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ORIGINAL PAPER

Amelioration of human osteoarthritis symptoms with topical 'biotherapeutics': a phase I human trial

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Abstract Osteoarthritis (OA) treatments presently rely on analgesics, which manage pain but fail to restore imbalances between catabolic and anabolic processes that underlie OA pathogenesis. Recently, biologic (biotherapeutic) drugs, which alter the activity of catabolic agents such as nitric oxide and inflammatory cytokines in ways, allowing tissue regeneration, were evaluated for efficacy in OA treatment. These studies failed to demonstrate dramatic abatement of OA symptoms by these drugs, but suggested strategies by which biologic agents might be used to treat OA. The present review summarizes current understanding of OA pathogenesis and evolving treatments. Preliminary evaluations of a novel biotherapeutic strategy are presented here. Twenty OA patients receiving sour topical cherry seed extract (SCE), an inducer of heme oxygenase-1 (HO-1), a major physiological protectant against oxidative stress exhibited significantly decreased joint pain and activation of CD4+ T cells expressing inflammatory cytokines ($p<0.05$), significantly decreased peripheral blood C-reactive protein (CRP), and increased leukocyte HO-1 ($p<0.05$) in comparison with ten placebo-treated patients. SCE inhibits joint-damaging inflammatory mediator production. This agent therefore meets the main criterion for classification as a "biotherapeutic," or "biologic" agent. The negligible toxicity and low cost of such materials make them promising contributors to OA treatment, sustainable within resource limitations of a wide range of patients.

Keywords Biotherapeutic agent · Osteoarthritis · Heme oxygenase-1 · Sour cherry · Cytokines · Inflammation

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Introduction

Osteoarthritis (OA), a degenerative age-related disease that affects the joints, is the most common human musculoskeletal disorder and a leading cause of disability in elderly populations worldwide (Aggarwal et al. 2013). OA onset is typically triggered by sustained biomechanical trauma, resulting in chondrocyte-mediated cartilage destruction.

Oxidative stress, created by this degradative process, promotes emergence of senescent osteoarthritic osteoblasts, which in turn enhance dysregulation of proinflammatory signaling and apoptotic depletion of functional joint cells, causing insufficient cartilage repair and aberrant remodeling of the extracellular matrix (Chevalier et al. 2013; Clerigues et al. 2012, 2013). Tissue damage is exacerbated by trauma-related dysregulation of normal maintenance of healthy joint homeostasis (Dieppe and Lohmander 2005). This disruption promotes increasingly severe inflammation (synovitis) (Volpi and Maccari 2005), leading to adverse changes in joint fluid composition, breakdown of extracellular matrix material, and impairment of normal tissue repair.

The pathomechanisms of OA are facilitated by progressively elevated levels of the inflammatory cytokines tumor necrosis factor alpha (TNF-α), and the interleukins (IL) IL-1β, IL-6, and IL-8, produced primarily by macrophages and T lymphocytes, systemically and in affected joint tissue (Attur et al. 1998). Downstream signaling cascades of these

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cytokines also increase expression of nitric oxide (NO) by mesenchymal cells (Volpi and Maccari 2005). Collectively, each of these factors contribute to joint capsule thickening, along with loss of cartilage, chondrocyte apoptosis, progressive articular dysfunction, and extreme chronic pain (Simanek et al. 2005).

Evaluation of "biotherapeutic" agents in OA treatment Several well-known features of the OA disease process that present very attractive therapeutic targets are summarized in Fig. 1. For example, the production of inflammatory cytokines by activated CD3+ T lymphocytes offers an excellent "choke point" for intervention in OA pathogenesis. This is due to critical roles for these mediators in disease-associated pain and articular tissue destruction (Attur et al. 1998). Many treatments interfere with inflammatory cytokines at the level of their interaction with their normal physiological receptors and block downstream signaling processes, including dysregulated inflammation (Chevalier et al. 2013). However, a class of agents known as "biologic" or "biotherapeutic" drugs are distinguished by mechanisms that modulate cellular signaling pathways to interfere with disease progression, promoting activities that contribute to healthy homeostasis (Chevalier et al. 2013). These approaches differ from use of analgesics and related drugs currently favored in clinical practice, which may ameliorate pain and other symptoms but have a negligible effect on the fundamental pathomechanisms of OA (Chevalier et al. 2013).

Disappointing performance of biotherapeutic treatments for OA Previous clinical trials of inflammatory cytokine inhibitors reported by other investigators failed to produce dramatic improvement of OA prognoses (Chevalier et al. 2013). Inhibitors of both TNF and IL-1β were constructed as fusion products of a synthetic genetic element containing a portion of the gene for cognate receptors of TNF or IL-1β, spliced to the Fc (constant) portion of the immunoglobin G1 (IgG1) antibody. The resulting fusion protein binds to each cytokine, competitively reducing their physiological availability, thereby inhibiting their proinflammatory effects. In OA, this includes destruction of joint tissue (Fig. 1) (Chevalier et al. 2013). These agents, administered systemically or via intra-articular injection to OA patients affected in both knees and hands, failed to halt structural deterioration or severity of symptoms (Chevalier et al. 2013). Moreover, genetically engineered cytokine inhibitors are extremely costly. For instance, per-patient costs for a 1-year regimen of Etanercept, a TNF-inhibitory fusion protein with broad application in inflammatory disease, is approximately $20,000 (Pollack 2011). This class of drug and related products are also associated with severe side effects, including cancer and, occasionally, fatal immune impairment (Chevalier et al. 2013; Smith and Skelton 2001; Jain and Singh 2013).

HO-1-mediated inhibition of inflammation The lack of effective biopharmaceutical strategies for OA management has left clinicians heavily dependent on corticosteroids and nonsteroidal anti-inflammatories. Despite being highly effective in controlling inflammation, these drugs are often severely toxic, particularly over extended time periods (Wenham and Conaghan 2010). Conversely, biotherapeutic strategies avoid small molecule inhibitors of proinflammatory signaling cascades—which may be costly, toxic, and only marginally effective. Here, we demonstrate that a topically applied blend of phytochemicals interferes with OA pathogenesis by reducing the number of T lymphocytes activated to produce disease-associated inflammatory cytokines. Sour cherry seed extract (SCE) systemically increases the activity of heme oxygenase-1 (HO-1), a major naturally occurring antioxidant defense enzyme, ameliorates OA symptoms, and inhibits activation of CD3+ T cells to express inflammatory cytokines. HO-1 does not directly inactivate reactive oxygen molecules. Rather, its contribution to antioxidant defense occurs indirectly due to two major heme degradation products of HO-1 activity: bilirubin and carbon monoxide (CO). The enzyme metabolizes heme that accumulates in tissues as a result of red blood cell turnover to CO, free iron, and bilverdin. Biliverdin is rapidly reacted to bilirubin by ubiquitously expressed biliverdin reductase, and in nanomolar intracellular concentrations, acts as a powerful scavenger of reactive oxygen molecules (Haines et al. 2012; Morse and Choi 2002). CO exerts its cytoprotective effects through increases in the activity of guanylate cyclase to increase levels of the second messenger cyclic guanosine-3′5′-monophosphate (cGMP), which interacts with multiple cellular targets to reduce oxidative stress (Haines et al. 2012; Morse and Choi 2002). Previously, the authors showed that SCE-induced HO-1 inhibits the representation of CD3+ TNF-α+ and CD3+ IL-8+ cells in cultured leukocytes from human rheumatoid arthritis and diabetes type 2 patients at significantly greater levels than in cells from healthy control subjects (Mahmoud et al. 2013, 2014). These findings are highly encouraging, as both rheumatoid arthritis and type 2 diabetes are chronic inflammatory diseases with many features of pathogenesis—particularly inflammatory tissue destruction—common with OA (Velasquez and Katz 2010).

Topically administered SCE may be considered a "biopharmaceutical" because it directly inhibits the activity of inflammatory cytokines, which are core contributors to the disease. Components of this product curtail cytokine expression by T cells via increased tissue activity of HO-1 (Mahmoud et al. 2013, 2014). Results of a preliminary evaluation of the efficacy of a topical HO-1 inducer for OA treatment are described below. The outcomes of this study suggest that phytochemical inducers of this enzyme are potentially superior to fusion protein biopharmaceuticals, which inhibit cytokine activity at the level of interaction with their cognate receptors (Chevalier et al. 2013).

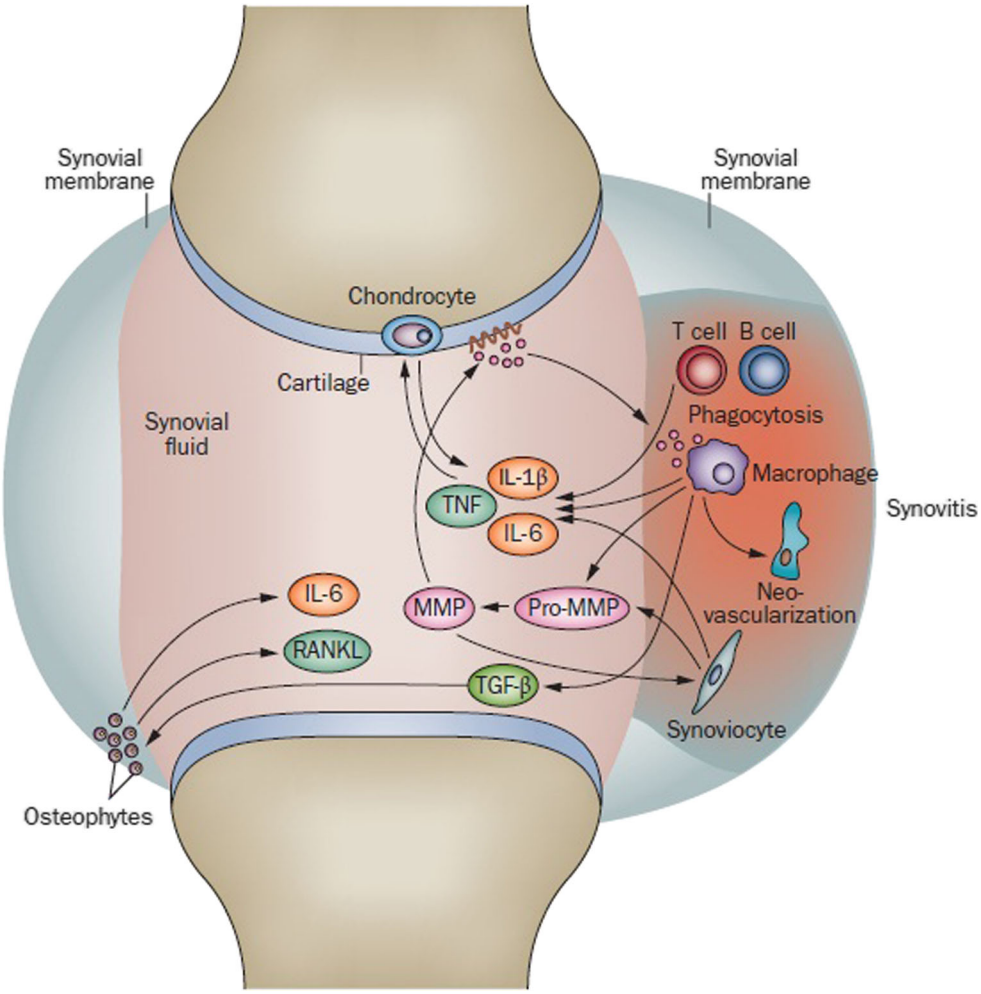


Fig. 1 Major pathomechanisms contributing to OA-associated articular tissue damage. Mechanical trauma and endogenous oxidative stressors increase synoviocyte, T cell, and macrophage expression of the inflammatory cytokines IL1-β, TNF-α, and IL-6, resulting in aberrant chondrocyte activity and damaged cartilage. Increased macrophage TGF-β expression stimulates bone tissue osteophytes, promoting joint inflammation. Pathologically elevated macrophage activity also increases neovascularization, increasing production of pro-matrix metalloproteinase (*ProMMP*), which activates its collagenase function by autocatalytic cleavage, allowing it to contribute to OA-associated collagen matrix destruction. From: Chevalier et al. (2013) [Reproduced with permission from Nat Rev Rheumatol 9, 2013]

Materials and methods

This section describes a representative clinical use of a topical biotherapeutic agent in the treatment of OA.

Study participants

Participants included 30 patients aged 40 years or older, diagnosed with inflammatory OA of the knee, according to criteria for OA set by the American College of Rheumatology (Altman et al. 1986). Patients selected for participation in the study were under treatment regimens that included nonsteroidal anti-inflammatory agents. Persons with medical conditions which might affect outcome measures independent from OA pathogenesis or routine treatment for the condition were excluded. The present study met Kuwait University's human subject protection criteria and was approved by the University's Institutional Review Board (IRB)/ethics committee. Informed consent agreements were obtained from all participating subjects.

Each subject was treated topically with 5 ml of SCE emulsion twice daily for 2 months. Treatments were conducted by applying 2.5 ml of the cream to both knees of each participant, followed by dispersal across the kneecap and surrounding skin in a circular motion, continuing until the full 5 ml had been absorbed. Patients in the placebo group were administered 5 ml of a variety of the skin cream created from the seed oil without the flavonoid fraction. The SCE emulsion was prepared from seed kernels as previously described (Mahmoud et al. 2013, 2014; Bak et al. 2010).

Treatment groups and outcomes assessed

The participants were randomly assigned into one of two treatment groups defined as follows: SCE group: 20 subjects, administered SCE emulsion twice daily for 2 months; control (placebo) group: 10 subjects, administered a SCE oil-based vehicle without the flavonoid fraction (placebo) with no SCE twice daily for 2 months.

Patients were assessed at baseline and at week 8 of treatment for: (i) index knee pain using the WOMAC pain subscale (Bellamy et al. 1988); (ii) serum HO-1; (iii) serum C-reactive protein (CRP); and (iv) activation of T lymphocytes to express the inflammatory cytokines IL-8, TNF-α, interferon gamma (IFN-γ), IL-1α, IL-1β, and IL-6. Blood collection and analysis was conducted according to previously published methods used by this laboratory (Mahmoud et al. 2013, 2014). Statistical analyses of data were performed using Windows Norusis/SPSS version 17. A p value of <0.05 was considered statistically significant.

Clinical and laboratory outcomes Prior to study enrollment, each patient was screened for compliance with inclusion/exclusion criteria. Parameters evaluated included medical history, current health status (by physical examination), laboratory and radiographic evaluations, and a review of current medications. The methodology for measurement of each of these outcomes is summarized below:

Pain assessment Self-assessed pain in the index knee was measured using the 100-mm visual analog scale (VAS) on the WOMAC pain subscale (Bellamy et al. 1988).

Phlebotomy and extraction of peripheral blood mononuclear cells Ten-milliliter samples of sodium heparin-anticoagulated peripheral venous blood were collected from study participants in vacutainer collection tubes (Becton Dickinson Biosciences Inc., Rutherford, NJ, USA) and diluted 1:1 in sterile phosphate-buffered saline (PBS) followed immediately by isolation of peripheral blood mononuclear cell (PBMC) using density centrifugation on Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) and a Centra-CL-2 centrifuge (MidAtlantic Diagnostics, Inc., Mount Laurel, NJ 08054 USA).

Cell culture PBMC were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The cells were washed and suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) at density of 1×10(6) cells/ml; 200 μl cultures in 96-well plates were incubated under humidified conditions for 6 h at 37 ° C in an atmosphere of 5 % CO_2. PBMC were stimulated in the presence of 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), 1 ng/ml of ionomycin (Sigma), and 2 mM monensin (Sigma). Here, monensin is added to cells as a glycoprotein export inhibitor, which allows intracellular accumulation of each target cytokine, thus enhancing its signal during flow cytometric analysis.

Flow cytometric analysis for inflammatory cytokines Expression of IL-8, IFN-γ, IL-1α, IL-1β, and IL-6 in CD3+ CD4+ lymphocytes in freshly collected peripheral blood or for each cell culture stimulation condition was analyzed as previously described (Mahmoud et al. 2013). Briefly, cells harvested from each culture were first incubated for 15 min at room temperature with fluorescein-isothiocyanate (FITC) anti-human CD3+ (Dakopatts, A/S, Glostrup, Denmark), then fixed and permeabilized using the Fix and Perm cell permeabilization kit (Life Technologies Inc., Eugene, OR, USA). Intracellular labeling of permeablized cells for inflammatory cytokines was accomplished by 30-min incubations at room temperature, with phycoerythrin (RD1)-conjugated monoclonal antibodies to human IL-8 IFN-γ, IL-1α, IL-1β, and IL-6 (BD PharMingen, Heidelberg, Germany). PBMC were then washed and evaluated by two-color flow cytometry for expression of each selected cytokine using the FC-500 flow cytometer (Beckman Coulter Corporation, Hialeah, FL, USA). Isotypic controls for the antibody used to detect cytokine expression were established for each cell preparation. Positive analysis regions for cells expressing selected cluster of differentiation (CD) immunophenotypic markers and cytokines were set against controls, and specific binding of fluorophore-conjugated antibodies was analyzed according to standard methods recommended by the manufacturer. Lymphocyte subpopulations were identified by position on forward and side-scatter plots. Staining of cell surface and internal antigens of interest in preparation for flow cytometry was conducted according to the manufacturer's protocol (Invitrogen Molecular Probes manual: *Detection of intracellular antigens by flow cytometry* (Rev 03/10) DCC-10-0815 (PN 624923BD). Issue A Initial Issue, 8/03 Rev Issue BD 10/11, Cytomics FC 500 CXP Software IFU Manual, running samples Sec 4.1 and creating protocols section 1–19).

ELISA analysis for HO-1 expression Measurement of lymphocyte expression of HO-1 was made using the StressXpress™ Human HO-1 ELISA Kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). Briefly, lysates made from cells were incubated in 96-well microtiter plates coated with anti-human HO-1 antibody, followed by treatment with secondary/detect antibody and related reagents provided with kits. Cell-associated HO-1 expression was evaluated during the absorbance of the developed kit reagents at 450 nm in a Biotek ELX 808 Microplate Reader. Results are reported as mean values in nanograms per milliliter of HO-1 in lymphocyte lysates of each patient group±standard error of the mean (SEM).

ELISA analysis for CRP levels Measurement of CRP was made by using Active US® CRP ELISA Kit (Diagnostic System Laboratories, Inc. Webster, Texas, USA). Briefly, peripheral blood was collected in non-anticoagulated vacutainer tubes (Becton Dickinson Inc.) and allowed to stand at room temperature for 2 h to form clots, from which serum was extracted with sterile applicators. Serum samples were

subsequently incubated in 96-well microtiter plates coated with anti-CRP antibody, followed by treatment with horseradish peroxidase (HRP)-cojugated anti-CRP. CRP concentration in each sample which was proportional to HRP-mediated conversion of a colorimetric substrate was estimated by monitoring of dual wavelength absorbance at 450 and 620 nm using a Synergy HT Multi-Mode Micro plate Reader.

Statistical analysis

Wilcoxon signed ranks test was used to compare variables in each group before and after treatment. Correlations between variables within each group were performed using Spearman rank correlation test. The analyses were performed using the SPSS for Windows statistical package version 17 (Norusis/SPSS, Inc.). A p value of <0.05 was considered statistically significant.

Results

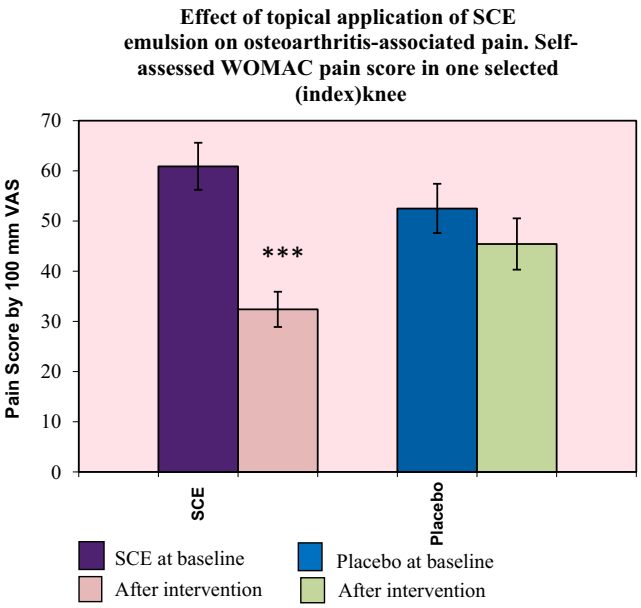
Clinical outcomes

The effect of topical SCE treatment on joint pain, self-assessed by OA patients in a selected index knee is shown in Fig. 2. When compared with pain scores measured before treatment (baseline values), SCE-treated patients reported significantly decreased pain following 2 months of daily application of the product ($p<0.001$). By contrast, comparison of pain scores reported by patients in the placebo group at baseline, with scores following 2 months of treatment with the placebo skin cream, revealed only nonsignificant differences ($p=0.139$).

Figure 3 shows expression of leukocyte-associated HO-1 in the test and control participants. Measurement of HO-1 content of PBMC lysates taken from both groups reveals significant increases in lymphocyte content of the enzyme in cells from SCE-treated OA patients evaluated by ELISA following 2 months of treatment versus baseline levels ($p<0.05$). A nonsignificant difference in baseline versus post-treatment PBMC HO-1 levels was revealed for the placebo patients ($p=0.220$).

Evaluation of CRP in peripheral blood serum of OA patients after 2 months of topical SCE treatment revealed significantly lower levels of this inflammatory biomarker than in samples taken in the same group at baseline ($p<0.005$; Fig. 4). Comparison of serum CRP in placebo-treated subjects showed only nonsignificant differences between baseline and posttreatment values ($p=0.957$).

The effect of SCE treatment on activation of peripheral blood leukocytes is shown in Fig. 5. Here, the effect of treatments on CD4+ T cells is shown, since these cells are a major source of the inflammatory cytokines contributing to



****p<0.001 versus baseline group values.*

Fig. 2 Suppression of OA-associated pain with topical SCE preparation. Outcomes are shown for two groups of OA patients: 20 administered 5 ml SCE-containing skin cream and 10 receiving placebo emollient applied topically to skin on the suprapatellar portion of index knee (the knee selected for pain assessment) twice daily for 2 months. Pain was self-assessed at baseline, and following the 2-month treatment regimen, with results reported as pain score±SEM on the 100-mm visual analog scale (*VAS*) WOMAC pain subscale

OA pathogenesis (Attur et al. 1998). It is nevertheless anticipated that SCE also alters proinflammatory signaling by macrophages and other tissue. Ongoing studies are evaluating the scope of SCE-mediated effects in other cell types. Relative to baseline measurements, PBMC from SCE-treated OA patients cultured with PMA/I, contained significantly lower post treatment representation of CD4+ IL-8+ ($p<0.001$), CD4+ TNF-α+ ($p<0.005$) and CD4+ IFN-γ+ ($p<0.005$) (Fig. 5a), CD4+ IL-1α+ ($p<0.005$), and CD4+ IL-1β+ ($p<0.005$) but not CD4+ IL-6 ($p=0.494$) (Fig. 5b). In these experiments, comparison of cells from blood of OA patients taken at baseline, versus placebo-treated patients show non-significant differences in representation of CD4+ T cells activated to express IL-8 ($p=0.730$), TNF-α ($p=0.165$), and IFN-γ ($p=0.160$) (Fig. 5a), IL-1α ($p=0.620$), IL-1β ($p=0.406$) and IL-6 ($p=0.240$) (Fig. 5b).

Discussion

Implication of preliminary human trials

The representative investigation described above was a double-blind clinical trial, evaluating a hypothesis that SCE,

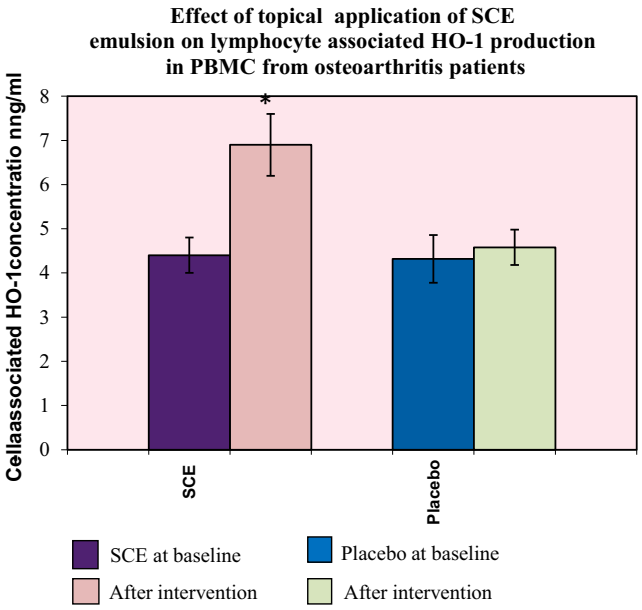
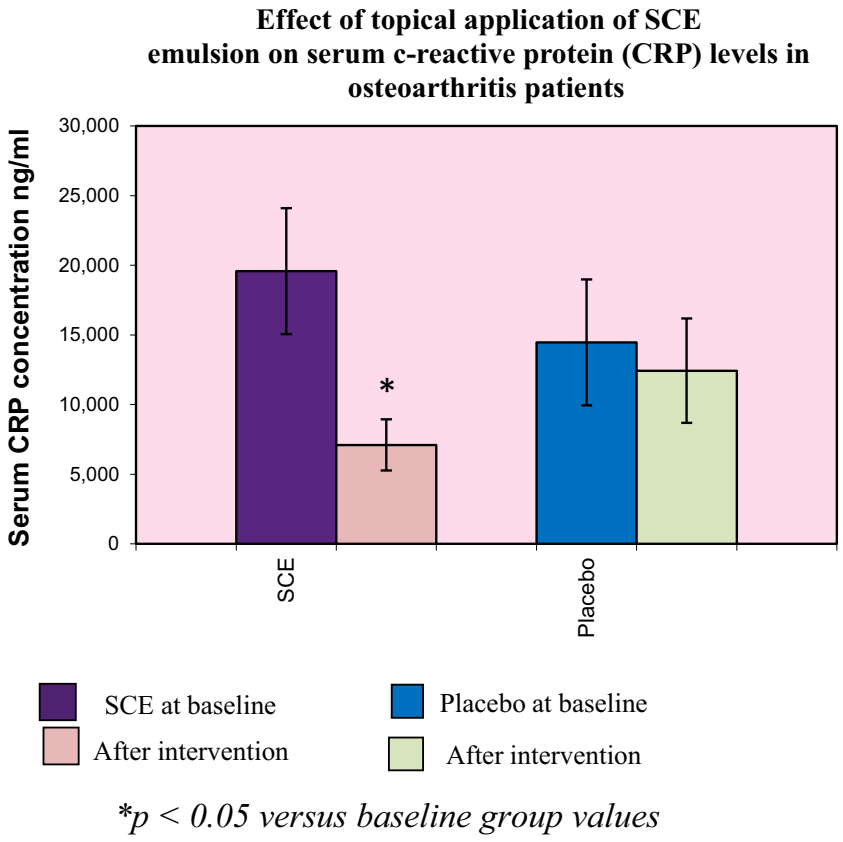


Fig. 3 SCE treatment-associated heme oxygenase-1 (*HO-1*) production increase by PBMC. Lysates made from PBMC Ficoll-Hypaque extracted from freshly collected peripheral blood of topical SCE-treated subjects (n=20), and placebo-treated subjects (n=10) at baseline and following 2 months of treatment, are evaluated by ELISA for average content of the enzyme, reported in nanograms per milliliter HO-1 protein±SEM

administered topically, reduces joint pain and systemically inhibits expression of inflammatory cytokines that promote OA pathogenesis; along with reduction in CRP, and numbers of T cells activated to express IL-8, TNF-α, IFN-γ, IL-1 α, and IL-1β. These results were obtained by persons administered extracts of the sour cherry seed kernel, but not the fruit. Interestingly, sour cherry juice is also shown to affect some of the same parameters as described in the present report. In that study, 58 nondiabetic patients with Kellgren grade 2e3 OA, each consumed 28 oz bottles of sour cherry juice or placebo, daily for 6 weeks. WOMAC pain scores and high sensitivity C-reactive protein (hsCRP) were significantly reduced in patients receiving juice but not placebo (P<0.01) (Schumacher et al. 2013). The mechanisms by which the juice ameliorated OA symptoms remain undefined. Future studies by our group will include efforts to isolate and characterize phytochemical agents produced by the sour cherry plant contributing to strengthening of processes contributing to healthy tissue homeostasis—including HO-1 activity.

Based on previous investigations and the significantly increased lymphocyte HO-1 expression noted in SCE-treated patients relative to baseline and placebo (Fig. 3), the therapeutic mechanisms likely include HO-1-mediated quenching of proinflammatory reactive oxygen species. In this study, we observed that topical treatment of OA patients with a

Fig. 4 SCE treatment-associated decrease in serum C-reactive protein (*CRP*). Defibrinated serum extracted from non-anticoagulated peripheral blood collected at baseline and following 2 months of treatment was evaluated by ELISA for CRP content in test subjects treated topically with SCE-containing skin cream (n=20), and a control group (n=10) treated with a placebo emollient. Outcomes are reported as average concentration of CRP in serum±SEM



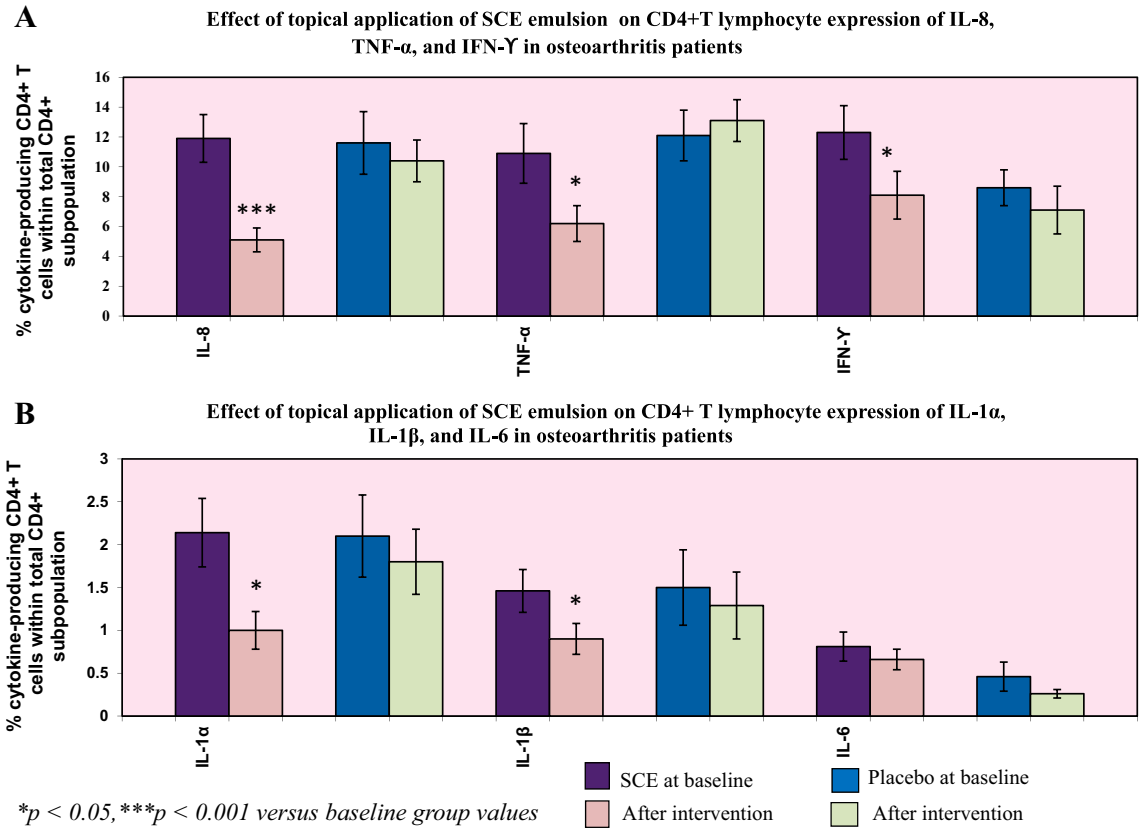


Fig. 5 SCE-mediated suppression of CD3+ CD4+ IL-8+, CD3+ CD4+ TNF-α+, CD3+ CD4+ IL-1α, and CD3+ CD4+ IL-1β+ representation in peripheral blood mononuclear cells (PBMC). PBMC from 20 OA patients treated with SCE emulsion and cells from 10 placebo-treated OA patients at baseline, and after 2 months of SCE treatment, were cultured 6 h, 37 °C with 50 ng/ml PMA/I, 1 ng/ml ionomycin, 2 mM monensin, stained using internal cell fluorophore labeling, then analyzed by two-color flow cytometry for IL-8, TNF-α, and IFN-γ (**a**) and IL-1α, IL-1β, and IL-6 (**b**). Results are reported as percentage of T helper (CD3+ CD4+) cells activated to express selected cytokines within the CD3+ CD4+ population ±SEM

preparation of sour cherry flavonoids that include HO-1 inducers which therapeutically diminish oxidative stress burden on tissue (Mahmoud et al. 2013; Mahmoud et al. 2014), significantly abated major OA symptoms. These effects occurred via inhibition of CD3+ T cell expression of inflammatory cytokines, thus demonstrating the capacity of this plant material to ameliorate OA severity by altering a major underlying contributor to disease progression. The specific identity of compound(s) responsible for this effect remain to be identified, however, since the HO-1-inducing ability of the seed kernel is localized in the solid fraction but absent from the oil, it is likely that the phytochemical(s) responsible for the effect are flavonoid in nature (Bak et al. 2010).

The outcomes reported here provide compelling validation for the clinical value of SCE, but must nevertheless be qualified by an acknowledgement that they are descriptive data and cannot be used to define underlying mechanisms. Whereas the therapeutic effects correlate with HO-1 expression, data presented here do not unambiguously show them to be HO-1-dependent. Proposed resolution for this challenge is summarized below in *Limitations and proposed solutions*. We previously demonstrated that SCE, administered orally to animals, induces HO-1 expression at levels that inhibit and reverse ischemia-reperfusion injury to the retina (Szabo et al. 2004) and heart (Bak et al. 2006; Juhasz et al. 2013), with topical application exhibiting powerful photoprotective effects on skin and negligible toxicity, even at whole-body dosage in excess of 200× therapeutic levels (Bak et al. 2011).

Human-used studies of orally delivered SCE show that subclinical dosage of the extract, consumed daily for 2 weeks by human volunteers, resulted in small but significant changes in the magnitude of mean cell volume, mean peroxidase index, ferritin, and transferrins, and predicted possible beneficial effects at therapeutic dosages. They also revealed an absence of trends toward significant increases in toxicity-associated enzyme activity, such as glutamic-oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) levels (Lekli et al. 2014). These encouraging findings further suggest future potential for clinical use of this plant material.

Emerging applications of HO-1 inducers

The results of ongoing work by our laboratory (Mahmoud et al. 2013, 2014; Bak et al. 2006, 2010, 2011; Szabo et al. 2004; Juhasz et al. 2013; Lekli et al. 2014) and others (Haines et al. 2012; Morse and Choi 2002) are consistent with recent demonstrations that inducers of HO-1 may radically improve prognoses of many diseases in which oxidative stress is a primary pathomechanism, particularly disorders of the cardiovascular system, lung, neurological tissue, and kidney (Haines et al. 2012). Strategies for therapeutic induction of the enzyme are increasingly viewed by clinicians as a primary approach to prevention and management of an expanding range of serious chronic illness (Morse and Choi 2002).

The capacity of HO-1 to specifically counteract inflammatory cytokine-mediated impairment of joint tissue homeostasis and oxidative stress-induced increases in senescent osteoarthritic osteoblasts (Clerigues et al. 2012, 2013) is also very intriguing. Senescent cells, which form as a result of cellular damage or replicative exhaustion, are major sources of inflammatory mediators, creating progressively proinflammatory tissue environments. Their age-dependent accumulation in all tissues is a primary contributor to age-related physical debilitation, including susceptibility to increasingly severe OA (Haines et al. 2014).

By acting to strengthen native endogenous countermeasures against oxidative stress and reduce the destructive effect of senescent cells, HO-1 inducers function as "biopharmaceuticals" rather than simply analgesic agents. Their activity specifically inhibits major OA-associated degenerative tissue remodeling, rather than temporarily abating pain. Interference with inflammatory signaling cascades is the mode of action for most small molecule drug-based OA countermeasures and is also a major contributor to drug toxicity (Morse and Choi 2002; Haines et al. 2011, 2014). For these reasons, therapeutic strategies which avoid interference with physiologic signaling and instead augment naturally occurring immunoregulatory mechanisms offer potent abatement of symptoms with greatly reduced risk of adverse side effects.

Phytochemicals are particularly attractive as HO-1-inducing biotherapeutic agents due to their "generally regarded as safe" (GRAS) status and ability to directly inhibit OA-associated tissue damage. Some of these, such as turmeric extract, which, like SCE, contains a phytochemical HO-1 inducer (curcumin) (Aggarwal et al. 2013), have long historical traditions as components of the human diet. Curcumin is particularly interesting because this polyphenolic component of turmeric spice may act as a biotherapeutic agent by inhibiting IL-1β-mediated articular cartilage destruction (Clutterbuck et al. 2013).

The specific role of HO-1 in mediating effects described in this and other reports remains to be clearly defined. SCE is a complex mixture of phytochemicals. Thus, it is possible that one or more of the components of this material mediates the observed effects independently of increased HO-1 activity. This possibility will be evaluated in future studies.

Relevance HO-1 increases in SCE-treated subjects In the representative human study described above, the significantly increased expression of HO-1 by PBMC, isolated from the blood of SCE-treated subjects ($p<0.05$) shown in Fig. 3, is consistent with the expected effect of transdermal delivery of HO-1-inducers at levels sufficient to mediate systemic upregulation of the enzyme. Indeed, increased activity of HO-1 in immune cells (and possibly other tissue) may account for the significant SCE treatment-associated reduction in serum CRP shown in Fig. 4 ($p<0.05$), as CRP level is a well-known correlate of the severity of inflammatory diseases, including OA (Jin et al. 2013; Kitamura et al. 2011). The relevance of increased HO-1 expression and CRP inhibition to OA and other inflammatory diseases is strengthened by earlier studies that demonstrate a close correlation between severity of joint inflammation and levels of HO-1, CRP, and matrix metalloproteinase (a tissue degradative enzyme) in synovial fluid of rheumatoid arthritis patients (Jin et al. 2013; Kitamura et al. 2011; Bak et al. 2003). These observations further underscore the role of disease- or trauma-related HO-1 expression as a general adaptive response to dysregulated inflammation with therapeutic potential in many clinical venues (Morse and Choi 2002). It is therefore tempting to speculate that SCE-induced increases in HO-1 expression is the critical mediator of significant treatment-related decreases in the representation of CD3+ subpopulations activated to express the inflammatory cytokines IL-8, TNF-α, IFN-γ, IL-1α, IL-1β, and IL-6 ($p<0.05$), shown in Figs. 5a, b. Our previous work and those of other investigators has demonstrated a clear correlation between HO-1 activity and its effects on disease-related outcome variables in a wide range of in vitro and in vivo studies in both humans and animals (Lekli et al. 2014; Clutterbuck et al. 2013; Jin et al. 2013; Kitamura et al. 2011).

Limitations and proposed solutions Many previous evaluations of whole plant extracts such as SCE, containing HO-1 inducers, to ameliorate inflammatory tissue damage cannot be considered definitive due to the complex composition of these materials. For instance, in the example provided as part of the present review, SCE is demonstrated to increase HO-1 expression, however pain abatement and decreased OA-associated biomarker expression was not shown to be directly dependent on HO-1 increases. Indeed, it is likely that many bioactive components of the material contributed to the effects observed. Ongoing work by the authors addresses the degree to which HO-1 mediates therapeutic effects via two major experimental strategies: First, the findings of previous studies using transgenic animals (Bak et al. 2003; Juhasz et al. 2011),

we will evaluate the capacity of HO-1 in mediating the physiologic effects of SCE. In these studies, HO-1 inhibitors and gene silencing protocols will determine the capacity of SCE to mediate selected effects independent of the enzyme. Both in vitro and animal HO-1 transgenic models are being used to assess its effects in SCE-stimulated systems. Second, SCE will be tested for its ability to cause epigenetic changes in chondrocytes, which may diminish the severity of OA symptoms. Experimental strategies that have provided significant insight into this are described in a report of hypomethylation in promoter sites for genes that contributed to its pathogenesis in patients' chondrocytes (Haseeb et al. 2014). Ongoing studies in our laboratory use these results and related emerging findings to optimize epigenetic effects of SCE, which contribute to improved prognoses of OA. It is acknowledged that the small sample size of this investigation limits the extent to which SCE use may be predicted to impact broad strategies for clinical management of OA. Nevertheless, the product's ability to significantly ameliorate joint pain following topical application suggests that it may become an attractive addition to regimens for treatment of the disease. The significant increases in leukocyte-associated HO-1 and decreased serum CRP, along with reduced representation of T cells activated to express inflammatory cytokines, suggest that it may act by diminishing the effect of core destructive processes that promote progressive joint tissue damage.

Conclusions The emerging therapeutic application of HO-1 inducers at outcomes of the preliminary human OA trial shown here suggest that this class of biotherapeutic agents might be useful as a stand-alone anti-arthritic therapy, especially for remediation of mild symptoms. However, we acknowledge that SCE and related products may not be sufficiently potent to be an independently curative OA treatment. The wide availability and low cost of SCE (these seeds are currently discarded as an agricultural byproduct), and lack of toxicity even at very high dosage (Bak et al. 2011), make it attractive as an adjuvant to other OA countermeasures—and may enable management of symptoms with lower dosages of other drugs than are currently prescribed.

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Author contributions The author contributions may be summarized as follows: Dr. Fadia Mahmoud made the concept and design of the study, data acquisition and data analysis. Dr. David Haines identified the chemical composition of emulsifying material required to stabilize sour cherry seed components of differing solubility in a carbamide vehicle. Drs. Mahmoud and Haines contributed equally to data interpretation and written presentation of the results. Overall supervision of patient care and monitoring was provided by Prof. Adel Al-Awadhi M.D.

Conflicts of interest The authors declare no competing interests.

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