EFFECTS OF COCOA-ENRICHED DIET ON OROFACIAL PAIN IN A MURINE MODEL

By

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To my family for supporting me through this process
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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

EFFECTS OF COCOA-ENRICHED DIET ON OROFACIAL PAIN IN A MURINE MODEL

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Studies have shown that flavonoids, polyphenols derived from fruit, vegetables, cocoa, green tea, red wine and many other foods, suppress the production of cytokines that promote inflammation and have many beneficial effects on health. Inflammation is one of the main causes of pain sensation/sensitivity, therefore, if inflammation is reduced then pain sensation, in an acute inflammatory state, should also be reduced/diminished. Despite the investment of immense resources, pain remains a significant societal issue in terms of costs and suffering.

There is limited research available to quantify pain sensation and if certain dietary supplements help reduce the perception of pain. We investigated the effects of cocoa on orofacial pain and the use of behavioral assays and animal modeling for the preclinical stages of analgesic development.

Using the Orofacial Pain Assessment Device (OPAD), we assessed pain sensation of a group of experimental male and female rats (N=20/group) that were fed a 10% cocoa diet for 2 weeks before neurogenic inflammatory pain induction.
Inflammation was induced by Capsaicin cream on the animal’s buccal region and the TMJ.

There was a significant increase (*P<0.05) in the pain ratio (an indication of pain-relief) for the cocoa treated animals. Rats fed the cocoa diet had significantly less pain as compared to the animals fed the control diet. Capsaicin-induced hyperalgesia was inhibited and we observed that the females were more sensitive to both thermal and capsaicin stimuli. Capsaicin inflammation produced a significant decrease in this pain ratio for the control-diet animals (P = 0.001).

Animals fed a 10% cocoa-enriched diet demonstrated inhibition of capsaicin-induced hyperalgesia when tested on the OPAD at 44 °C. This has implications for the use of novel alternative therapies such as diet modification for the control of pain.
CHAPTER 1
BACKGROUND

Pain is one of the most prevalent maladies afflicting the human condition and for a long time it was seen as a symptom of other physiological problems, rather than treated as a medical issue in its own right. Pain is invisible, difficult to articulate, therefore, developing viable long-term treatment options is challenging. Pain has historically been under-diagnosed and ineffectively managed. The prevalence of chronic pain affects not only the patient but also society. Financially, the direct medical expenses and indirect costs in the form of lost productivity is estimated to be between $215 and $635 billion annually [1]. It is only recently, that the causes and treatments for chronic pain have been brought to the fore of medical professionals’ critical attention. It is now understood that pain is not merely a side effect of underlying injuries/diseases. Management of pain both during and after any period of healing is what determines the success of the patient's recovery and the impact that recovery will have on the social, economic, and psychological spheres of that patient. Managing chronic pain is often many times more complicated than treating its underlying medical condition. Most pain management approaches are multi-faceted. Patients respond differently to pain depending on various social, economic, and psychological levels. Pain treatments options often depend on a patient's relative health and specific physiological capabilities and treatment models for chronic pain are highly individualized. Preexisting factors such as genetics, gender, and experience can also cause “variability in pain sensitivity, perception and tolerance” [2, 3]. Methods of pain management vary greatly depending on the patients’ needs and the physician’s philosophy on treating pain. Oral pharmaceuticals have been the most visible form of pain-management in society. Other
methods of treatment are usually dovetailed into a patient’s treatment plan to augment the inadequate relief achieved by pharmacotherapeutics [4]. Acupuncture has been shown to have greater-than-placebo level effects on chronic pain, and is used by several million Americans to manage chronic pain despite the controversy regarding the reasons for its efficacy [5]. Physical therapy such as massage, meditation and exercise are also frequently prescribed additions to a pain-management regimen, provided that activity is paced appropriate to individual patients’ needs [6, 7]. Psychological therapy are often a necessary part of pain management for a multitude of inter-related reasons. Psychological conditions such as depression and chronic pain have a high level of comorbidity, in part due to the negative effects various medications for managing pain can have on the body and spirit over time. The social and economic consequences of living with chronic pain, such as losing one’s job or being unable to continue activities one previously enjoyed, can lead to depression and other psychological issues. On the unconscious level, too, prolonged pain can have an impact on a patient’s psychological health, corrupting cognitive pathways and introducing pain related attention biases that over-emphasize the presence of pain and make it significantly harder to manage physiologically [8].

Orofacial pain constitutes a mean of 13% of all pain conditions according to a systematic review and meta-analysis by Macfarlane, et al., 2001 [9]. There is a strong relationship between orofacial pain, psychological distress and pain throughout the body suggesting that the etiology of orofacial pain is not specific to this regional pain. Many orofacial pain conditions have overlapping symptomatic presentations, with symptoms including heightened sensitivity to temperature and touch in the orofacial region [10].
Inflammation is one of the main causes of pain. The inflammatory process is crucial in maintaining tissue health during injury and infections. The recruitment of inflammatory cytokines and the release of reactive oxygen species (ROS), help eliminate foreign pathogens. This cascade of inflammatory mediators like (tumor necrosis factor alpha, Interleuking-1 Beta and Interleukin 8) signal other inflammatory mediators and the process can overreact to an inflammatory pathologic condition. When there is an imbalanced oxidative stress tissue damage occurs. Oxidative stress triggers immune response via Nuclear Factor Kappa B and Activator Protein-1, which in turn result in the production of more inflammatory cytokines. This cycle leads to chronic inflammation and a plethora of chronic disorders including cardiometabolic disorders, neurologic disorders and cancers [11-14]. Glucocorticoids from the adrenal gland activate transcription of anti-inflammatory genes and suppress pro-inflammatory genes. Oxidative stress reduced the anti-inflammatory potency of cortisol and causes cortisol resistance leading to chronic inflammation [15].

Diet is one of the key lifestyle factors involved in the prevention and control of disease. Diets rich in polyphenols from fruit, vegetables, cacao, red wine, coffee and green tea have significant health benefits in reducing inflammation [16, 17]. A promising polyphenol that has been studied in the literature is cacao. Cacao is a complex plant consisting of over 300 components including procyanidins, theobromine, (−)-epicatechin, catechins, and caffeine [18]. The tropical tree Theobroma cacao originated in Central America. The name cacao is derived from the Aztec Nahuatl word xocolatl, meaning bitter water. Raw cacao powder is just cold-pressed unroasted cocoa beans. This cold-press process keeps the living enzymes in the cocoa and removes the cacao
butter fat. Cocoa powder is raw cacao that has been roasted at high temperatures [19]. Cocoa-containing foods are a rich source of a subclass of polyphenolic flavonoids called flavanols also known as flavan-3-ols or catechins. Two monomers found within flavanols are epicatechin and catechin. They serve as building the blocks for oligomers and polymers known as procyanidins or condensedtannins. Procyanidins are linked (oligomeric) flavanols with chain lengths of over 10 units [20]. These linked (oligomeric) flavanols can vary in size, and are typically referred to by the number of linked flavanols. For example, two linked flavanols are dimers and three linked flavanols are trimers, and so on. Cocoa flavanolins are the sum of the simple (monomeric) flavanols, including epicatechin, and catechin, as well as procyanidins. The term cocoa flavanols represents the collective sum of monomer flavanols up to and including decamers (ten linked flavanols). There are four forms of flavanols that occur in cocoa; (+)-epicatechin, (-)-epicatechin, (+)-catechin and (-)-catechin. These four forms (stereoisomers) occur both naturally or can be transformed from one form to another through food processing. The type of flavanols considered to be responsible for both the beneficial health effect and bitter taste are the (-)-epicatechins [20]. Cocoa is particularly rich in (-)-epicatechin, and research demonstrates that (-)-epicatechin is highly bioavailable (readily absorbed) as compared to the other isomers. Research to date has also indicated that the positive cardiovascular results are attributed to (-)-epicatechin. The cocoa flavanol (-)-epicatechin prevents cortisol resistance and reduces intracellular oxidative stress [22]. Cocoa products containing flavonol have been shown to have potential in preventing cardiometabolic disorders [23]. Higher levels of dark chocolate consumption were associated with a 1/3 decrease in risk for developing cardiometabolic disorders [23, 24].
Cocoa effects on cardiovascular health is by enhancing endothelium-dependent vasodilation and decreasing arterial stiffness, which is associated with atherosclerosis [25]. Cocoa can be beneficial for maintaining cardiovascular health, without adverse effects on body weight or body composition [27]. Cocoa flavonol lowers the adherence capacity of leukocytes and helps regulate hyperglycemia [21, 26, 28]. It has been shown to have anti-inflammatory properties and anti-carcinogenic potential [29, 30]. Recent research has demonstrated that the use of dietary cocoa affects expression of proteins that are involved in the development of central sensitization. A 10% cocoa diet suppressed basal calcitonin gene-related peptide (CGRP) levels in the spinal trigeminal nucleus (STN) of the inflamed trigeminal nerves of rats [31, 32]. There is limited research on orofacial pain and diet modification, the studies of Cady and Durham are pioneering the way for new pain therapy using diet modification with cocoa flavanols. This is an important step in the progression of seeking alternative treatment for orofacial pain.
Quantifying the amount of pain an individual in enduring has been challenging for the medical profession. Most of the translational pain research has been on animals. Most animal reflex pain measure assays lack the ability to reliably assess pain in vivo for several reasons. Bias during manual control of the stimulus and the fact that animals are usually restrained are some of the reasons why the traditional pain assays are not reliable. The animals also experience stress or anticipation during the process. Since many orofacial pain conditions have heightened sensitivity to temperature and touch, most of the research is in replicating these pain conditions. This orofacial hypersensitivity to thermal and mechanical stimuli can be demonstrated in rodent orofacial pain models. Accurate quantification of pain-related behaviors in rodent orofacial pain models is crucial for the preclinical evaluation of potential therapeutics and analgesics [33]. A novel behavioral model for assessment of orofacial pain is the Orofacial Pain Assessment Device (OPAD). The Orofacial Pain Assessment Device (OPAD, Stoelting Co) was developed to acquire, save, process, and export data. OPAD has rudimentary statistical capabilities that are sufficient for most analyses [34]. It has the ability to generate mechanical and thermal stimuli that are not experimenter initiated and generate behavior that is indicative of pain intensity after cerebral processing. With the OPAD the animal controls the amount of nociception it receives and the animal can decide on withdrawing from the aversive stimulus or receiving the reward [35].
Materials and Methods

Hairless male and female (N=20 each) Sprague-Dawley rats (250 - 300 g, Charles River) 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 were tested in the mornings at the same time and a recovery day from fasting was included between testing sessions to minimize nutritional differences. Water and standard laboratory chow were available ad libitum when not being tested. The animal weights were recorded and monitored weekly. The University of Florida Committee for the Care and Use of Animals approved all experimental procedures.

Pure cocoa powder was obtained from Askinosie Chocolate, Springfield, MO. and sent to Research Diets (New Brunswick, NJ) for fabrication of the modified cocoa diet AIN-76A. The cocoa group was fed a 10% g/g cocoa research diet (Modified AIN-76A, Research Diets) that contained 95.5g cocoa powder/kg pellet weight or 6.7% of total energy intake. The control animals were fed an isocaloric standard rodent diet (D10001, Research Diets) [32].

The Orofacial Pain Assessment Device (OPAD) was used to assess pain as described in previously published papers [34, 35]. Briefly, unrestrained animals were placed individually into testing chambers that has an adjustable slit opening lined on the surface with metal that can be heated or cooled under peltier control. 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 at temperatures of 18ºC - 44ºC. We evaluated the pain index (reward/stimulus) ratio as the primary
outcome measure. This pain index has been validated as a sensitive and reliable measure for orofacial pain in rodents [36, 37].

The mechanical OPAD was designed to function on a reward-conflict paradigm just as the traditional temperature OPAD but measures heightened tactile sensitivity in the orofacial regions of the animal. This devise uses a mechanical stimulus, which is increased during the testing session to measure tactile sensitivity in the orofacial region instead of temperature stimulation. It consists of a 7.5”x7.75”x5.75” acrylic testing chamber with a 2.185”x2” window and removable metal floor. A stainless steel looped wire in a 360° array is used to partially block the window access to the reward bottle. The opening is 0.7” in the center of the array. The reward bottle consists of 2 Water: 1 Sweetened Condensed Milk as the traditional OPAD. The 360° array of wires would contact the orofacial regions of the animals while they reached for the reward bottle. Initially, the reward bottle nozzle is inside the array, so that the initial contact to access to the reward bottle does not require contact with the mechanical stimulus. This initial contact completes an electric circuit between the cage floor, animal, and bottle. This contact time is measured using 100Hz, custom LabVIEW 2013 module, National Instruments. Continuous contact with the reward bottle causes the bottle to move further away from its start position inside the wire array at a rate of 5.0 inches/minute. This is accomplished by a stepper motor underneath the cage and the movement of the water bottle slowly increases the distance between the reward bottle and cage. As the reward bottle moves away from the rat and the distance from the cage increases, the pressure exerted by the mechanical stimulus (wire arrays) on the face increases. The animal has to engage the wire arrays with more force on the orofacial region in order to gain access
to the reward. This cycle occurs every 2 minutes and the tolerated bottle distance is recorded then the bottle returns to the start position. This two minute cycle is considered a drinking event and indicates that the animal is trained and is participating. This process is repeated for five cycles (10 minutes) and tolerated bottle distance measurements is recorded and average tolerated distance is calculated each testing session [38].

After baseline training to ensure consistent participation the animals were randomly divided into control-diet and cocoa-diet groups (N=10/group) and tested with the same stimuli. The mechanical sensitivity device consisted of a testing chamber and a reward bottle and impeding access to the reward bottle was an array of eight stainless steel wires. Every two minutes (5 displacements/10 min) the bottle returned to the start position and the displacements were averaged for a tolerated bottle average distance for each animal.

Acute neurogenic inflammation was induced with capsaicin cream as describe previously [38]. Animals were lightly anesthetized (2.5% isoflurane) and capsaicin cream (0.075%, Thomson Micromedix) was applied bilaterally to the cheeks and over the area of the TMJ and masseter muscle. Capsaicin, a compound in hot chili peppers, works by activating the Transient Receptor Potential Vanilloid 1 (TRPV1). The TRPV1 family of ligand-gated ion channels are expressed on nociceptors and are activated by endogenous lipids like arachidonic acid or by exogenous substances such as capsaicin [39]. Capsaicin produce a hot burning pain when applied to the skin and causes an acute inflammatory condition. Capsaicin applied to the buccal regions of the animals does not damage the tissue but induces facial allodynia and hyperalgesia. Capsaicin
was removed after 5 minutes and animals were all tested 30 minutes later at 44°C using the OPAD, with the males tested on the mechanical device.

Unless otherwise stated, data are presented as the mean ± standard error of the mean (S.E.M.) and probability (p) values less than 0.05 were considered statistically significant. Student’s T-test, ANOVA, and general linear model multivariate measures were completed and when significant, appropriate post-hoc comparisons were completed. All statistical analyses were made using IBM SPSS Statistics 23 and Microsoft Excel.
CHAPTER 3
RESULTS

All rats were fed ad libitum in their home cage the standard ACS food during training and baseline sessions before changing the in cage food to the cocoa or control diets. 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.

![Figure 3-1](image)

Figure 3-1. Effects of diet on thermal sensitivity

In the Modified/Naïve male rats (Figure 3-1, top left), cocoa-enrichment of the diet produced a significant effect on the pain index during the neutral part of the test session (F2, 67 =3.867, P=0.026), but there was no effect of the control diet at 37°C (F2, 69=0.428, P=0.654). In the Modified/Naïve male rats (Figure 3-1, bottom left) there
was no significant effect at 44°C for either the cocoa- (F2, 67 = 1.092, P=0.342) or control-groups (F2, 69=2.583, P=0.083). In the Modified/Naïve female rats (Figure 3-1, top right and bottom right), cocoa-enrichment of the diet did not significantly affect the pain index during either neutral (top right, F2, 54 =2.711, P=0.076). Female rats on the control diet had a significant effect during the 44°C test segment (Figure 3-1, bottom right) (F2, 47 =4.927, P=0.012), but not the neutral 37°C segment (Figure 3-1, top right) (F2, 46 =1.102, P=0.341). There was a significant treatment effect at the 44°C test segment (P<0.05) during the Modified/Naïve test session, with the Cocoa-group having a significantly higher pain index (2.1±0.3) as compared to control animals (1.0±0.2).

Capsaicin was applied topically to the face to produce an acute neurogenic-inflammatory pain stimulus and animals were tested 30 min post-application. Male rats treated with the cocoa-enriched diet demonstrated significantly higher (P<0.05) reward/stimulus ratios as compared to cocoa-enriched females and control-diet male and female animals at both 37°C (Fig. 3-2, top left) and 44°C (Fig. 3-2, bottom left).

Following capsaicin application, Modified/Cap cocoa-treated male rats had a significantly higher pain index as compared to control-treated male rats at both 37°C and 44°C (*P<0.05). Female Modified/Cap rats treated with cocoa had a significantly higher pain index at 44°C. Male rats treated with cocoa had a significantly higher pain index as compared to cocoa-treated females for both 37°C and 44°C (*P<0.05).

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 alldynia. Cocoa-enriched female rats similarly demonstrated inhibition of capsaicin-
induced thermal hyperalgesia at 44°C, as indicated by a significantly higher pain index (P<0.05) (Figure 3-2, bottom left).

Figure 3-1. Effects of diet on thermal hyperalgesia and mechanical hyperalgesia

Tolerated bottle average distance was used to assess mechanical sensitivity. Baseline was established over three trials, then a capsaicin cream trial was conducted. For mechanical sensitivity, capsaicin cream significantly decreased tolerated bottle distance in both cocoa and control fed animals (one-way ANOVA, *p=0.037), but there was no difference between cocoa and control diet groups (Figure 3-2, top right).
Female rats on the cocoa-enriched diet had a significantly higher (*P<0.05) pain index at 18°C as compared to their baseline (Standard/Naïve) values and compared to control-animals (Figure 3-3). Animals fed the control-diet had a significant decrease at 18°C. Taken together, this indicates that there is sensitization in the operant behavior to the cool stimulus and cocoa-enrichment inhibited the development of this sensitization.
Evidence shows that diets rich in flavanols from foods like cocoa can reduce the incidence of non-communicable diseases such as cardiovascular diseases, diabetes, obesity, strokes and even cancers. Many challenges exist in the development of novel therapeutics for pain management, including the choice of pain assessment techniques at the preclinical stage. There is limited research available to quantify pain sensation while on certain diets and there is no current research on the effects of Flavanols from cocoa on pain sensitivity. Also, patients are motivated to try new options for pain management and they inquire about possible new options to control their pain symptoms.

Traditionally, reflex-based measures (e.g., tail-flick) have been used, but the consensus for their use as a stand-alone and predictive outcome has come into question due to the complexity of the pain experience that integrates both nociceptive input with more central processing that cannot be adequately accounted for with a reflex measure [33]. Our group has adopted and developed operant assessments, including the Orofacial Pain Assessment Device (OPAD), which better reflect responses predicated on the integration of the nociceptive and central inputs [34]. For a more in depth summary of the use of the OPAD in comparison to other pain measures, refer to a review by Murphy et al [41]. Briefly, 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. This design allows for an investigator-independent
assessment of pain outcome measures that more closely simulate human pain conditions, which are influenced by motivation and emotional states. The use of appropriate preclinical behavioral assays allows for clinically-relevant assessment of novel therapeutics. As such, we utilized 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 compared to males [36] 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. Male rats did not display sensitivity at 44°C over the course of the study, but animals treated with cocoa had an increase response at the 37°C lead-in segment. This increase at the neutral temperature may represent an enhancement of the rewarding aspects of the sweetened milk by the cocoa diet. Previously, we reported that female hairless rats have a greater aversion for cold, with males being more sensitive to nociceptive heat. In the cohort reported above in this study, the females had a lower pain index that decreased over the course of the study, while the males’ outcomes remained constant at the 44°C stimulus temperature after changing to either the cocoa or control diets.

We used capsaicin, the active ingredient of red chili peppers, as a neurogenic-inflammatory stimulus. Previously, we demonstrated that topical capsaicin produces a reversible, non-damaging thermal hyperalgesia [40]. Capsaicin produced a significant decrease in the pain ratio at 44°C for both females, as compared to baseline values (Figure 3-1). The cocoa-enriched diet significantly inhibited the thermal hyperalgesia for
both male and females. The male animals treated with cocoa had higher pain ratios as compared to cocoa treated females following capsaicin treatment. This highlights additional sex differences noted during the study. The male rats on the cocoa diet also had a significant increase in their pain ratio at 37°C following capsaicin application, as compared to the control diet animals. This may indicate inhibition of contact allodynia produced by the capsaicin cream. Additionally, the male rats in both the treatment groups developed mechanical hyperalgesia when tested on the operant mechanical assay, but there was no difference in response between the cocoa vs. control groups. This may be related 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 as well [43].

Final Thoughts

Cocoa’s benefits have been well documented in the literature from cardiovascular disease, diabetes, obesity, immune support, allergy support, improves intestinal microflora, improvement in mood and cognition, and in decreasing inflammation [44]. Our findings that cocoa can inhibit nociception and sensitization of trigeminal neurons is in agreement with the ability of a cocoa enriched diet to modulate the expression of proteins in trigeminal neurons and glia implicated in peripheral and central sensitization. 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 [45]. CGRP is known to
promote inflammation and nociception. CGRP regulates blood flow in the brain, causing vasodilation of dural vessels, and causes release of inflammatory agents. It is involved in the transmission of painful stimuli from the meninges to the central nervous system [46]. A substance that can block CGRP like cocoa may be effective in treating orofacial pain and may suppress nociception. The inclusion of Beta - Sitosterol and cocoa as a dietary supplement we suspect would suppress activation of trigeminal neurons, and thus may be beneficial in the treatment of orofacial pain.


BIOGRAPHICAL SKETCH

Laura Bowden was born in Romania but raised in Boca Raton, Florida. She received her bachelor’s degree in biology from Florida Atlantic University and attended the University of Florida, College of Dentistry (UFCD). After graduation, she started a private practice in Kansas City with her husband, a fellow UFCD graduate. During her ten years as a general dentist, she was committed to lifelong learning and achieved Fellowship status in the Academy of General Dentistry. She also became board-certified in Craniofacial Pain and Dental Sleep Medicine. Having a special interest in orofacial pain and dentofacial orthopedics, she returned to UFCD in 2014 to further advance her education by becoming a specialist in orthodontics. During her time at UF she had the privilege of training under nationally renowned professors and being involved in cutting-edge research. She is forever proud to be a Florida Gator.