Accepted: 26 February 2017

DOI: 10.1111/ocr.12149

ORIGINAL ARTICLE

WILEY Orthodontics & Craniofacial Research

Effects of cocoa-enriched diet on orofacial pain in a murine model

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Structured Abstract

Objectives: To investigate and discuss the effects of cocoa on orofacial pain.

Setting and Sample Population: The Department of Orthodontics at the University of Florida (UF). Male and female hairless rats (N=20/group) were tested.

Materials and Methods: Rats were tested using the Orofacial Pain Assessment Device (OPAD) before and after changing their food from the standard chow to a cocoa-enriched or control-equivalent diet.

Results: Male rats fed the cocoa diet had a significantly higher operant pain index when tested at 37°C as compared to control diet-fed animals. Female rats on the cocoa diet had a significantly higher pain index when tested at 18°C and 44°C, as compared to animals fed the control diet. Capsaicin-induced pain was inhibited, with cocoa-diet male rats having a significantly higher pain index than control-diet male rats and cocoa-diet female rats at both 37°C and 44°C. Cocoa-diet female rats had a significantly higher pain index at 44°C than control-diet females. Mechanical sensitivity was affected following capsaicin cream, with a significantly decreased tolerated bottle distance in both cocoa- and control-diet animals, but there was no difference between cocoa- and control-diet groups.

Conclusion: Using the OPAD operant system, we demonstrated that a diet rich in cocoa was effective in inhibiting neurogenic inflammatory pain in rats. This has implications for the use of novel alternative therapies such as diet modification for pain control.

KEYWORDS
analgesia, cocoa, murine, operant, pain

1 | INTRODUCTION

Orofacial pain is one of the most prevalent maladies afflicting humans, and despite the investment of immense resources, it remains a significant societal issue in both cost and suffering. This has a significant impact on both individuals and society, and the development of viable long-term treatment options is challenging. Pain therapies include drugs, physical and psychological therapies and a combination of these techniques, with pharmaceuticals representing the major area of novel therapeutics development. But because many patients are averse to using drugs due to inefficacy or unfavourable side effects, patients and clinicians have turned to alternative therapies, including diet modification.

Consumption of foods rich in flavanols, such as green tea and cocoa, is thought to have health benefits, most notably decreased blood pressure and reduced risk of cardiovascular disease (CVD).[1,2]

Cocoa is rich in the flavanol isoform (-)-epicatechin, and in addition to CVD benefits, has been shown to prevent cortisol resistance and produce anti-inflammatory and antioxidative effects.[3] Recent studies have demonstrated that cocoa can inhibit activation of trigeminal neurons and modulate expression of proteins in the ganglion and spinal cord implicated in nociception.[4-6] A cocoa-enriched diet was found to suppress expression of proteins that promote inflammation and neuronal sensitization while increasing the levels of several anti-inflammatory and antinociceptive proteins. Specifically, cocoa stimulated an increase in the basal expression of the anti-inflammatory protein MKP-1 and glutamate transport protein GLAST, which would suppress activation of nociceptive neurons in the trigeminal ganglion and spinal trigeminal nucleus. Furthermore, results from biochemical analysis of cocoa extracts provided evidence that inhibition of neuropeptide release from trigeminal neurons was mediated by the anti-inflammatory compound beta-sitosterol.[7] Taken together, these cellular study results support the notion that dietary cocoa is likely to be a therapeutic option for chronic orofacial pain conditions that involve activating and sensitizing trigeminal neurons.

The aim of this study was to investigate the effects of a cocoa flavanols on pain control. We hypothesized that a cocoa-enriched diet would be effective in reducing pain in a model of capsaicin-induced pain. The rationale was that the cocoa could modulate the inflammatory cascade and inhibit hyperalgesia and allodynia. This study also highlights the use of clinically relevant operant pain models as a way of evaluating novel therapies.

2 | MATERIALS AND METHODS

2.1 | Animals

Hairless male and female (N=20 each) Sprague-Dawley rats (250-300 g; Charles River, Wilmington, MA, USA) were used and housed in pairs in a standard 12:12-hour light/dark cycle. Animals were fasted for 15+/−1 hours before each experimental testing day and allowed a recovery day between sessions to minimize nutritional differences. Animals were tested at the same time in the morning and had water and food available *ad libitum* when not being tested. Animal weights were monitored weekly. The UF Committee for the Care and Use of Animals approved all procedures.

2.2 | Thermal facial operant testing

The OPAD was used to assess pain behaviour, as described in detail previously.[8,9] Unrestrained animals were placed into the testing chambers that had a peltier-stimulus thermode that uses electric currents between two materials to either emit or absorb heat to provide hot and cold stimuli, respectively. A bottle containing diluted (1:2 with water) sweetened condensed milk solution was positioned such that access to the reward was possible contingent on facial contact with the thermode. Reward-licking events and facial-stimulus-contact events were recorded.[10] Animals were considered trained (~2 weeks) on the OPAD when they achieved >1 000 licking events with the thermode set at 33°C. We evaluated the "pain index" (reward-licking events/stimulus-contact events) ratio as the primary outcome measure.[9]

The animals completed two more weeks of testing denoted as "Standard/Naïve" in Figures 1 and 3. We defined this as a "baseline session," as it was completed prior to diet modification at 18°C (females) on Mondays, 33°C (males, females) on Wednesdays and 44°C (males, females) on Fridays of each week. During the 18°C and 44°C test sessions, a 1-minute lead-in at 33°C was provided before ramping up or down to the final stimulus temperature. This period provided a neutral stimulus within the overall session for comparison with the aversive stimuli.

2.3 | Mechanical facial operant testing

For the male rats, we used another reward/conflict operant-based device to assess mechanical pain. This included a testing chamber

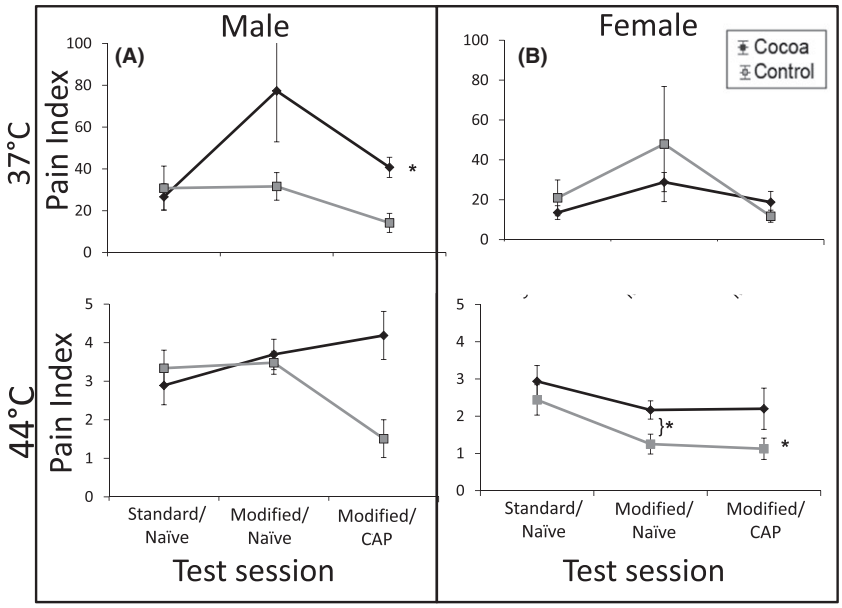

FIGURE 1 Diet effects on thermal sensitivity. (A). Male: There was a significant treatment effect (*P<.05) for rats fed the cocoa-enriched diet when tested at the neutral (37°C) stimulus temperature, but not for those fed the control diet. Testing animals in either group at the hot (44°C) stimulus temperature had no significant effects. (B). Female: Female rats demonstrated no effect at 37°C (A), but there was a significant treatment effect for the control-treated animals at 44°C (*P<.05). Cocoa-treated females had significantly higher reward/stimulus pain ratios at both the "Modified/Naïve" and "Modified/Capsaicin" test sessions at 44°C (*P<.05)

with an array of eight stainless steel wires that impeded access to the reward bottle, as described previously by Riley et al.[11] Contact with the reward bottle activated a motor to move the bottle away from the cage at a rate of 5.0 inches/min, requiring increased pressure exerted by the mechanical stimulus device on the face to reach the reward bottle. Every two minutes a maximum displacement was recorded (five displacements/10 minutes) and the bottle returned to the start position. The displacements were averaged for a tolerated bottle average distance for each animal. Due to availability of equipment, we tested female rats each week at 18°C and male rats using the mechanical assessment device.

2.4 | Diet modification

All animals initially were fed the standard UF Animal Care Service (ACS) food, and data acquired with animals fed this diet are denoted as the "Modified/Naïve" test session in Figures 1 and 3. The male and female groups were then randomly divided into "**cocoa-diet**" and "control-diet" groups (N=10/group). The cocoa-diet group was fed a 10% g/g cocoa research diet (AIN-76A; Research Diets) and the control-diet group was fed an isocaloric, cocoa-free rodent diet (D10001; Research Diets). As the control-diet differed calorically from the standard ACS food, we also investigated the effects of diet between the control-diet and ACS-diet groups (N=6/group).

2.5 | Inflammation induction

Acute neurogenic inflammation was induced with capsaicin cream as described previously[12]; this test session is denoted as "Modified/CAP" in Figure 1. Briefly, animals were lightly anaesthetized (2.5% isoflurane), and capsaicin cream (0.075%; Thomson Micromedex) was applied bilaterally to the cheeks over the area of the masseter muscles. Capsaicin was removed after 5 minutes, and animals were tested 30 minutes later at 44°C using the OPAD. Males were also tested on a different session using the mechanical device following capsaicin application.

2.6 | Data and statistical analyses

The pain index data are presented as the mean±standard error of the mean (SEM), and probability (*P*) values less than .05 were considered statistically significant. For heat sensitivity (44°C) assessment (Figure 1), we compared the effects of "Test Session" between males and females. Heat and mechanical sensitivity (Figure 2) were assessed between the cocoa- and control-diet groups, and the effects of sex were evaluated. The effects of diet on cold sensitivity (18°C; Figure 3) were also analysed between test sessions. We used Student's *t* test, ANOVA and general linear model multivariate measures for comparisons, and when significant, Scheffe post hoc analysis was completed. All statistical analyses were made using IBM SPSS Statistics 23 and Microsoft Excel.

3 | RESULTS

3.1 | Effect of ACS diet and control Diet

There was no significant difference (*P*>.05) in the pain index between the ACS-diet and control-diet groups at either 37°C (52±20 vs 12±3) or 44°C (8.5±1.5 vs 8.6±1.9). All animals started within the same weight range (250-300 gm), and there were no significant differences in weight gain between the cocoa-diet and control-diet groups (*data not shown*).

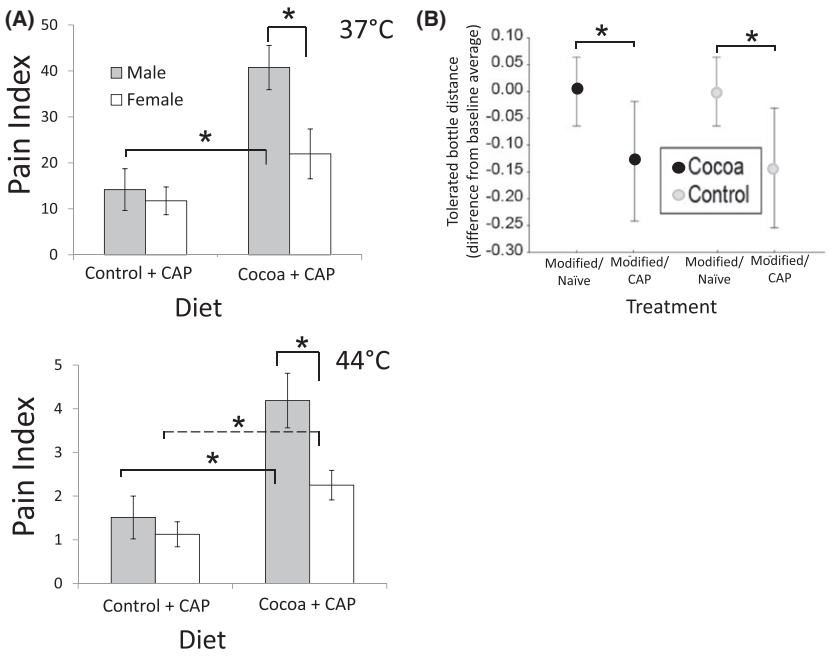

FIGURE 2 Modified-diet effects on thermal hyperalgesia (A) and mechanical hyperalgesia (B) following capsaicin application. Male rats on the cocoa diet had a significantly higher pain index than control-diet male rats at both 37°C and 44°C (*P<.05) and cocoa-treated females at both 37°C and 44 °C (*P<.05). Cocoa-diet females had a significantly higher pain index at 44°C. For mechanical sensitivity, capsaicin cream significantly decreased tolerated bottle distance in both cocoa- and control-diet animals (*P<.05), but there was no difference between the cocoa- and control-diet groups

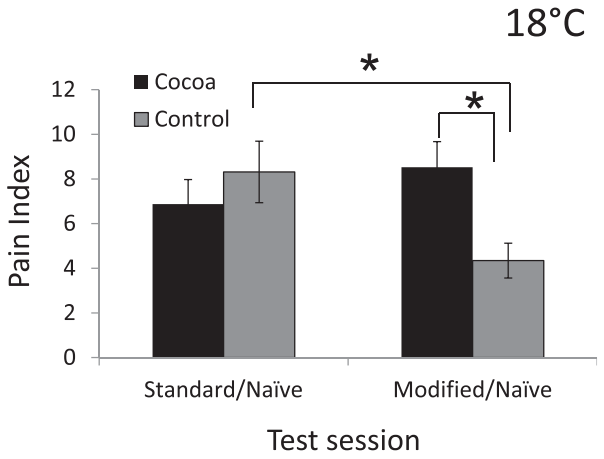

FIGURE 3 Diet effects on cold sensitivity. Cocoa-diet female rats experienced a significant increase in their pain index at 18°C as compared to control-diet animals (*P<.05). Control-diet rats had a significant decrease in their pain index at 18°C (*P<.05)

3.2 | Effect of cocoa-enriched diet on thermal sensitivity and hyperalgesia

We evaluated the pain index (reward/stimulus ratio) during both the neutral lead-in period and hot or cool stimulus periods, depending on the test session.

In male rats (Figure 1A), cocoa enrichment of the diet produced a significant effect on the pain index during the neutral lead-in period ($F_{2,67}$ =3.867, P=.026), but there was no effect of the control diet at 37°C ($F_{2,69}$=0.428, P=.654) (**top**). There was no significant effect at 44°C for either the cocoa ($F_{2,67}$=1.092, P=.342)- or control-diet group ($F_{2,69}$=2.583, P=.083) (bottom). In female rats (Figure 1B), there was no significant effect for the cocoa-diet group on the pain index during either neutral lead-in (top, $F_{2,54}$ =2.711, P=.076) or 44°C (bottom, $F_{2,55}$=1.437, P=.247) segments. Female rats on the control diet had a significant pain index decrease during the 44°C test segment (bottom, $F_{2,47}$ =4.927, P=.012), but not the neutral lead-in segment (top, $F_{2,46}$ =1.102, P=.341). There was a significant treatment effect at the 44°C test segment (P<.05) during the "Modified/Naïve" test session, with the cocoa-diet group having a significantly higher pain index (2.1±0.3) as compared to control-diet animals (1.0±0.2).

Following capsaicin application, male rats in the cocoa-diet group demonstrated a significantly higher (P<.05) pain index than cocoa-diet females and control-diet male and female animals at both 37°C (Figure 2A, top) and 44°C (Figure 2A, bottom). Cocoa-diet females similarly demonstrated inhibition of capsaicin-induced thermal hyperalgesia at 44°C, as indicated by a significantly higher pain index (P<.05).

3.3 | Effect of cocoa-enriched diet on cold sensitivity and capsaicin-induced mechanical hyperalgesia

Female rats in the cocoa-diet group had a significantly higher (*P<.05) pain index at 18°C as compared to baseline ("Standard/Naïve") values and compared to control-diet animals (Figure 3). Animals fed the control diet had a significant decrease at 18°C. Tolerated bottle average distance in male rats did not show a difference between capsaicin and "Modified/Naïve" treatments for either cocoa- or control-fed animals (*data not shown*). Capsaicin cream decreased the tolerated bottle distance in both cocoa- and control-diet animals (P<.05; Figure 2B), but there was no diet effect.

4 | DISCUSSION

Challenges exist in the development of novel therapeutics, including the choice of pain assessment techniques. We have developed operant assessments, such as the OPAD, that better reflect responses predicated on the integration of the nociceptive and central inputs.[9,13–15] For a more in-depth summary of the use of the OPAD in comparison with other pain measures, refer to a review by Murphy et al.[10] The OPAD uses a reward-conflict paradigm that, unlike for reflex-based responses, involves assessment of higher-level cognitive processing, whereby the animal must decide whether it will complete the task to obtain a reward, based on its pain level. The use of appropriate preclinical behavioural assays allows for clinically relevant assessment of novel therapeutics. We used the OPAD to evaluate the effects of cocoa on orofacial pain sensitivity.

Studies on sex differences in pain sensitivity of humans show that females are generally more sensitive than males,[11] and we see this phenomenon in this rodent study. With repeated testing, female rats were more sensitive to both hot and cold stimuli, and the cocoa-diet appeared to inhibit the development of this temperature sensitivity (Figures 1 and 3). Male rats did not display sensitivity at 44°C over the course of the study, but animals treated with cocoa increased their response at 37°C. This increase at the neutral temperature may represent an enhancement of the rewarding aspects of the sweetened milk by the cocoa-diet. The females had a lower pain index that decreased during the study, while the males' outcomes remained constant at the 44°C stimulus temperature after changing to either the cocoa or control diet. Previously, we reported that female hairless rats have a greater aversion for cold, with males being more sensitive to nociceptive heat.[16]

We used topical capsaicin as a neurogenic inflammatory stimulus that previously we demonstrated produced a reversible, non-damaging thermal hyperalgesia.[12] Capsaicin caused a significant decrease in the pain ratio at 44°C for both females and males, as compared to baseline values (Figure 1). The cocoa-diet animals had a significantly inhibited response and higher pain index to the thermal hyperalgesia for both males and females. The increased ratio at 44°C indicates inhibition of thermal hyperalgesia caused by the capsaicin, and the significant effect at 37°C is likely due to inhibition of contact allodynia. Following capsaicin treatment, the male cocoa-diet animals had higher pain indexes than cocoa-diet females. This may relate to inherent sex differences in the capsaicin response, with females having a greater sensitivity to capsaicin. This capsaicin-sex difference has also been reported in humans.[17] Additionally, the male rats in both treatment groups developed mechanical hyperalgesia when tested on the operant mechanical

assay, but there was no difference in response between the cocoa- vs. control-diet groups. This indicates that cocoa treatment did not affect mechanical hyperalgesia.

Our finding that cocoa can inhibit nociception and sensitization of orofacial pain is in agreement with the ability of a cocoa-diet to modulate the expression of proteins in trigeminal neurons and glia implicated in peripheral and central sensitization.[5,18] While the concept of modifying diet to change pain sensitivity is promising, a limitation of the current study includes the use of acute pain models. Future studies evaluating chronic conditions will be needed to further characterize the use of cocoa in the clinical setting as an alternative therapy.

5 | CONCLUSIONS

Using clinically relevant operant pain outcome measures, we demonstrate that a diet rich in cocoa was effective in inhibiting neurogenic inflammatory pain in rats. This has implications for patients suffering from pain, and future studies are needed to evaluate the effects of cocoa as an alternative therapy for chronic pain.

ACKNOWLEDGEMENTS

We thank the American Association of Orthodontists Foundation for its support in funding this project.

CONFLICT OF INTERESTS

JKN and RMC are employees of Velocity Laboratories, a company that provides fee-for-service behavioral testing using operant pain assays.

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How to cite this article: Bowden LN, Rohrs EL, Omoto K, et al. Effects of cocoa-enriched diet on orofacial pain in a murine model. *Orthod Craniofac Res*. 2017;20(Suppl. 1):157–161. https://doi.org/10.1111/ocr.12149