based on our previous studies (Zhai, Haney et al. 2007; Zhai, Liu et al. 2007), we hypothesized that early treatment with *Echinacea* can improve wound healing through the modulation of classical and alternative macrophage function.

Chronic stress of restraint was introduced in this wound healing model to test the effects of *Echinacea* on delayed wound healing. Chronic stress is mostly immunosuppressive in the periphery and exacerbates existing disease (Padgett and Glaser 2003; Munhoz, Lepsch et al. 2006). It is well documented that chronic restraint stress significantly impairs wound healing (Padgett, Marucha et al. 1998; Rojas, Padgett et al. 2002; Sheridan, Padgett et al. 2004; Head, Farrow et al. 2006). This study confirmed previous observations as the RST and RST + vehicle

groups showed slower wound closure and longer healing time in comparison to the non-stressed controls. Interestingly, stress-induced negative effects on wound healing could be prevented by EPA extract in the early phases of wound healing, the initial 5 days when neutrophil and macrophage function are most important.

There was no improvement of wound healing in the non-stressed mice given EPA (FWD + EPA) compared to the non-stressed controls. However, the FWD + EPA group did not exhibit differences in rates of wound closure as found with the FWD + vehicle group, suggesting EPA may modulate the moderate stress of gavage. The alcohol extract of EPA contains high levels of echinacoside (Table 1) which has been shown to benefit wound healing with its antihyaluronidase activity in animal models when topically applied (Speroni, Govoni et al. 2002; Rousseau, Tateya et al. 2006). However, the improved healing effect of our study may not be the result of oral administration of echinacoside because pharmacokinetic data do not support absorption of caffeic acid derivatives in the gastrointestinal tract (Matthias, Blanchfield et al. 2004; Matthias, Addison et al. 2005; Matthias, Penman et al. 2005). In this regard, alkamides, the second major constituent of alcohol extracts, are relatively orally bioavailable (Matthias, Blanchfield et al. 2004; Matthias, Addison et al. 2005; Matthias, Penman et al. 2005; Woelkart, Koidl et al. 2005). *In vitro*, alkamides have been demonstrated to strongly inhibit LPS-induced end points (i.e. NO and TNF- α) of macrophage cell line and human whole blood (Woelkart and Bauer 2007). However, it is unclear as to what extent these *in vitro* effects can readily translate into *in vivo* systems with oral administration.

In addition to alkamides and caffeic acid derivatives, ketones are also found in alcohol extract of EPA. Like alkamides, ketones are lipophilic and easily absorbed by the intestines (Chicca, Pellati et al. 2008). Because of their usually concomitant appearance, the interaction between ketones and alkamides is unknown. Ketones have been suggested to have antifungal activity (Binns, Purgina et al. 2000) and direct cytotoxicity on cancer cells (Pellati, Calo et al. 2006; Chicca, Pellati et al. 2008). The biological activities of ketones need to be further identified.

Restraint stress caused decreased body weight and increased plasma glucocorticoid (GC) levels. They both appear to be relative to stress-induced activation of corticotrophin-releasing factor receptors (Harris, Zhou et al. 2002), which is involved in the modulation of circulating GC levels and food intake. Higher levels of GCs released by hypothalamic-pituitary-adrenal (HPA) axis have been directly correlated with stress-impaired wound healing. Upon binding of GCs to their cytosolic receptors, the newly formed ligand-receptor complexes strongly inhibit inflammatory NF- κ B function (Leung and Bloom 2003). The subsequent weakened inflammatory response decreases cellular recruitment and proliferation at the wound site and therefore slows wound healing (Rojas, Padgett et al. 2002; Sheridan, Padgett et al. 2004).

It remains unclear how EPA reverses restraint stress-delayed wound healing. Since restraint stress displays an anti-inflammatory nature through GC-dependent mechanism (Head, Farrow et al. 2006), we proposed that *Echinacea* might have anti-GC activity resulting in enhancement of the immune system. Immunostimulatory effects of oral administration of multiple *Echinacea* species have been reported (Goel, Chang et al. 2002; Zhai, Liu et al. 2007) in healthy non-stressed animals and are possibly attributed to the more bioavailable alkamides. In recent years, alkamides are suggested to be involved in activation of the endocannabinoid system (Gertsch, Schoop et al. 2004; Woelkart, Xu et al. 2005). There are two cannabinoid (CB) receptors: CB1 receptors are predominantly, but not exclusively found in the brain, while CB2 receptors occur mainly in the immune system (Klein, Newton et al. 2003). Evidence suggests that the *in vitro* immunomodulatory effects of alkamides are due to their binding to CB2 receptors (Woelkart, Xu et al. 2005). The *in vivo* relevance of the CB2 receptor-involved molecular mechanism is still not clear. Evidence suggests that the *in vitro* immunomodulatory

effects of alkamides are due to their binding to CB2 receptors (Woelkart, Xu et al. 2005). The *in vivo* relevance of the CB2 receptor-involved molecular mechanism is still not clear.

The endocannabinoid system can modulate HPA axis function (Rademacher and Hillard 2007). Activation of CB1 receptors inhibits restraint stress-induced HPA axis activation, whereas blockade of CB1 receptors increases the plasma GC levels (Patel, Roelke et al. 2004). Alkamides from *Echinacea* have shown some affinity to the CB1 receptors though the binding is much weaker compared to the CB2 receptors *in vitro* (Raduner, Majewska et al. 2006). Alkamides are highly lipophilic and, therefore, may readily pass through the blood-brain barrier and the plasma membrane of many types of cells in the central nervous system, where they could bind to and activate CB1 receptors resulting in changes to GC secretion. We determined both plasma corticosterone levels and splenocyte sensitivity to inhibition by corticosterone. Our speculation was not supported as the alcohol extract of EPA showed no inhibitory effects on the HPA axis activation. On the contrary, the stressed mice treated with EPA showed higher levels of plasma corticosterone and increased splenocyte resistance to corticosterone inhibition when compared to the RST + vehicle control, but were not different from the RST group. Because elevated plasma IL-6 is also an indicator of stress (Nukina, Sudo et al. 2001), we measured plasma IL-6 and found a pattern of IL-6 concentration similar to GC concentration. Taken together, these data (GC, IL-6 and corticosterone resistance) indicate that EPA in restraint stressed animals does not modulate GC responses directly in order to improve wound healing. Blockade of GC receptors with an antagonist RU486 could only partially reverse stress-delayed wound healing (Padgett, Marucha et al. 1998), suggesting that in addition to activation of the HPA axis to release GCs, stress may impair wound healing through other mechanisms (Padgett and Glaser 2003). *Echinacea* may act through one of these other mechanisms.

In summary, oral administration of EPA extract in a cutaneous wound healing mode exhibited a beneficial healing effect when the animals were exposed to a chronic restraint stressor. Although stress can delay wound healing through glucocorticoid related mechanisms, our work indicates that *Echinacea* does not modulate corticosterone, but can improve wound healing in the presence of high levels of corticosterone. The positive effect of oral administration of *Echinacea* may be attributable to its readily bioavailable alkamides or ketones.

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Fig. 1. HPLC chromatograms of alcohol extract of EPA: (A) lipophilic alkamides and ketones detected with UV absorbance at 210 nm (upper line) and 260 nm (lower line), and (B) water soluble caffeic acid derivatives detected with UV absorbance at 330 nm. HPLC analysis was performed as described previously by Wu et al. (2004) and Lalone et al. (2007).

Fig. 2.

Effects of RST and EPA on body weight loss. A sterile 3.5 mm punch biopsy was used to make duplicate wounds on the dorsum on day 0. The stressed animals were administrated three cycles of 12-h interval restraint (RST) stress prior to wounding and 4 cycles post wounding. The non-stressed mice were deprived of water and food (FWD) when the stressed mice were under restraint cycles except the food *ad lib* group, which had free access to food and water throughout the experimental procedure. Mice were gavaged with three doses of alcohol extract of EPA (130 mg/kg) prior to and four doses post wounding, one dose per day or an equal volume of vehicle. Body weight was recorded on days as indicated and then was adjusted by the weight at the start of the experiment to reflect change in body weight. Data represent the mean $\pm$ SE of each treatment group (n= 6–7 mice/group).

Fig. 3.

Effects of RST and EPA on wound closure of (A) the non-restraint stressed groups and (B) the stressed groups with the non-stressed FWD group. The animals were treated with RST and EPA as described in Fig. 1. Wounds were photographed immediately after wounding and then daily until healing. Individual wound size was calculated as a ratio in comparison to a standard-sized dot placed next to the wound and expressed as the percentage of the original size. Data represent the mean $\pm$ SE of each treatment group ($n=6-7$ mice/group).

The *P* values of the ANOVA analysis of wound size

ANOVA contrasts	Days post wounding									
	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10
Food <i>ad lib</i> vs FWD										
[Food <i>ad lib</i> + FWD] vs (FWD + vehicle)	0.009	0.031			0.005	0.009				
[Food <i>ad lib</i> + FWD] vs (FWD + EPA)						0.026				
RST vs (RST + vehicle)										
[Food <i>ad lib</i> + FWD] vs [RST + (RST + vehicle)]	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.001	0.009	0.037
[Food <i>ad lib</i> + FWD] vs (RST + EPA)						0.010	0.012			
(RST + EPA) vs [RST + (RST + vehicle)]	0.000	0.001	0.000	0.014	0.015

Fig. 4.

Effects of RST and EPA on healing time. Healing, assessed daily by application of 3% hydrogen peroxide beginning 7 days after wounding, was defined as the absence of enzymatic reaction. Data represents the mean $\pm$ SE of each treatment group (n=6–7 mice/group). The healing time of each individual animal is the average of its two wounds. * p <0.001 vs the Food *ad lib* and FWD groups; # p <0.001 vs the RST + EPA group.

Fig. 5.

Effects of RST and EPA on (A) spleen/body weight ratio, (B) proliferation of splenocytes treated with LPS ($1 \mu\text{g/ml}$) and corticosterone, and plasma concentrations of (C) corticosterone and (D) IL-6. Animals were administrated four consecutive 12-h interval restraint stress cycles (during light phase) and at the same time treated with an alcohol extract of EPA (130 mg/kg) body weight once daily for 4 doses or an equal volume of vehicle. Two hours after the last stress cycle, animals were euthanized and body weight and spleen weight were recorded. Corticosterone resistance, plasma corticosterone and IL-6 were measured as described in the Material and methods. Dashed line in B indicates cell viability of splenocytes of each treatment group stimulated with LPS alone and referred to 100%. Data represent mean $\pm$ standard error of the mean of 3–4 mice. * $p < 0.05$; ** $p < 0.001$.

Table 1

The amounts of alkamides, ketones and caffeic acid derivatives in EPA preparation as determined using HPLC.

Group	Metabolite	Concentration*
Alkamide	amide 2	1.01
	amide 3	0.58
	amide 5	0.45
	amide 8	2.35
	amide 9	0.94
Ketone	ketone 20	1.34
	ketone 21	0.17
Caffeic acid derivative	caftaric acid	0.61
	chlorogenic acid	0.67
	cichoric acid	1.07
	echinacoside	7.78

* Values are µg metabolite/mg dried extract