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Research report

Tart cherry anthocyanins suppress inflammation-induced pain behavior in rat

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Abstract

The use of complementary and alternative medicine (CAM) has increased in the United States and more patients are seeking CAM therapies for control of pain. The present investigation tested the efficacy of orally administered anthocyanins extracted from tart cherries on inflammation-induced pain behavior in rats. Paw withdrawal latency to radiant heat and paw withdrawal threshold to von Frey probes were measured. The first set of experiments examined the effects of tart cherry anthocyanins (400 mg/kg) on the nociceptive behaviors and edema associated with inflammation induced by intraplantar injection of 1% carrageenan. These studies also included tests of motor coordination. The second set of experiments determined if tart cherry anthocyanins (15, 85, and 400 mg/kg) dose-dependently affected the inflammation induced by intraplantar injection of 25% complete Freund's adjuvant. We found that tart cherry extracts reduce inflammation-induced thermal hyperalgesia, mechanical hyperalgesia and paw edema. The suppression of thermal hyperalgesia was dose-dependent and the efficacy of highest dose (400 mg/kg) was similar to indomethacin (5 mg/kg). The highest dose anthocyanin (400 mg/kg) had no effects on motor function. These data suggest that tart cherry anthocyanins may have a beneficial role in the treatment of inflammatory pain. The antihyperalgesic effects may be related to the anti-inflammatory and antioxidant properties of anthocyanins. A better understanding of the modulatory role of dietary constituents and phytonutrients on pain will offer further therapeutic options for treating patients with persistent and chronic pain conditions.

Keywords: Anthocyanins; Tart cherry; Inflammatory pain; Complementary and alternative medicine; Carrageenan; Complete Freund's adjuvant; Rat; Hyperalgesia

1. Introduction

The use of complementary and alternative medicine (CAM) has increased in the United States and a recent survey indicates that 40% of Americans use CAM for the treatment of persistent or chronic conditions [11]. CAM has been frequently used for pain control when prescribed medications were ineffective [27]. Persistent or chronic pain is a common symptom among disease states ranging from cancer to arthritis. To alleviate suffering from unmitigated pain, patients often seek CAM therapies such as herbal medicines, acupuncture, and mind-body modalities. Dietary constituents may also play a role in the treatment of pain [13,37].

There is a great interest in determining the role of compounds of plant origin, phytonutrients, in promoting improved health. Natural antioxidants, such as the flavonoids, found in foods may play an important role in preventing the formation of free radicals and the subsequent formation of lipid peroxides [21,39]. Anthocyanins, a flavonoid subclass, are the primary pigments in flowers and fruits [32] and are widely present in various plant species. Anthocyanins are predominantly associated with fruits but have also been found in vegetables, roots, tubers, legumes, and cereals [3]. Anthocyanins are heterocyclic flavonoids, composed of two or three moieties—an aglycone anthocyanidin, sugar(s) and an acyl acid [26]. Anecdotal reports indicate that consumption of tart cherries can alleviate the pain of gout and arthritis [16]; this prompted our laboratory to investigate tart cherries for biologically active compounds.

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Previous in vitro investigations of tart cherry anthocyanins have found potent anti-inflammatory and antioxidant properties comparable to over-the-counter preparations [29,30,42–44]. Anthocyanins isolated from tart cherries significantly inhibited lipid peroxidation [44] and display anti-inflammatory activity [44]. Further, anthocyanins are absorbed and are bioavailable to humans and rats following oral administration [5].

The present investigations tested the in vivo efficacy of anthocyanins extracted from tart cherries on inflammation-induced edema and pain behavior in rats. These studies include tests of motor coordination following the gastric administration of tart cherry anthocyanins. Additional experiments were conducted to determine if tart cherry anthocyanins dose-dependently affect complete Freund's adjuvant-induced inflammation. To the author's knowledge, this is the first study of the in vivo efficacy of tart cherry anthocyanins on inflammatory pain. Preliminary findings have been previously presented in abstract form [38]. Our observations suggest that dietary supplements of tart cherry anthocyanins may dose-dependently decrease the mechanical and thermal hyperalgesia associated with acute inflammation. Further studies are needed to determine the mechanism of action of the antihyperalgesic effects of tart cherry anthocyanins.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 200–400 g, were acclimated to housing for at least 1 week prior to investigation. Rats were on a 12/12-h day/night cycle, with food and water provided ad libitum. Animals were randomly assigned to each treatment group and all testing was performed between 10:00 a.m. and 3:00 p.m. The Institutional Animal Care and Use Committee at The Johns Hopkins University approved the experimental protocol and the studies were performed according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [46].

2.2. Models of inflammation

Two models of acute inflammation were employed in these studies. In both models, each rat was injected with 100 μl of the inflammatory agent under brief isoflurane anesthesia (3%).

To assess the effects of high dose tart cherry anthocyanins on acute inflammatory pain behaviors, 1% carrageenan suspension (w/v with 0.9% saline) was injected into the left hindpaw. To determine if tart cherry anthocyanins dose-dependently affect acute inflammation, 25% complete Freund's adjuvant (CFA) suspension (v/v with 0.9% saline) was injected into the left hindpaw.

2.3. Tart cherry anthocyanin preparations

Anthocyanins were prepared from Balaton^{TM} tart cherries according to the previously published method [6,40] with the exception that XAD-16 resin (mesh size 20–60; Supelco, Bellefonte, PA) instead of XAD-16 resin (mesh size 20–50; Sigma Chemical Co., St. Louis, MO) was used. Briefly, the flavonoids and anthocyanin mixture obtained from the XAD-16 resin column was further fractionated by Medium Pressure Liquid Chromatography (MPLC) on a C-18 column (40 mm × 500 mm), eluting with increasing amounts of MeOH (0.1% HCl) in H_2O. The fraction identified by HPLC to have a high content of anthocyanins was lyophilzed and used for these studies.

2.4. Drugs

Gastric administration of all drugs was accomplished via oral gavage. Tart cherry anthocyanins were delivered as a solution with 0.9% saline and delivered at doses of 15, 85, and 400 mg/kg. These doses of tart cherry anthocyanins are based on previous laboratory work [6,30,44] and pilot studies in mice (data not shown). Indomethacin (Calbiochem-Novabiochem Corp., San Diego, CA), a potent non-steroidal anti-inflammatory agent, was suspended in a 1.5% carboxymethylcellulose (Sigma, St. Louis, MO) [23] solution at a dose of 5 mg/kg. This dose of indomethacin reduces pain and inflammation associated with acute inflammation [8,12,19] and serves as a positive control in these investigations. Saline was used as a negative control.

2.5. Behavioral assessment of mechanical and thermal hyperalgesia

Animals were acclimated to laboratory environment, investigator handling and behavioral equipment during at least two training sessions. A single investigator injected all animals and collected all behavioral data. To blind the investigator during the behavioral measurements, multiple drugs doses were used in each experimental session and the animals were randomly placed onto the testing apparatus. Room temperature of the behavioral testing facility was maintained at 22 °C.

2.5.1. Thermal stimuli

Paw withdrawal latencies (PWL) to radiant heat stimuli were recorded from the left and right hindpaw of each rat [17]. Rats were placed into plexiglass chambers on a glass surface that was heated to 28–30 °C. Following a 20-min acclimation period, a radiant heat stimulus was alternately applied to each hindpaw, and the time to paw withdrawal measured. Each hindpaw was tested five times. A 20 s exposure limit was imposed to prevent tissue damage, and a 5-min interval was maintained between trials to avoid sensitization. The PWL of each rat was calculated as the median

of five trials, and data across animals are presented as the mean ± S.E.M.

2.5.2. Mechanical stimuli

The 50% paw withdrawal threshold (PWT) to a static mechanical stimulus was assessed using the up-and-down method [7,9]. Animals were placed under a plexiglass chamber on a wire mesh floor and allowed to acclimate for 20 min. A series of eight calibrated von Frey filaments, ranging from 0.41 to 15.8 g in log increments, were used. Testing was initiated with the 2.0 g von Frey filament, the middle of the series of filaments, applied to the plantar surface of each hindpaw for 7–10 s. A positive paw withdrawal response was recorded if the animal briskly lifted the hindpaw. Questionable responses, such as the animal shifting body weight or lifting following the removal of the stimulus, were not recorded as a positive response and the trial was repeated. In the absence of a paw withdrawal response to the initially selected filament, the next stronger stimulus in the series was presented; however, in the event of a positive withdrawal response, the next weaker stimulus in the series was used during the next trial. The test continued until (a) the responses of five more stimuli after the first change in response had been obtained or (b) the upper/lower end of the von Frey set was reached. In cases where continuous positive or negative responses were observed to the exhaustion of the filament set, the paw withdrawal threshold was assigned to be 26.5 and 0.3, respectively. The resulting pattern of positive and negative responses was used to calculate the 50% PWT using the following equation:

$$50\%\,\mathrm{PWT} = 10^{[F+k\delta]}$$

where F is the force of the final von Frey filament used in log units; k is the tabular value for the pattern of positive and negative responses [9]; δ is the mean difference between stimuli in log units. Since the Dixon threshold procedure produces data that are not normally distributed, the data are presented as the median ± 25–75 quartile.

2.6. Measurement of edema

Paw thickness was used as a measurement of inflammation-induced edema [4,10,12,20,45]. Briefly, the dorsoventral thickness of each hindpaw was measured using a caliper placed at the border of the phalanges and the metatarsals. The measurement was taken when each edge of the caliper was just touching the dorsal and ventral surface of the hindpaw (i.e. the caliper was not squeezed onto the hindpaw). Data are expressed as the mean paw thickness±S.E.M.

2.7. Rota-rod protocol

An accelerating rota-rod (AccuScan Instruments, Inc., Columbus, OH) was used to determine if oral administration of tart cherry anthocyanins affect motor coordination of rats. Rats were tested one at a time, and each animal was acclimated to the rota-rod apparatus during three training sessions prior to the day of data collection. The rat was placed onto the rotating bar (diameter = 7 cm) and the rota-rod was switched on to accelerating mode. The rota-rod was set to accelerate, in a linear manner, from 0 to 40 revolutions per min (rpm) over a time of 2.5 min [35]. On the day of data collection, each rat was given a single training session. Baseline data were collected followed by the gastric administration of tart cherry anthocyanins (400 mg/kg). Three trials were performed at intervals of 10 min, and data were collected at 0.5, 2, and 5 h post-anthocyanin gavage. Data are expressed as the mean of the maximum rpm ± S.E.M.

2.8. Experimental design

2.8.1. Effects of high dose tart cherry anthocyanins on carrageenan-induced inflammation

Two separate groups of rats were used in PWL and PWT assessment of high dose tart cherry anthocyanins due to the time constraints of multiple behavioral testing methods.

The first series of experiments examined the effects of high dose tart cherry anthocyanins (400 mg/kg) on carrageenan-induced thermal hyperalgesia. Rats were randomly assigned to each treatment group (n = 14–16 rats per treatment group). Baseline PWL data were collected, and rats were pretreated for 3 days via gavage with tart cherry anthocyanins (400 mg/kg), indomethacin (5 mg/kg), or 0.9% saline. On day 3 of pretreatment, PWL was measured at 30 min after gavage in order to assess if daily administration of tart cherry anthocyanins affected basal nociceptive thermal responses in rats. On day 4, each animal received the daily gavage dose and an intraplantar injection of 1% carrageenan (100 μl). Behavioral testing was performed 0.5 h before and 0.5, 1.5, 2.5, 4.5, and 24 h after the carrageenan injection. In order to assess inflammation-induced edema, paw thickness measurements were taken just prior to and at 2, 5, and 24 h after the carrageenan injection.

The second series of experiments examined the effects of high dose tart cherry anthocyanins (400 mg/kg) on carrageenan-induced mechanical hyperalgesia. Rats were randomly assigned to each treatment group (n = 12 rats per treatment group). Rats were not pretreated in this investigation as statistical analyses revealed that gavage pretreatment with either tart cherry anthocyanins or indomethacin did not significantly affect pre-inflammation behavioral responses (see Section 3). Baseline PWT data were collected, and rats were given tart cherry anthocyanins (400 mg/kg), indomethacin (5 mg/kg), or 0.9% saline via gavage. Analogous to thermal hyperalgesia testing, PWT testing was performed 0.5, 1.5, 2.5, 4.5, and 24 h after the 1% carrageenan (100 μl) injection.

2.8.2. Dose–response of tart cherry anthocyanins on complete Freund's adjuvant-induced inflammation

In order to determine if tart cherry anthocyanins are effective in multiple inflammatory pain models and if a

dose–response relationship exists, a series of experiments were performed using CFA-induced inflammation. Prior to these investigations, a series of pilot experiments were performed to determine a dose of CFA that produced significant nociceptive behavior of shorter duration and less severe edema than that produced with 100% CFA. This pilot study used male Sprague–Dawley rats ($n = 3$–5 rats per treatment group), and each animal was injected with 100 μl of CFA at varying concentrations (12.5, 25, and 50%). Our data revealed that 25% CFA reproducibly caused significant inflammation-induced nociceptive behaviors with less edema as compared to our previous experience with 100% CFA [36]. As a result, the 25% CFA concentration was used in the present investigations.

Rats were randomly assigned to each treatment group ($n = 6$ rats per treatment group). Baseline PWL and PWT data were collected. Each animal received an intraplantar injection of 25% CFA (100 μl) and tart cherry anthocyanins (15, 85, and 400 mg/kg), indomethacin (5 mg/kg) or 0.9% saline via gastric administration. Behavioral testing was performed 1, 4, and 24 h after the CFA injection. In order to assess inflammation-induced edema, paw thickness measurements were taken just prior to and at 2, 5, and 24 h after the CFA injection.

2.9. Statistical analyses

All data were analyzed using Statistica version 5.5 from StatSoft, Inc. Paw thickness and PWL measurements were analyzed using a repeated-measures analysis of variance (ANOVA) followed by paired *t*-tests (including Bonferroni's correction for multiple comparisons), and data are presented as the mean ± S.E.M. Friedman ANOVA or Kruskal–Wallis ANOVA followed by a Wilcoxon matched pair test or a Mann–Whitney *U*-test was used to analyze the PWT data and data are presented as the median ± 25–75 quartile. Rota-rod data were analyzed via one-way ANOVA. A probability level <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of high dose tart cherry anthocyanins on carrageenan-induced inflammation

The mean paw withdrawal latency after 3 days of treatment with tart cherry anthocyanins (9.8 ± 0.2 s) was not significantly different from the PWL after saline treatment (9.6 ± 0.3 s; $P > 0.6$) or from the PWL before treatment (9.8 ± 0.3 s; $P > 0.8$). Thus, daily treatment with anthocyanins does not influence basal nociceptive behaviors in rats.

Each rat received saline, anthocyanins (400 mg/kg), or indomethacin (5 mg/kg) via oral gavage, directly followed by a hindpaw injection of 1% carrageenan (100 μl). A two-way ANOVA revealed that the carrageenan-induced thermal hyperalgesia was significantly suppressed by indomethacin and tart cherry anthocyanins ($P < 0.01$). Post hoc statistical testing revealed that the PWL of animals treated with tart cherry anthocyanins or indomethacin were significantly greater than the PWL of saline-treated rats at 1.5, 2.5, and 4.5 h after the carrageenan injection ($P < 0.02$; Fig. 1A). There was no significant difference between the anthocyanin- and indomethacin-treated animals. These results indicate that anthocyanins were as effective as indomethacin in reducing inflammation-induced thermal hyperalgesia.

In the next series of experiments, we examined the effects of high-dose tart cherry anthocyanins on carrageenan-induced mechanical hyperalgesia. Rats were not pretreated in these experiments as the statistical analyses described above revealed that gavage pretreatment with either tart cherry anthocyanins or indomethacin did not significantly affect pre-inflammation behavioral responses. Immediately following oral gavage of saline, anthocyanins (400 mg/kg), or indomethacin (5 mg/kg), the hindpaw was injected with 1% carrageenan (100 μl). The paw withdrawal threshold to mechanical stimuli (PWT) decreased significantly after the carrageenan injection. However, the PWT data from rats that received tart cherry anthocyanins and indomethacin were significantly higher than the PWT for animals administered saline at 0.5 and 1.5 h after the carrageenan injection (Fig. 1C; $P < 0.02$).

Indomethacin and high dose tart cherry anthocyanins (400 mg/kg) significantly suppress the carrageenan-induced increases in paw thickness (Fig. 1B; $P < 0.02$), as compared to saline controls. Although all rats displayed a significant increase in paw thickness, the edema was significantly less in rats that received indomethacin or anthocyanins as compared to the saline-treated animals at all time points after the carrageenan injection. Further, anthocyanins displayed a similar efficacy as indomethacin in the suppression of inflammation-induced increases in paw thickness.

3.2. Dose–response of tart cherry anthocyanins on CFA-induced inflammation

In another series of experiments, animals randomly received different doses of tart cherry anthocyanins (15, 85, or 400 mg/kg), indomethacin (5 mg/kg), or saline immediately before intraplantar injection of 25% CFA (100 μl). In saline-treated animals, the CFA injection led to a dramatic decrease in the paw withdrawal latency to radiant heat that was obvious at the first time point tested (1.5 h) and that lasted for more than 24 h (Fig. 2A). In the anthocyanin- and indomethacin-treated animals, the decrease in paw withdrawal latency was less than in the saline-treated animals. At 1.5 h after the intraplantar injection of CFA, the tart cherry anthocyanins produced a dose-dependent increase in the paw withdrawal latency (Fig. 2B; $P < 0.05$) with an ED_{50} of 87 mg/kg. The paw withdrawal latency at the highest dose of anthocyanin was comparable to the paw withdrawal latency of indomethacin-treated rats. The paw withdrawal latency at

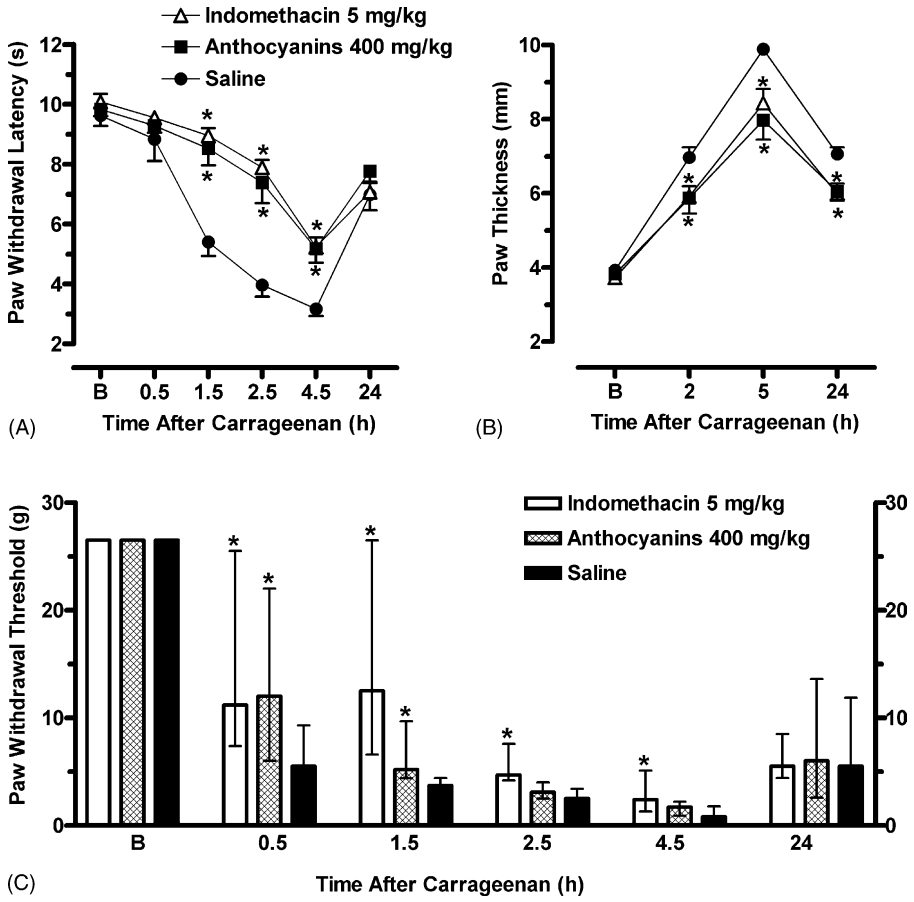

Fig. 1. The effects of tart cherry anthocyanins on carrageenan-induced inflammation. (A) Paw withdrawal latency to a radiant heat stimulus is plotted vs. time after intraplantar injection of 1% carrageenan (100 μl). Immediately before the carrageenan injection, tart cherry anthocyanin (400 mg/kg), indomethacin (5 mg/kg), or saline was administered by gavage. Compared to saline-treated rats, animals treated with tart cherry anthocyanins and indomethacin exhibited significantly longer paw withdrawal latencies at 1.5, 2.5, and 4.5 h following carrageenan injection. The PWL of each rat was calculated as the median of five trials and data are presented as the mean ± S.E.M. (B) Paw thickness is plotted as a function of time after carrageenan injection. All rats displayed a significant increase in paw thickness at 2, 5, and 24 h following carrageenan injection; however, the edema was significantly less in rats that received oral indomethacin and anthocyanins. Data are presented as the mean ± S.E.M. ($n = 14$–16 rats per treatment group). B: baseline ($^{*}P < 0.02$ vs. time-matched saline-treated rats). (C) Paw withdrawal threshold to von Frey probes is plotted as a function of time after the carrageenan injection. Orally administered tart cherry anthocyanins and indomethacin suppressed the development of carrageenan-induced mechanical hyperalgesia at post-injection times 0.5 and 1.5 h. Data are presented as the median ± 25–75 quartile.

the high dose anthocyanin and indomethacin continued to be significantly greater than saline at 4.5 h post-CFA (Fig. 2A; $P < 0.01$).

In saline-treated animals, the 25% CFA injection led to a dramatic decrease in the paw withdrawal threshold to von Frey probes that was obvious at 1 h after injection, reached a minimum at 4 h and lasted for more than 24 h (Fig. 2C). A Kruskal–Wallis ANOVA of the anthocyanin data revealed a significant dose-dependent difference in paw withdrawal thresholds at the 1 h time point ($P < 0.05$). A post hoc analysis revealed that the paw withdrawal thresholds in animals treated with indomethacin or higher dose anthocyanins (85 or 400 mg/kg) were significantly greater than in animals treated with saline or low dose anthocyanins (15 mg/kg) ($P < 0.05$; Mann–Whitney *U*-test).

In saline-treated animals, the 25% CFA injection led to a dramatic increase in the paw thickness that was apparent at 2 h after injection and reached a maximum at 24 h (Fig. 2D). In the anthocyanin- (15, 85, and 400 mg/kg) and indomethacin-treated animals, the increase in paw thickness was less than in the saline-treated animals at the 2 h time point ($P < 0.05$). At 5 h post-CFA injection, the paw thickness was significantly less in rats treated with the higher dose anthocyanins (85 and 400 mg/kg) and indomethacin compared to the time-matched saline animals ($P < 0.05$). Only the largest dose of anthocyanins (400 mg/kg) and indomethacin significantly suppressed CFA-induced increases in paw thickness at the 24 h time point ($P < 0.03$).

3.3. Motor effects of anthocyanins

Oral administration of high dose tart cherry anthocyanins (400 mg/kg) did not affect the maximum rpm that the rats could withstand during the accelerating rota-rod experi-

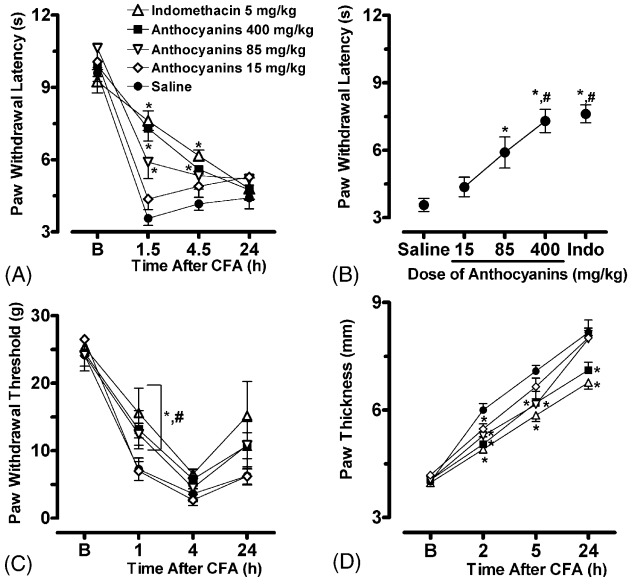

Fig. 2. Effects of different doses of tart cherry anthocyanins on inflammation induced by complete Freund's adjuvant (CFA). (A) Paw withdrawal latency to radiant heat is plotted as a function of time after intraplantar injection of 25% CFA (100 μl). Immediately before the CFA injection, tart cherry anthocyanin (15, 85, or 400 mg/kg), indomethacin (5 mg/kg), or saline was administered by gavage. Paw withdrawal latency decreased markedly after the injection. At 1.5 and 4.5 h after the injection, animals administered the anthocyanin (400 mg/kg) or indomethacin had a significantly longer latency than animals administered saline. At 24 h after CFA injection, the paw withdrawal latency data were not significantly different among treatment groups ($^{*}P < 0.02$ vs. time-matched saline-treated rats). (B) Dose–response function for the anthocyanins. Paw withdrawal latencies measured at 1.5 h after CFA injection are plotted as a function of dose. The paw withdrawal latency was significantly greater in rats treated with indomethacin or high dose anthocyanins (400 mg/kg) as compared to low dose anthocyanins (15 mg/kg; $^{\#}P < 0.05$) or saline treatment ($^{*}P < 0.02$). Rats treated with a dose of 85 mg/kg anthocyanins displayed a paw withdrawal latency that was significantly greater than the saline-treated animas ($^{*}P < 0.02$). The ED_{50} for anthocyanin was 87 mg/kg. $^{*}P < 0.05$ vs. time-matched saline-treated rats; $^{\#}P < 0.05$ vs. time-matched, low dose (15 mg/kg) anthocyanins. (C) Paw withdrawal threshold to von Frey probes is plotted as a function time after the CFA injection. Paw withdrawal threshold decreased markedly after the injection. At 1 h after injection, the paw withdrawal thresholds were significantly greater in rats treated with anthocyanins (85 and 400 mg/kg) compared to the saline-treated ($^{*}P < 0.02$) and low dose anthocyanin-treated ($^{\#}P < 0.05$) animals. Data are presented as the median ± 25–75 quartile. $^{*}P < 0.05$ vs. time-matched saline-treated rats; $^{\#}P < 0.05$ vs. time-matched, low dose (15 mg/kg) anthocyanins. (D) Paw thickness is plotted as a function of time after CFA injection. Paw thickness increased in all animals following the CFA injection. However, at 2 h after injection the paw thickness in the animals treated with anthocyanins (15, 85, and 400 mg/kg) or indomethacin was significantly smaller than saline-treated animals. Tart cherry anthocyanins (400 mg/kg) and indomethacin continued to suppress the increase in paw thickness for up to 24 h following CFA injection ($^{*}P < 0.05$ vs. time-matched saline-treated rats; $n = 6$ rats per treatment group). B: baseline; Indo: indomethacin.

ment (Fig. 3; $P = 0.8$). Thus, the effects of tart cherry anthocyanins on inflammation-induced thermal and mechanical hyperalgesia are not caused by impaired motor functions.

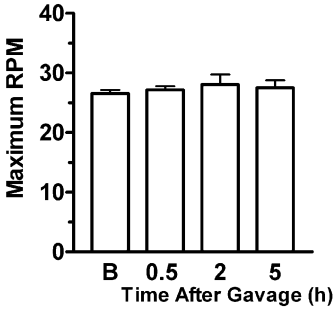

Fig. 3. Tart cherry anthocyanins (400 mg/kg) do not affect motor function. An accelerating rota-rod protocol was used, and three trials were performed at each time point. Oral administration of anthocyanins did not affect motor coordination at all time points tested ($P = 0.82$; $n = 9$ rats per time point). Data are expressed as the mean of the maximum rpm ± S.E.M.

4. Discussion

The use of CAM has increased in the United States [11] and more patients are seeking CAM therapies for control of pain [27]. Dietary constituents, such as sucrose, glucosamine, and products developed from soybeans, may play a role in the treatment of pain [13,37]. The present studies demonstrate that anthocyanins extracted from tart cherries reduced inflammation-induced thermal and mechanical hyperalgesia in rats.

Our data indicate that anthocyanins extracted from tart cherries do not have an analgesic effect in the absence of inflammation; however, in the presence of inflammation oral administration of anthocyanins significantly reduced thermal and mechanical hyperalgesia. The onset, duration, and magnitude of action of anthocyanins on thermal hyperalgesia were similar to that of indomethacin. Like indomethacin, tart cherry anthocyanins were also effective in suppressing the carrageenan-induced increase in paw thickness. Our results indicate that tart cherry anthocyanins are as effective as indomethacin in suppressing carrageenan-induced thermal hyperalgesia and edema, but have a shorter-lasting effect on mechanical hyperalgesia. Also, the beneficial effects of anthocyanins on reducing inflammation-induced edema displayed a longer duration of action, as compared to the positive effects observed with the decrease in thermal hyperalgesia. Further, our studies demonstrate that high dose anthocyanins do not affect motor coordination; therefore, the antihyperalgesic properties of anthocyanins are not due to indirect effects on motor control.

Similar to carrageenan-induced inflammation, tart cherry anthocyanins suppress nociceptive behaviors and edema following the intraplantar injection of CFA. Anthocyanins dose-dependently suppressed the early stages of CFA-induced thermal hyperalgesia (at 1.5 h following CFA injection); however, only high dose anthocyanins (400 mg/kg) and indomethacin were effective at later stages of inflammation (at 4.5 h following CFA injection). We found that anthocyanins displayed a similar trend towards a dose-dependent

attenuation of hyperalgesia to mechanical stimulation. Also, only higher dose anthocyanins and indomethacin significantly suppressed the CFA-induced increase in paw thickness during the later stages of inflammation. This finding supports previous work showing that edema following intraplantar injection of an inflammatory agent is significantly suppressed only after the administration of a high dose proanthocyanidin (200 mg/kg), with no effect of low dose treatment (100 mg/kg) [33]. These data indicate that tart cherry anthocyanins and indomethacin are less effective in suppressing CFA-induced inflammation, as compared to carrageenan-induced inflammation in rats. This finding may be related to a difference in the severity or duration of the inflammatory response elicited by each of these models of acute inflammatory pain. To the authors knowledge, there are no definitive studies comparing the severity of the inflammatory response of intraplantar injection of carrageenan versus CFA in rat; however, limited reports suggest that CFA produces an inflammation that is more severe and possesses a longer duration of action as compared to carrageenan [19,28].

Reports from other laboratories [24] have demonstrated that anthocyanins are found in plasma samples following a single orally administered dose, thus possessing oral bioavailability in rat [22,24]. Study of the time-course of changes in the concentration of anthocyanins in the plasma following oral administration have shown that anthocyanins are detected for up to 4 h [24] and these data are consistent with the duration of observed effects on pain behavior in our studies.

Our results suggest that tart cherry anthocyanins suppress nociceptive behaviors associated with acute inflammation and may have a beneficial role in the treatment of inflammatory pain. Although further studies are needed to determine the precise mechanism of action of the antihyperalgesic effects of tart cherry anthocyanins, we hypothesize that anthocyanins are acting like the non-steroidal anti-inflammatory agents by inhibiting the cyclooxygenase (COX)-mediated synthesis of prostglandins. Prostaglandins are synthesized and released following tissue injury, and sensitize peripheral nociceptors leading to a facilitated state of nociceptive processing in the dorsal horn of the spinal cord (for reviews see [18,34]). Our laboratory has shown that tart cherry anthocyanins possess potent anti-inflammatory effects in vitro, with equivalent efficacy on the inhibition of COX-1 and -2 activity [30,44]. Cyanidin, the aglycone form of anthocyanins, showed better anti-inflammatory activity than aspirin using the in vitro system that monitors the ability to inhibit cyclooxygenase conversion of arachidonic acid to prostaglandins [44]. Additional in vitro investigations from our laboratory have shown that tart cherry anthocyanins possess cyclooxygenase-1 and -2 inhibitory activity and cyanidin exhibited comparable inhibitory activity to non-steroidal anti-inflammatory agents, naproxen and ibuprofen [41,44]. Anthocyanins have been shown to be effective anti-inflammatory and analgesic agents in rodents, as a significant reduction in the acetic acid-induced writhing responses and carrageenan-induced plantar edema was found following the administration of a proanthocyanidin [33]. Thus, one mechanism of the antihyperalgesic affects of tart cherry anthocyanins may be related to inhibition of COX-mediated prostaglandin synthesis following acute inflammatory insult.

The study of phytonutrients in promoting improved health and potential therapeutic applications has increased as growing epidemiological evidence suggests that the incorporation of fruits and vegetables into the diet provides beneficial effects [1,31]. Much of this renewed interest is due to the findings that the generation of free radicals, due to oxidative stress, is implicated in a number of pathological processes including aging, atherosclerosis, cancer, and inflammation [15]. Antioxidants protect living systems from damage related to the production of reactive oxygen species; however, when free radical production exceeds the antioxidant capacity of the system, lipid peroxidation can occur. The prevention or reduction of lipid peroxidation reduces cross linkage of proteins, damage to DNA, and the release of inflammatory mediators [14].

Tart cherry anthocyanins belong to a large group of natural phenolic molecules called the flavonoids, which possess therapeutic actions thought to be related to their effective antioxidant activity in biological systems [25]. Our laboratory has shown that anthocyanins and their aglycone, cyanidin, have potent antioxidant properties [30,42–44]. Using an in vitro model of Fe(II)-induced peroxidation of liposome [2], anthocyanins isolated from tart cherries significantly inhibited lipid peroxidation and had antioxidant activities comparable to *tert*-butylhydroquinone, butylated hydroxytoluene, and Vitamin E [30,44]. Thus, an additional mechanism of the antihyperalgesic affects of tart cherry anthocyanins may be related to reduction of oxidative stress following acute inflammatory insult.

Although preclinical and clinical research has only begun to reveal how diet influences pain processes, there is an emerging literature that suggests that constituents of the diet may play a modulatory role in pain mechanisms resulting in attenuation of pain. A better understanding of how dietary constituents and phytonutrients influence pain will offer further therapeutic options to physicians treating patients with persistent and chronic pain conditions.

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