Authentic tart cherry juice reduces markers of inflammation in overweight and obese subjects: a randomized, crossover pilot study

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Subclinical inflammation is frequently noted in chronic diseases such as diabetes, cardiovascular disease (CVD) and obesity. Accumulating epidemiological evidence demonstrates that diets rich in vegetables and fruits, e.g., cherries, may significantly reduce the risk of chronic disease, in part, via antioxidant and anti-inflammatory activities. In this randomized, placebo-controlled crossover study, we recruited 10 at-risk participants (38.1 ± 12.5 years; 8 females, 2 males) with BMI >25.0 kg m⁻² (32.2 ± 4.6 kg m⁻²; 5 obese, 5 overweight) to consume 240 mL (8 ounces) daily of either 100% tart cherry juice (TCJ) or an alternate placebo beverage, for 4 weeks with a 2-week intervening washout period before switching to the alternate beverage for four weeks. Fasting blood samples were collected at the beginning and end of each arm for measurement of biomarkers of inflammation. The erythrocyte sedimentation rate (ESR), an indicator of chronic inflammation, was significantly (p < 0.05) lower with TCJ than with the placebo beverage, which increased ESR by 19%. Mean baseline hsCRP, an indicator of acute inflammation, was 7.0 ± 5.2 mg L⁻¹ and consumption of TCJ did not affect hsCRP levels. The chemokine MCP-1 and cytokine TNF-alpha were lower in participants after consuming TCJ compared to those consuming the placebo beverage. Plasma IL-6 and IL-10 were not different between treatments. Collectively, the data suggest that authentic 100% TCJ may reduce biomarkers of inflammation often noted in chronic disease and may be a preferable dietary selection compared to artificially flavored beverages with added sugars.

1. Introduction

Subclinical inflammation and oxidative stress occur frequently with the onset and progression of chronic diseases such as cardiovascular disease (CVD), diabetes, obesity, and cancer as well as conditions such as metabolic syndrome (MetS), which places individuals at risk for chronic disease. 1-5 Numerous studies support that consumption of fruits and vegetables rich in nutritive and non-nutritive bioactive components is inversely associated with the occurrence of chronic diseases. This protection is due, in part, to the anti-inflammatory and anti-oxidant activities of the plethora of molecules, both nutritive and non-nutritive, in these foods. 4

Tart cherries are rich in polyphenolic anthocyanins, as well as many other nutritive and non-nutritive bioactive components that exert antioxidant and anti-inflammatory activities. 5 Cherry-rich components such as powders and individual components have been shown in several animal and cell culture models to significantly reduce oxidative stress and inflammation. 5-8 In human studies, consumption of sweet cherries (227 g d⁻¹, 3 months) significantly reduced the occurrence of pro-inflammatory arthritis and has also been shown in fasted healthy women to reduce, within hours, circulating pro-inflammatory markers including high-sensitivity C-reactive protein (hsCRP) and nitric oxide (NO). 9,10 Other studies have demonstrated that tart cherry juice (TCJ) can decrease oxidative stress and inflammation induced by exercise in older individuals and elite athletes, e.g., marathon runners. 9,10 In younger college students, both sweet and tart cherries decreased strength loss and perceived pain after post-exercise-induced oxidative stress and inflammation. 11 Collectively, there are adequate data supporting the capacity of cherries, both sweet and tart, to reduce oxidative stress and inflammation associated frequently with the onset and elaboration of chronic disease although additional studies in at-risk humans are needed to determine the efficacy of TCJ.

In this study, we conducted a 10-week randomized, placebo-controlled 2 × 2 crossover dietary intervention using at-risk overweight (25.0–29.9 kg m⁻²) and obese (≥30.0 kg m⁻²) participants. We randomized participants to consume 240 mL (8 ounces) daily of either placebo (artificial fruit-flavored, anthocyanin-free beverage) or 100% TCJ (equivalent to ~50 tart
obese participants (BMI ≥ 25.0 kg m\(^{-2}\)) as the primary inclusion criterion) as shown in Fig. 1. Participants were ≥18 years of age, not pregnant, not diabetic, with no unresolved infections or diseases (diabetes, CVD, IBD, cancer and liver disease), and nonsmokers. Histories of medication and dietary supplement use were collected and those taking anti-inflammatory or lipid-lowering medications were excluded. This study was approved by the Institutional Review Board at Arizona State University and informed consent was obtained from each respondent prior to entering the study. This study has been registered with ClinicalTrials.gov with identifier NCT03638362.

Participants. The study population was recruited from the Phoenix, AZ metropolitan area via flyer/handbill dissemination, word-of-mouth notification, poster displays, etc. as reviewed and approved by the ASU IRB. Individuals were screened via telephone call and onsite interviews at the ASU Polytechnic Campus (Mesa, AZ) to ensure a generally healthy state and absence of significant chronic disease including diabetes, IBD, arthritis, etc. During the screen, individuals were weighted and height measured (stadiometer) to determine whether the respondent was overweight or obese. A total of 17 respondents were screened (assuming 20% attrition) with 4 not meeting inclusion criteria for BMI >25 kg m\(^{-2}\).

Throughout the study, three from the remaining 13 participants (23%) withdrew due to illness, infection, and new prescription of lipid-lowering medication. Remaining participants were weight-stable for the previous 6 months and not on current weight loss regimens. Participants were instructed to refrain from consuming any other significant anthocyanin-containing fruits and juices during the study period including cherries, red grapes, pomegranates, berries (blueberries, cranberries, raspberries, blackberries, etc.) and their juices, red wine, or dark chocolate. Respondents were provided a recording form and oral and/or written instructions to assist each participant record relevant details for all foods and beverages consumed, such as brand name, preparation method, and location consumed. Portion size was either estimated, using food models, pictures, or other visual aids, or measured, using weight scales or volume measures. To increase the quality of the data, a trained interviewer reviewed the completed record with the respondent at each visit. Dietary, medical, and physical activity questionnaires and records were collected weekly, coded and entered into software by trained personnel, and analyzed using Food Processor Nutrition and Fitness Software (ESHA, version 8.5; Salem OR).

Treatment and placebo beverages. After enrollment, subjects were randomly assigned using an online, automated randomizer program (http://www.graphpad.com/quickcalcs/index.cfm) for 10 participants and two arms to consume either 240 mL (8 ounces) of 100% TCJ (R.W. Knudsen, Chico, CA) or a generic, color-matched (to mask beverage), anthocyanin-free fruit punch (Great Value fruit punch, Bentonville, AR) for 4 weeks. The placebo was selected to have similar total carbohydrate, caloric, acidic, and visual properties (red color) as the TCJ but without polyphenols, e.g., anthocyanins, or other potentially confounding nutritive or non-nutritive antioxidant or anti-inflammatory components. After a 2-week washout period, subjects were switched to the alternate beverage for an additional 4 weeks. At the start of each study arm, participants were provided at the metabolic kitchen at the ASU Polytechnic Campus (Mesa, AZ) the placebo or TCJ beverages in plastic containers (polyethylene terephthalate bottles) labeled only with the

**Fig. 1** Experimental design of dietary intervention study. Ten participants were randomly assigned to either a placebo (n = 5) or TCJ (n = 5) arm and consumed 8 ounces for four weeks. After a 2-week washout, each participant consumed the alternate beverage for 4 weeks generating n = 10 for each of 4 groups: (1) pre- and (2) post-placebo or (3) pre- and (4) post-TCJ consumption.
study name and accompanied with instructions for use including shaking before opening (to minimize sedimentation) and contact information of the principal investigator and relevant research personnel. At each subsequent visit after the first visit, participants returned empty beverage bottles and submitted diet records, both of which were used to ensure compliance.

2.2 Biomedical analysis and anthropometric measurement

After a ≥12-hour fast, blood samples were drawn in standard venipuncture protocols into each of 3 evacuated tubes (Fisher Scientific, Hampton, NJ) by a trained phlebotomist in the clinical laboratory at the ASU Polytechnic Campus. Plasma was separated by centrifugation at 1100g at 4°C for 20 min and archived in 0.5 mL aliquots at −80°C until analysis for chemokines, cytokines and hsCRP. ESR was determined immediately after each blood draw. Anthropometric measurements including body weight, height and body composition (body fat percentage, fat mass, fat-free mass, total body water, and basal metabolic rate) were measured at each visit by stadiometry and bioelectrical impedance (TFB 300A Tanita Body Composition Analyzer, Tokyo, Japan). Dietary records and physical activity questionnaires were collected and reviewed at each visit.

2.3 Analysis of total polyphenols

Total polyphenols were quantified using the Folin–Ciocalteu method as described previously. Briefly, water (0.3 mL), Folin–Ciocalteu reagent (0.2 mL), and 0.2 mL of diluted sample or external standard (gallic acid) were added to test tubes and mixed (3 s; low speed). Tubes were incubated at 25°C for 10 min followed by addition of 0.6 mL of 20% (w/v) sodium carbonate. Tubes were then incubated in a water bath at 40°C for 20 min, cooled for 30 min to 25°C, and analyzed at 755 nm using purified gallic acid as the external standard. The coefficient of variation (CV), a measure of precision, for the Folin–Ciocalteu method for total polyphenols was <9.8% for inter-assay analysis.

2.4 HPLC analysis of anthocyanin profile in TCJ to determine authenticity

The anthocyanin profile of TCJ was analyzed by HPLC to determine authenticity using the method of Spanos and Wrolstad. Briefly, single-strength TCJ (4 mL) was passed through an activated C18 cartridge (Sep-Pak; Waters, Milford, MA, USA), followed by 0.01% HCl (5 mL). Pigments were eluted with acidified methanol (3 mL) and passed through a 0.45 µm filter disk. Subsequently, samples were analyzed via binary-gradient HPLC using a 5 µm (2.5 cm x 4.6 mm) C-18 column at a flow rate of 1.5 mL min⁻¹ with detection at 515 nm. The mobile phases were (A) formic acid : water (90:10) and (B) formic acid : methanol : water (10:50:40). Profiles from samples were compared with authentic pigment profiles of tart cherries, and purified cyanidin-3-glucoside was used to generate a qualitative reference chromatogram in accordance with authentication protocols used commercially by fruit juice testing laboratories (CV <1%). Cyanidin 3-glucosylrutinoside, cyanidin 3-glucoside, cyanidin 3-sophoroside, and cyanidin 3-rutinoside were the most frequently occurring anthocyanins in TCJ, which is consistent with fresh tart cherries. Samples from the same three batches of TCJ used in this study were also analyzed for sugar profile, organic acids and titratable acidity, as well as BRIX (Krueger Food Labs, Chelmsford, MA).

For the bioavailability data, plasma samples from three individuals were analyzed independently for tart cherry-specific anthocyanin profiles at baseline and two hours post-consumption of 240 mL (8 ounces) of TCJ (used in this study) to determine absorption and potential bioavailability (Eurofins Craft Technologies, Wilson, NC).

2.5 Cytokine and chemokine analysis

Frozen plasma samples (−80°C) were thawed and levels of TNF-alpha, MCP-1, IL-6, and IL-10 were analyzed by ELISA using Single Analyte ELISA Kits (SABiosciences Corporation, Frederick, MD). Briefly, serial dilutions (31–2000 pg mL⁻¹) of antigen standards or samples (50 µL) were added to antibody-coated wells of a 96-well plate, shaken for 10 s, and incubated for 2 h at 25°C. After washing, secondary detection antibodies were added and the plate incubated for 1 h at 25°C followed by addition of avidin-HRP (100 µL) for 30 min at 25°C. After washing wells, the colorimetric endpoint was developed over 15 min at 25°C and absorbance read at 450 nm. CV for the colorimetric methods were as follows: TNF-alpha (<12%), MCP-1 (<6.7%), IL-6 (CV <17.8%), and IL-10 (CV <14.6%).

2.6 hsCRP analysis

Frozen aliquots of plasma were thawed and the level of hsCRP was measured by ELISA using a human CRP quantikine kit (CV <10%) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

2.7 Erythrocyte sedimentation rate (ESR)

ESR was analyzed using EDTA-treated whole blood and an ESR kit (Fisher Scientific, Waltham, MA) based on the Westergren method. Briefly, an aliquot (1 mL) of blood was transferred to a reagent tube containing saline. After mixing by gentle inversion, a graduated dispette was inserted to establish a vertical, graduated column and the distance traveled by the packed elements at one hour was measured (CV <6.8%).

2.8 Statistical analyses

Sample size and statistical analysis. The sample size for this study was based on the pro-inflammatory marker hsCRP. Previous studies have shown with similar intervention protocols a significant 20% reduction in hsCRP after TCJ consumption. Based on the SD of the difference in hsCRP pre- and post-intervention with TCJ, it was estimated that, with a sample of 10 subjects, a 20% difference in hsCRP could be detected between the placebo and cherry juice trials (p < 0.05; power, 80%).

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 17.0.2, 2009. All values expressed within this research are shown as mean ± standard deviation (SD) or SEM. Data were tested for normality using the Shapiro–Wilk statistic and histograms. If data were
not normally distributed, values were transformed by calculating the inverse, logarithm, or square root. Data were then analyzed using two-way analysis of variance (ANOVA). Differences were considered significant at $p \leq 0.05$. Repeated measures and multiple paired-samples $t$-tests were conducted. All data were tested for normality and transformed by the Friedman Test and Wilcoxon Signed Ranked Test if required.

3. Results

3.1 Authenticity, composition, and total polyphenols of TCJ

We first determined the authenticity of TCJ by analysis of the anthocyanin-specific profile in three different lots, or batches, of TCJ using an external testing laboratory (Krueger Food Labs, Chelmsford, MA). Comparisons of results were made to standardized reference samples, which demonstrated that test samples exhibited a typical characteristic tart cherry profile with identification of cyanidin-3-2G-glucosylrutinoside (12.01 min), cyanidin-3-glucoside (13.52 min), cyanidin-3-rutinoside (13.99 min), peonidin-3-rutinoside (16.95 min), and an acylated anthocyanin, cyanidin aglycone, and a non-acylated cyanidin compound eluted at approximately 19.6 min (Fig. 2, upper panel). Additional analyses of TCJ components indicated no hydrolysis syrups (as oligosaccharides), characteristic BRIX (% sugar) of 13.9 for single-strength juice, and normal values for organic acids, sugar profile, and titratable acidity (Table 1). Analysis of TCJ (triplicate for each of 3 juices) indicated a total polyphenol concentration of 1827 ± 113 gallic acid equivalents compared to negligible levels in placebo. Although we did not measure anthocyanins quantitatively, we used published composition data for tart cherries, e.g., Montmorency, Balaton, etc., to calculate that TCJ contains 9.9–241.2 mg (24.4-fold difference) total anthocyanins per 8 ounces TCJ (from ~300 g tart cherries).16–18 If we limit to Balaton and Montmorency cherries, the range is 9.9–23.7 mg per 8 ounces TCJ. These calculations are based on ~16 tart cherries per 100 g fresh fruit and ~50 cherries in 8 ounces of TCJ. Comparison of nutritional information for test beverages revealed 113 kcal and 31 total carbohydrates per 8 ounces (240 mL) placebo and 140 kcal and 34 g total carbohydrate for TCJ.

3.2 Determination of absorption of TCJ anthocyanins

We conducted a pilot experiment to determine qualitatively the absorption, appearance and increase of anthocyanins in plasma from TCJ consumption since studies of overall absorption and pharmacokinetics in humans are limited and anthocyanins are purported to have extremely low bioavailability. Maximum plasma concentrations in humans range from 1.4–592.0 nM and appear at 0.5–4 hours post-consumption.19 Based on this information and after baseline blood draws, three individuals each consumed 240 mL (8 ounces) of TCJ over 30 min. Two hours later, blood was collected, plasma was separated, and samples analyzed. Fig. 2 (lower panel) shows a representative HPLC chromatogram of a baseline plasma sample, plasma 2-hours post TCJ consumption, and a reference trace where plasma was spiked with TCJ. The chromatogram shows the appearance of TCJ anthocyanins in plasma supporting absorption, detection, and increases of dietary anthocyanins in plasma from TCJ (Fig. 2, lower panel).

3.3 Baseline characteristics, anthropometric measurements, dietary intake, and physical activity

We collected and analyzed data for anthropometric indices in participants and noted no significant differences between any of the parameters between groups (Table 2). The average body weight was 89.3 ± 0.4 kg (196.5 ± 0.4 pounds) and average BMI was 32.3 ± 0.1 kg m$^{-2}$ indicating the group was overweight ($n = 5$; BMI 25.0–29.9 kg m$^{-2}$) and obese ($n = 5$; ≥30.0 kg m$^{-2}$). The
average percentage fat mass was 39.8 ± 0.2%, where values >25% for males and >37% for females indicate obesity. In this study, there were 8 females and 2 males.

Dietary records were collected and analyzed at each visit and, upon analysis, indicated no significant differences in dietary intake patterns of macronutrients, energy, or fatty acids between groups (Table 3). Data expressed as percent of total caloric intake further indicate that participants were consuming an average 68.0 ± 1.1%, 16.7 ± 1.7%, and 15.3 ± 2.0% of kcal from carbohydrate, protein, and fat, respectively. Current acceptable macronutrient distribution ranges (AMDR) have been recommended by the Food and Nutrition Board of the Institutes of Medicine (IOM) for carbohydrate (45–65% of energy), protein (10–35% of energy), and fat (20–35% of energy). Participants in this study averaged marginally above the AMDR for carbohydrate and below the AMDR for fat. Lipid panels and indirect calculated indices of insulin resistance were previously reported. Level and intensity of physical activity were not different between placebo and TCJ groups.

### 3.4 Cytokines and chemokine

We measured three acute-phase markers of inflammation and one anti-inflammatory marker, viz., IL-10, since anti-inflammatory effects by bioactive agents can either occur by suppression of pro-inflammatory mediators and/or induction of anti-inflammatory mediators. IL-10 is a cytokine with numerous, pleiotropic effects during inflammation and used frequently to indicate anti-inflammatory effects of dietary exposures. We noted no significant differences between pro-inflammatory IL-6 (0.58 ± 0.06 versus 0.59 ± 0.06 pg mL$^{-1}$) or anti-inflammatory IL-10 (0.25 ± 0.02 versus 0.27 ± 0.02 pg mL$^{-1}$) levels between placebo and TCJ groups, respectively (Fig. 3). In those consuming TCJ, TNF-alpha was reduced marginally by 5% ($p = 0.19$) from (5.83 ± 1.14 to 5.53 ± 0.94 pg mL$^{-1}$) and no differences were observed in the placebo groups (5.56 ± 1.07 to 5.47 ± 0.94 pg mL$^{-1}$). MCP-1, a pro-inflammatory chemokine, was significantly reduced by ~5.8% from 200.4 ± 46.1 to 168.6 ± 0.40 pg mL$^{-1}$ compared to a slight increase in levels from those in the placebo group (Fig. 3).

### 3.5 hsCRP and ESR

Additional measures of inflammation used routinely in clinical settings are hsCRP and the ESR. Participants entered the study with an elevated level of hsCRP (7.0 ± 0.8 mg L$^{-1}$); low risk

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**Table 1** Analysis of tart cherry juice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanin profile</td>
<td>Normal</td>
</tr>
<tr>
<td>Oligosaccharides (%)</td>
<td>No hydrolysis syrups detected</td>
</tr>
<tr>
<td>BRIX (%)</td>
<td>13.9</td>
</tr>
<tr>
<td>Sugar profile</td>
<td></td>
</tr>
<tr>
<td>Fructose (g L$^{-1}$)</td>
<td>31.9</td>
</tr>
<tr>
<td>Glucose (g L$^{-1}$)</td>
<td>60.3</td>
</tr>
<tr>
<td>Sucrose (g L$^{-1}$)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Organic acids</td>
<td></td>
</tr>
<tr>
<td>Quinic (g L$^{-1}$)</td>
<td>1.61</td>
</tr>
<tr>
<td>Malic (g L$^{-1}$)</td>
<td>17.7</td>
</tr>
<tr>
<td>Citric (g L$^{-1}$)</td>
<td>0.23</td>
</tr>
<tr>
<td>Pumaric (g L$^{-1}$)</td>
<td>7</td>
</tr>
<tr>
<td>Malic acid (D isomer)</td>
<td>&lt;0.020</td>
</tr>
<tr>
<td>Formol (g L$^{-1}$)</td>
<td>28.3</td>
</tr>
<tr>
<td>Sorbitol (g L$^{-1}$)</td>
<td>20.9</td>
</tr>
<tr>
<td>Potassium (mg L$^{-1}$)</td>
<td>2480</td>
</tr>
<tr>
<td>Proline (mg L$^{-1}$)</td>
<td>45</td>
</tr>
<tr>
<td>Titratable acidity (mg L$^{-1}$)</td>
<td>247.3</td>
</tr>
<tr>
<td>As malic acid (g L$^{-1}$)</td>
<td>16.6</td>
</tr>
<tr>
<td>Total polyphenols (g L$^{-1}$)</td>
<td>1827 GAЕ</td>
</tr>
</tbody>
</table>

**Table 2** Effect of placebo and TCJ interventions on anthropometric parameters$^a$

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Placebo Pre</th>
<th>Placebo Post</th>
<th>TCJ Pre</th>
<th>TCJ Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>89.1 ± 13.4</td>
<td>89.0 ± 13.9</td>
<td>89.1 ± 12.1</td>
<td>89.8 ± 12.8</td>
</tr>
<tr>
<td>BMI$^b$, kg m$^{-2}$</td>
<td>32.2 ± 4.8</td>
<td>32.2 ± 5.1</td>
<td>32.2 ± 4.6</td>
<td>32.5 ± 4.8</td>
</tr>
<tr>
<td>BMR, kcal</td>
<td>1689 ± 186</td>
<td>1688 ± 188</td>
<td>1696 ± 184</td>
<td>1696 ± 184</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>35.6 ± 10.8</td>
<td>35.5 ± 11.1</td>
<td>35.2 ± 10.2</td>
<td>35.8 ± 10.0</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>78.3 ± 23.8</td>
<td>78.1 ± 24.3</td>
<td>77.5 ± 22.4</td>
<td>78.8 ± 22.1</td>
</tr>
<tr>
<td>Total body water, kg</td>
<td>86.1 ± 14.1</td>
<td>86.1 ± 13.2</td>
<td>86.8 ± 13.3</td>
<td>86.9 ± 14.0</td>
</tr>
</tbody>
</table>

$^a$ Data analyzed using two-way ANOVA. Values are mean ± SD, $n = 10$. No significant difference for the interaction (time and treatment) or main effects of time and treatments. $^b$ Data were inversely transformed (1/variable) prior to statistical analysis; values reported untransformed; mean ± SD.

$^b$ Data analyzed using two-way ANOVA. Values are mean ± SD, $n = 10$. No significant difference for the interaction (time and treatment) or main effects of time and treatments. $^c$ Data transformed by taking the square root (square root variable) prior to statistical analysis; values reported untransformed, mean ± SD. $^d$ Data logistically transformed (log variable) prior to statistical analysis; values reported untransformed, mean ± SD.

**Table 3** Effect of dietary intervention with TCJ on dietary intake$^e$

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Placebo Pre</th>
<th>Placebo Post</th>
<th>TCJ Pre</th>
<th>TCJ Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal d$^{-1}$</td>
<td>2014 ± 571</td>
<td>1728 ± 646</td>
<td>1742 ± 642</td>
<td>1714 ± 664</td>
</tr>
<tr>
<td>Total fat, g d$^{-1}$</td>
<td>65 ± 28</td>
<td>50 ± 26</td>
<td>63 ± 26</td>
<td>54 ± 28</td>
</tr>
<tr>
<td>Carbohydrate$^e$, g d$^{-1}$</td>
<td>291 ± 87</td>
<td>259 ± 102</td>
<td>227 ± 96</td>
<td>254 ± 94</td>
</tr>
<tr>
<td>Protein, g d$^{-1}$</td>
<td>70 ± 23</td>
<td>63 ± 30</td>
<td>66 ± 19</td>
<td>54 ± 25</td>
</tr>
<tr>
<td>Omega-3 fatty acids$^e$, g d$^{-1}$</td>
<td>0.29 ± 0.40</td>
<td>0.56 ± 1.11</td>
<td>0.79 ± 0.93</td>
<td>0.25 ± 0.16</td>
</tr>
<tr>
<td>Omega-6 fatty acids$^e$, g d$^{-1}$</td>
<td>0.24 ± 0.51</td>
<td>0.19 ± 0.64</td>
<td>0.51 ± 0.45</td>
<td>0.31 ± 0.37</td>
</tr>
</tbody>
</table>

$^e$ Data analyzed using two-way ANOVA. Values are mean ± SD, $n = 10$. No significant difference for the interaction (time and treatment) or main effects of time and treatments. $^d$ Data transformed by taking the square root (square root variable) prior to statistical analysis; values reported untransformed, mean ± SD. $^c$ Data logistically transformed (log variable) prior to statistical analysis; values reported untransformed, mean ± SD.
<1.0 mg L\(^{-1}\); average risk 1.0–3.0 mg L\(^{-1}\)) indicating underlying inflammation although the source was unclear, but likely associated with overweightness and obesity. We noted no significant differences between pre- and post-intervention samples for either intervention arm. Interestingly, we noted a significant difference between ESR rates but not necessarily a marked suppression of ESR by TCJ (Fig. 4). ESR from those in the placebo group increased ~20%, whereas ESR in the TCJ group decreased by 5% (Fig. 4).

4. Discussion

In this 10-week placebo-controlled crossover study, we have shown in at-risk overweight and obese participants that daily consumption of TCJ for four weeks significantly reduced MCP-1, a pro-inflammatory marker associated with the initiation and development of CVD. We noted a trend for reduction of TNF-alpha levels, a second pro-inflammatory mediator. Although we noted no significant differences in plasma CRP or IL-6 between participants, we did observe a significant difference between the ESR between the two groups. Collectively, these results support the notion that daily consumption of authentic, polyphenol-rich TCJ reduces markers of inflammation associated with chronic disease and may be a better dietary choice than artificially flavored beverages with added sugars.

We initially conducted a qualitative demonstration that TCJ anthocyanins (from the specific juice used in this study) and by extension its metabolites are indeed absorbed and potentially bioavailable (reaching target sites). Dietary anthocyanins are purported to have extremely poor bioavailability based, in large part, on extremely low plasma levels found in plasma after ingestion.\(^{22}\) It is routinely posited that further research is required to resolve mechanisms associated with absorption, metabolism, and clearance of anthocyanins to determine more clearly the exact biological activities and health effects.\(^{19}\) In anticipation of significant effects on biological parameters, the investigators demonstrated feasibility and practical potential for an effect by TCJ negating the argument that absorption, hence bioavailability, was not meaningful to any extent. We noted numerous other peaks in the HPLC trace at approximately 7–10 minutes suggesting other anthocyanins and/or metabolites (intermediates) appeared. Anthocyanins are labile and metabolized quickly once absorbed and enterohepatic recycling is purported to be a contributing factor to myriad plasma metabolites. The authors assert, however, that cyanidin-3-glucoside and cyanidin-3-rutinoside, as key markers of TCJ ACN, are markedly increased in plasma (AUC) after TCJ consumption and support absorption and potential bioavailability.

Induction of chemokines such as MCP-1 (CCL-2, chemokine [C–C motif] ligand-2) plays a major role in selectively and potentially recruiting monocytes, neutrophils, macrophages, and lymphocytes to sites of inflammation and oxidative stress such as the aortic endothelium and adipose tissue.\(^{23}\) Thus, the observation in this study that TCJ significantly lowered MCP-1 supports its potential usefulness against pro-inflammatory stress associated with disease, as well as chronic, metabolic inflammation accompanying MetS.

Other investigators have shown anthocyanin-induced reductions of MCP-1. For example, in seven healthy volunteers receiving 12 g of an anthocyanin extract, the antioxidant...
capacity of the plasma was significantly increased and plasma MCP-1 significantly reduced despite low concentrations of anthocyanins, cyanidin, peonidin, etc. detected in plasma.24 In hypercholesterolemic patients (n = 160) consuming a purified anthocyanin mixture (320 mg d−1) or a placebo twice a day for 24 weeks, anthocyanin supplementation significantly decreased hsCRP in this double-blind, randomized clinical trial.25 Zhang et al. further showed that anthocyanin supplementation (320 mg d−1) for 24 weeks significantly decreased plasma MCP-1 levels (−11.83% versus 12.84%) compared with placebo in 146 hypercholesterolemic patients.26 In rodent studies, cyanidin-3-glucoside, a prevalent component of tart cherries, significantly reduced systemic levels and renal expression of TNF-alpha and MCP-1 in db/db mice.27 Oral gavage of apolipoprotein E-deficient mice with cyanidin-3-O-beta-glucoside and its metabolite protocatechuic acid significantly increased plasma levels and reduced CC chemokine receptor 2 in peripheral blood monocytes and MCP-1.28 Collectively, many studies have reported results supporting the capacity of anthocyanins, rich in tart cherries, to reduce pro-inflammatory MCP-1 expression.

Others have shown similar results on MCP-1 using functional foods rich in anthocyanins in the whole food matrix or other subgroupings of the polyphenol family of molecules. For example, in obese participants fed for 6 weeks mangoes rich in gallotannin-derived polyphenols, MCP-1 and pro-inflammatory IL-8 were significantly decreased.29 In two other studies, pro-anthocyanidin-rich cocoa and grape seed byproducts significantly and dose-dependently reduced MCP-1 mRNA expression.30 Numerous other polyphenolic components have been shown to reduce MCP-1 including phenolic acids (cafeic acid and its phenethyl ester, ferulic acid), flavonoids (luteolin, apigenin), flavanones (naringenin), stilbenes (resveratrol), and anthocyanins.31 These components also significantly, but not uniformly, decreased other pro-inflammatory cytokines including IL-1 beta, IL-6, IL-8, and TNF-alpha. Our observation suggests that TCJ may be an effective means of reducing pro-inflammatory MCP-1 and may be useful in mitigating and preventing the onset of various chronic, inflammatory diseases such as arthritis, atherosclerosis, and diabetes.

Numerous pro- and anti-inflammatory cytokines are increased during inflammation and, thus, are potential targets for bioactive agents and functional foods, e.g., TCJ. In studies of the effects of tart cherries on exercise-induced oxidative and inflammatory stress, pre-exercise plasma levels of IL-6, IL-8, and TNF-alpha did not differ between the placebo and TCJ groups, but post-exercise plasma concentrations were significantly lower in the cherry group suggesting less severe oxidative stress and/or inflammation.32,33 In another study, consumption of sweet cherries (n = 18; 280 g d−1 for 28 d) significantly decreased numerous pro-inflammatory and associated biomarkers including EN-RAGE (extracellular newly identified receptor for advanced glycation end-products), ferritin, PAI-1 (plasminogen activator inhibitor-1), EGF (epidermal growth factor), ET-1 (endothelin-1), and IL-18 and increased IL-1R antagonist, an anti-inflammatory inhibitor of IL-1.34 Interestingly, cherry consumption did reduce TNF-alpha by 14%, but not significantly, similar to the result in our study where TNF-alpha was reduced, but not significant due to considerable inter-subject variation (Fig. 3). We included anti-inflammatory IL-10 in our analysis since it is a cytokine with multiple, pleiotropic effects on inflammation and immune-regulation and can inhibit synthesis of pro-inflammatory cytokines, e.g., TNF-alpha. We observed no significant differences between the placebo and TCJ groups with mean levels of IL-10 (0.3 pg mL−1) in the lower range noted for human plasma (0.4–2.0 pg mL−1), which would be expected in unstimulated tissues.35 We also noted no differences in IL-6 levels between placebo and TCJ groups. This result is consistent with a study in highly-trained male athletes consuming 30 mL TCJ or placebo in the morning then 60 mL post-exercise for 7 days where IL-6 levels did not differ between groups.36 In a second randomized, parallel study, subjects (n = 49) consumed Bing sweet cherry juice or apple juice (200 mL d−1, 12 weeks) with no differences in fasting serum levels of IL-6 between groups.37 Conversely, in a study of male and female athletes, consumption of 240 mL TCJ or placebo 5 d before, 1 d during, and 2 d post-marathon significantly reduced exercise-induced increases in IL-6 in the TCJ group.38 In trained cyclists (n = 16), consumption of TCJ concentrate (30 mL for 8 d) significantly reduced exercise-induced (stochastic cycling) increases on days 5, 6, and 7 post-trial.33 Collectively, there are studies that support cherry-induced decreases in some cytokines but also studies that demonstrate no apparent effects on other cytokines including those used in the current study.

As an acute phase reactant, hsCRP is released during inflammation and thus is a potential target for anti-inflammatory dietary bioactive agents and functional foods such as tart cherries. In this study, the overall baseline level of CRP in participants was elevated at 7.0 ± 0.8 mg L−1 with a range of 1.3–23.7 mg L−1 (normal, <1.0 mg L−1; average risk 1.0–3.0 mg L−1). Two participants exhibited values at each of the four blood draws of >10 mg L−1 suggesting more severe chronic metabolic inflammation than the others. This is typical with some mild-to-moderate stressors where CRP levels can increase up to 50–100 mg L−1 within a few hours. Our initial telephone interviews, screening, and observation during laboratory visits indicated no overt pro-inflammatory source, e.g., disease, anxiety, etc., other than BMI >25.0 (overweight/obese), upon entry and participation in the study. Overall, despite all participants exhibiting elevated CRP levels, TCJ consumption had no significant impact in this study (n = 10) on levels after four weeks. This result is consistent with results of a study of 47 healthy adults where tart cherry concentrate (30 mL for 6 weeks) exerted no effect on CRP levels.39 An additional study demonstrated no changes in serum CRP after consumption of sweet cherry juice (200 mL d−1) for 6 and 12 weeks in older subjects (>70 years) with dementia although several cognitive outcomes were significantly improved.40 In highly trained, water-based sport athletes, consumption of TCJ for 6 consecutive days had no effect on the higher post-exercise plasma levels of CRP and IL-6. The authors suggested that this was

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due to the intermittent, non-weight bearing demands of water polo as a sport which did not induce significant oxidative stress or inflammation.40 In obese subjects, consumption of sweet cherries (three cups daily) for 4 weeks did not alter serum CRP or urinary prostaglandin E2 (principal mediator of inflammation) and thromboxane B2 (potent vasoconstrictor) when compared to baseline.31 A key limitation and potential confounder of this study was the use of eleven batches of fresh tart cherries with substantial variation in total ACN concentrations by cherry variety and batch ranging from 7.2 to 161.6 μg g⁻¹, a 22.5-fold difference. In another study, consumption of a cherry-based product twice daily significantly increased serum levels of IL-1β, TNF α, and IL-8. However, in this study of sleep patterns, samples were drawn at the acrophase of the naturally occurring melatonin rhythm (1 am) where metabolite-induced increases in IL-1 beta and TNF-alpha occurred.32

Others have demonstrated significant reductions of CRP after cherry consumption. In trained cyclists (n = 16), TCJ concentrated reduced serum CRP in post-exercise samples.31 In a study of healthy males and females (n = 12), TCJ at two doses (30 and 60 mL) reduced serum CRP at the lower dose within 3 hours post-consumption.43 In another study, males and females (n = 18) with BMI 20–30 kg m⁻² consumed pitted sour cherries (280 g for 4 weeks), which significantly decreased numerous pro-inflammatory and associated biomarkers including CRP.34 Collectively, there are numerous studies supporting significant reductions in CRP after consumption of cherries (tart and sweet), but also numerous studies suggesting equivocal outcomes.

The ESR is a routinely used clinical test for chronic inflammation typically caused by increased production of acute phase reactants such as CRP and fibrinogen. In this study, we did note a significant difference in ESR between placebo and TCJ groups, but largely due to an increase by 19% in the placebo group. ESR was, however, reduced marginally in the TCJ group by 6%, resulting in an overall 25% difference between the two intervention groups. This unexpected placebo-induced increase and subsequent difference may be due to the presence of added sugars since free fructose and its metabolites purportedly contribute to insulin resistance and MetS with consequent organ and tissue dysfunction leading to oxidative stress, chronic inflammation, endothelial dysfunction, autophagy and increased intestinal permeability.44 The amount of free fructose in the placebo beverage was 62 g L⁻¹, which translates to an extra 14.7 grams of free fructose per day.45 Analysis of TCJ used in this study demonstrated that it was authentic with no added sugar but with naturally occurring fructose (31.9 g L⁻¹) and glucose (60.3 g L⁻¹). Consumption of 240 mL d⁻¹, as done in this study, would correspond to 14.9 and 7.7 grams of extra fructose added to the diet for placebo and TCJ, respectively, with the former, but not the latter, as high fructose corn syrup (HFCS) as indicated on the product label. Additionally, the carbohydrate consumption of this cohort was 68% of energy (based on dietary recalls) exceeding, albeit marginally, the recommended upper limit of the AMDR of 45–65%. Given the prevalence of the western pattern diet, or standard American diet, it is likely that the participants already consumed considerable amounts of simple sugars over the study period.

The placebo-driven effect on ESR brings to attention the potential effect of placebo selection in dietary interventions although other factors may have contributed to this effect. The placebo beverage was an artificially colored (red), generic, anthocyanin-free fruit punch produced and distributed commercially and was obtained at a local supermarket (Phoenix, AZ). The dietary interventions were blinded, or masked, by distributing placebo or TCJ in plastic containers (polyethylene terephthalate) labeled only as “Tart cherry juice study” accompanied with a handout regarding use, e.g., shake before opening, and contact information for the study principal investigator and authorized research personnel if questions or concerns arose from the participant. The TCJ was filtered by the manufacturer but the filtering process allowed for a certain amount of sediment to pass through which could potentially be observed by participants although not reported. This strategy has been employed in numerous dietary beverage interventions including those involving TCJ where artificially flavored Kool-Aid fruit-flavored drink mix (Kraft Foods, Northfield, IL) was used as placebo by Howatson (2010), Traustadóttir (2009), Bell (2014), and Schumacher (2013).9,10,46 Moreover, in a meta-analysis of 12 randomized controlled trials specifically focusing on fruit juices, Wang et al. (2014) reports selections for placebo beverages included modified sports beverages, synthetic orange-flavored drinks, water, and a generic control drink matched for sugar composition.17 Based on this information, the investigators selected the placebo beverage and considered it an appropriate and adequate placebo for this study. Regarding selection of a true placebo, guidelines on conducting clinical trials of dietary interventions are not available making execution of placebo-controlled trials in dietary interventions considerably more challenging.

Since dietary fructose may have contributed to the ESR, other test parameters may have been modulated as well. However, accumulating evidence, although limited, does not support the notion that dietary fructose, as found alone or in HFCS, contributes more to subclinical inflammation than other dietary sugars including glucose and sucrose. Della Corte et al. conducted a systematic review and meta-analysis of intervention studies exploring the effects of sugar on hsCRP, IL-6, TNF-alpha, and MCP-1 and found no clear indication that fructose alone caused adverse effects differently than any other sugars.48 To clarify this conundrum regarding the confounding, pro-inflammatory effects of free fructose, additional human intervention studies are needed with increased sample sizes, more extended follow-up periods, more rigorous experimental designs, and with subclinical inflammation, characteristic of MetS, as a primary outcome.

We noted a possible discordance between ESR and hsCRP in this study. Both tests are widely used laboratory markers of systemic inflammation although both lack sensitivity and
specificity and both can be confounded by many factors causing an increase in one and a decrease, or no change, in the other. Typically, however, elevations are commensurate with inflammatory mediators (cytokines) and levels proportional to the intensity of inflammation although not occurring uniformly in all persons. Previous studies have reported up to 12% discordance rate (~1 in 8 patients), which may be attributable to various factors, including differences in cytokine stimulation, inherent differences in normalization, or false/positive false negative characteristics of individual acute phase reactants. Age, general health, gender, and adiposity are also considerations since ESR increases with age and age-associated connective tissue disorders in females and CRP levels generally increase in overweight and obese individuals. Any of these factors and/or combinations could have contributed to the observations in this study.

5. Conclusions

In this study, we have demonstrated a significant reduction in pro-inflammatory MCP-1, a trend for reduced TNF-alpha, and a significant difference in ESR compared to placebo after consumption of TCJ for four weeks in individuals at-risk or with chronic (metabolic) inflammation. These data potentially support the use of authentic, naturally occurring fruit juice, viz., TCJ, as an alternative to beverages artificially sweetened, particularly with added free fructose. Moreover, inclusion of TCJ may markedly reduce the risk of inflammation associated with many chronic diseases including CVD, diabetes, and obesity.

Author contributions

KRM initiated and designed the study. JB and LB recruited, screened, and provided informed consent to respondents under the supervision of KRM, as well as collected, processed, and analyzed, in part, data, samples and questionnaires (diet records and physical activity forms). KRM interpreted the data and prepared the manuscript. All authors critically reviewed the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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