Research Article

Study assessing the efficacy of herbal teas on bone health and quality of life in a population with osteopenia: rooibos actions on melatonin and tulsi actions on quality of life.

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ABSTRACT

The purpose of the OsTea translational study was to assess the efficacy of teas (tulsi, rooibos, oolong) compared to placebo (coriander) on markers of bone health and quality of life (QOL) in those with osteopenia and on human mesenchymal stem cell (hMSC) differentiation into osteoblasts to identify potential mechanisms of action. Following consumption of tea (3 times/day; 90 days), participants collected a urine sample during the night (10pm-6am) and filled in questionnaires before and after the study. Rooibos consumption demonstrated a significant decrease in urinary CTX levels vs placebo; trended towards increases in nocturnal melatonin levels ($p=0.06$); significantly decreased serotonin-producing microbes in the gut; and demonstrated trends towards improvements ($p=0.09$) in QUALIOST emotional parameters. Tulsi consumption primarily affected subjective measures, such as significantly improved scores for PSS, STAI-trait anxiety, and osteoporosis/osteopenia-related parameters in the QUALIOST. To further identify potential mechanisms underlying these actions of rooibos on CTX and melatonin (urinary and gut), rooibos and melatonin effects on human osteoblastogenesis were carried out for 21 days under oxidative stress conditions to mimic osteopenia. Although both rooibos and melatonin protected against oxidative stress-induced loss of osteoblasts *in vitro*, their underlying mechanisms were different. Melatonin, like tulsi and oolong, demonstrated the greatest protection against oxidative stress at days 10-11 of exposure, which was due to effects on hMSC viability and through melatonin receptors. Rooibos, on the other hand, demonstrated protection at days 10-11 and 20-21, which was through signaling mechanisms involved in differentiation processes and not on cell viability. These findings suggest that the clinical actions of rooibos on decreasing CTX levels in a population with osteopenia may be through a cooperative effort between melatonin and rooibos by protecting hMSC viability against oxidative stress-induced loss and by promoting osteoblast differentiation, respectively. This study also supports the use of tulsi for improving quality of life in a population susceptible to osteoporosis.

Key words: Rooibos tea, tulsi tea, oolong tea, coriander, osteopenia, melatonin, human mesenchymal stem cells, oxidative stress, osteoblasts.

1. INTRODUCTION

As humans age, changes in hormonal profiles, bone health, and quality of life occurs (1). Generally, these changes start silently in andropausal men and menopausal women (2, 3). This transition process is associated with loss of bone-protective hormones (e.g., estrogen, progesterone, testosterone, melatonin) (4, 5) and gains in bone-destructive hormones like cortisol (6), causing an imbalance in the bone remodeling process (i.e., faster bone resorption by osteoclasts than bone formation by osteoblasts) in both men and women (1). Moreover, oxidative stress either locally or systematically through cellular damage, promotes imbalanced bone remodeling leading to bone diseases like age-related osteoporosis (7, 8). Oxidative stress in postmenopausal osteoporosis, due to an estrogen deficiency, has been related to decreases in glutathione (GSH) levels, loss of defensive antioxidant abilities leading to increases in reactive oxygen species (ROS) (9-12). In fact, increases in ROS can inhibit osteoblast differentiation and mineralization (13), reduce osteoblastic differentiation marker expression, and induce death depending on the ROS levels (14). Moreover, oxidative stress may induce RANKL-induced osteoclastogenesis, shifting towards increases in osteoclast activity (11, 15). Excessive bone resorption by osteoclasts gradually leads to low bone density, osteopenia, osteoporosis, or an osteoporosis-related fracture (16).

Globally, more than 200 million women age 60 or older have osteoporosis with 8.9 million fractures occurring per year (17). Although fragility fractures are higher in women, men generally have higher rates of fracture-related mortality (17). Even though the medicines for osteoporosis are available, mortality due to fragility fracture is still on the rise (18, 19). For various reasons (e.g., stage of bone disease, adverse drug effects, co-morbidities, preference), many, especially menopausal women, are seeking alternative approaches like yoga, meditation, phytoestrogens (20), melatonin (18, 19), and herbal teas to manage their bone health (21). These alternative approaches are becoming popular options not only for their beneficial effects on bone but also to improve the quality of life in men and women (2, 22). Because bone disease is a chronic condition that has a high socio-economic burden that strongly impacts on one's health and mortality, strategies to slow and/or prevent bone loss are essential (23, 24).

Following water, the most commonly consumed beverage is tea. Tea has been around for thousands of years (21). The health benefits of tea are primarily due to its high polyphenols, especially flavanols, commonly known as catechins, part of the flavonoid family (25, 26). Flavonoids are reported to have many beneficial properties, including antioxidant, anticarcinogenic and hypolipidemic properties (25, 27). Tea has also demonstrated protective effects in bone (3, 28, 29). Conventionally, black, green, white, yellow, oolong, and puerh teas produced from the leaves of *Camellia sinensis* can be classified as a "true" tea, which are rich in polyphenols (30). Although herbal teas like tulsi, mint, chamomile, and rooibos are not from *Camellia sinensis*, they can have similar health benefits and have been commonly used in traditional medicine (21). Consumption of rooibos tea is associated with an increase in metabolic rate and fat oxidation in men (31) and an increase in BMD in women (32). Leaves harvested and dried from *Aspalathus linearis*, which produces green rooibos (unfermented) and red rooibos (fermented) tea, are rich in flavonoids and possess antioxidant, antitumor, anti-atherosclerotic, anti-inflammatory, and estrogenic actions in *in vitro* (i.e., leukocytes, embryo fibroblast) and in rodent (33-35) model. Consumption of rooibos tea or flavonoids contained in rooibos increases

osteoblast activity and mineralization (36-39) and decreases oseoclast formation and activity (39-41). The tea produced from the dried leaves of holy basil (tulsi) (*Ocimum sanctum* Linn) has been gaining much attention as a result of many psychological and physiological benefits observed from consumption of tulsi tea (42). Rich in many active phytochemicals and due to its antioxidant and anti-inflammatory properties, tulsi is recommended for the treatment of different diseases (i.e., bronchitis, malaria, GI symptoms, skin disease, arthritis, eye diseases, etc.) (43). Overall, past studies performed in preclinical and cell culture models demonstrated that all of these herbal teas have the potential to provide a significant benefit to bone, especially osteoblasts, through their actions on free radical scavenging, induction of antioxidant enzymes, or by upregulation of osteoprotegerin without untoward side effects (21).

Melatonin, the endogenous hormone to modulate circadian rhythms, is also well known for its antioxidant and free radical scavenging actions rending an additional layer of protection in multiple cell and tissue types (44-50). Melatonin, which is considered a multifunctional homeostatic factor (46) plays a significant role in bodily processes, including the skeletal system, through modulation of its synthesis and secretion endogenously or when given at higher doses exogenously (16, 49). Considering melatonin's myriad mechanisms of action especially those related to oxidative stress, herbal tea's effects on endogenous melatonin levels were also assessed.

The purpose of the translational OsTea Randomized Controlled Trial (RCT) (Herbal Teas on Bone Health in an Osteopenic Population; NCT03480126) was to assess the efficacy of tulsi, rooibos, and oolong) in a population with osteopenia. The endpoints of the OsTea clinical trial were to determine (1) the effects of these teas on markers of bone health (bone marker status, melatonin, cortisol, CRP) in a population of men and women with osteopenia (primary outcome), (2) to assess the effect of these teas on quality of life assessed through validated questionnaires and daily participant diaries (secondary outcome) and (3) to determine tea effects on hMSC viability and differentiation into osteoblasts under normal and oxidative stress conditions.

2. MATERIALS AND METHODS

2.1. Recruitment and enrollment.

The clinical component of the OsTea study was designed as a randomized, double-blind, placebo-controlled trial lasting for three months following the ethical standards and accordance with the Declaration of Helsinki and national and international guidelines. The study was approved by the Duquesne University Institutional Review Board on February 27, 2018 (IRB Grant protocol number 2018/02/7) and was registered on clinicaltrials.gov on March 29, 2018 (Identification no.: NCT03480126). Various strategies were employed for recruiting participants, including 1) publishing study-related articles in neighborhood and city newspapers (e.g., South Hills Living, Pittsburgh Catholic) and Duquesne University's advertising media (e.g., DU Daily website, Duquesne Alumni Magazine, Duquesne Duke, Duquesne University Times), 2) posting of flyers around Pittsburgh neighborhoods, and 3) advertising through "Pitt+Me" research recruitment program of the University of Pittsburgh (51). During the phone interview, the study's procedures and expectations were explained to the potential participants, followed by a screening process to determine their eligibility. Inclusion criteria consisted of age 18 years or older, having osteopenia (T-score between -1 to - 2.5), willingness to drink tea three times a day for 3 months, willingness to come to study location, willingness to undergo testing of bone markers and other biochemical parameters before and after the therapies, and willingness to provide a self-assessment on the

quality of life throughout the program. Once all the inclusion criteria were fulfilled, subjects were screened further and eliminated based on our exclusion criteria. Exclusion criteria were primarily based on the factors, which might have any positive or negative influence on bone health and quality of life. Because the goal of the OsTea study was to provide an intervention to prevent the progression of osteopenia to osteoporosis, people with osteoporosis were excluded as they require established pharmacotherapy to avoid serious consequences (e.g., fracture). Other exclusion criteria included: osteopenia due to other medical conditions such as hyperparathyroidism, metastatic bone disease, multiple myeloma, or chronic steroid use; and current use of prescription bone therapies that could potentially affect bone health and their quality of life. Such bone therapies included: hormone therapy (HT), birth control pills, prescription medications for bone loss such as bisphosphonates, calcitonin and steroids used either recently or chronically for the past 6 months. Individuals who satisfied both the inclusion and exclusion criteria were invited via email to schedule an initial visit with the study team at the study location, Duquesne University. A diagnosis of osteopenia was confirmed by DXA reports sent by the participants. After receiving confirmation of osteopenia and their signed consent forms, participants were then enrolled in the study. At the first visit, participants completed a baseline intake form, which consisted of questions relating to basic demographic information and use of prescription and nonprescription drugs and/or supplements. Participants were asked to fill out questionnaires assessing stress, depression, anxiety, and quality of life (See Section 2.6). Their blood pressure was taken (See Section 2.3) and they were given a 30-day supply of tea, which was determined prior to their visit through block randomization (See Section 2.2). They were also given 30 days of diary sheets (See Section 2.6) that they were asked to complete daily. Before consuming any of the teas, participants were asked to collect a urine sample (See Section 2.4), saliva samples (See Section 2.4) and a stool sample (See Section 2.7).

2.2. Randomization and treatment follow-up.

Randomization was conducted prior to participant recruitment based on an 8-block randomization scheme. Based on the standard deviations determined from serum bone marker data from the MOPS (19) and MOTS (52) clinical trials, we concluded a sample size of 10 per group would provide enough participants to detect a significant change in markers of bone health with 80% power. Because the recruitment process for the study took longer than expected, recruitment ended after 15 months. Eligible participants (n=35) were then randomly assigned (1:1:1:1 allocation ratio) to either the placebo (coriander) (n=9), tulsi (n=8), rooibos (n=9) or oolong (n=9) groups. Although coriander has been found to have positive effects on anxiety and insomnia in some studies (53), it was selected as the placebo to mimic tea with respect to taste, aroma, and appearance of tea and, to our knowledge, there are no reported effects on bone. Both the study subjects and principal investigator were blinded to the group assignments ensuring a double-blind structure and, upon enrollment, each participant received an identification number to maintain anonymity. Both the placebo (coriander) and herbal teas (rooibos, tulsi, oolong) were purchased from reputable sources. For the coriander and tulsi, an equal amount (1g) of each was weighed and placed in identical tea bags. The groups were coded as A, B, C, or D, and only the graduate student on the study team who was responsible for delivering the tea bags to the participants was unblinded to this coding procedure. The 30-day supply of tea (90 tea bags total) were placed in a zip-lock storage bag to maintain freshness. Detailed brewing instructions, which were prepared per manufacturer's instructions and kept identical for all teas, were provided, and explained to each

participant. Each participant was asked to consume 3 tea bags of tea per day morning, midday, and evening when possible. All information regarding tea consumption was recorded on the diary sheets and remaining tea bags from the prior month were counted to assess compliance.

2.3. Blood pressure measurement.

Each participant's blood pressure was measured at each visit (month 0, month 1, month 2, month 3) using an automated blood pressure cuff (GoWISE USA Advanced Control Arm Blood Pressure Monitor) to assess the effect of herbal teas on blood pressure. Systolic and diastolic blood pressure measurements were taken from the left arm. Mean (± SEM) blood pressure readings at each time point were calculated and compared within and between groups. To avoid experimental variation, the same blood pressure cuff was used in all assessments.

2.4. Collection and storage of urine, salivary samples.

To assess the status of markers related to bone health, participants' urine, saliva, and stool samples were collected at month 0 (baseline) and month 3 (final). For the collection of urine, participants were given one urine cup at their initial (month 0) visit and then again at their month 2 visit along with detailed instructions on how to collect and store their urine samples. Participants were asked to collect their urine samples between 10 pm and 6 am the night prior to initiating tea consumption for the baseline measurement and the night prior to their last visit for the 3-month collection and then to freeze them until their visits. For the collection of saliva, participants were given two vials (morning/AM, evening/PM) at the initial (month 0) visit and at the month 2 visit along with detailed instructions on how to collect and store their salivary samples making sure that the time of collections were the same at month 0 and month 3. Participants were asked to collect their saliva samples the day and night prior to initiating tea consumption for the baseline measurement and the night prior to their last visit for the 3-month collection and then to freeze them until their visits (e.g., baseline samples were given at one-month visit and final samples were given at 3-month visit). All collections were coded and stored at -20C until assessment. To minimize analytical variation, all samples were tested at the same time.

2.5. Biochemical assessments.

The bone markers, procollagen type 1 intact amino-terminal propeptide (total-P1NP) and type I collagen C-telopeptide (CTX-I), were assessed in the urine samples collected at months 0 and 3. The inflammatory marker, CRP, and hormonal markers, melatonin, and cortisol,) were assessed from urine (CRP and melatonin) or saliva (cortisol) samples.

*Bone markers***:** The bone formation marker, total-P1NP, was measured via sandwich enzyme-linked immunosorbent assay (ELISA) assay using the human total P1NP ELISA kit (CAT# MBS9314633, Mybiosource, CA, USA) according to the manufacturer's instructions. The bone resorption marker, CTX-I), was measured via sandwich ELISA assay using human Type I Collagen C-Telopeptide (CTX-I) ELISA Kit (CAT# 6033 Chondrex, Inc, Redmond, WA, USA) per kit instructions. Changes in the concentration of bone markers P1NP (in ng/mL) and CTX-1 (in ng/mL) were calculated for each time point and compared within and between groups. Ratios of bone formation to bone resorption (i.e., P1NP: CTX) were calculated over time and compared

within and between groups. All controls contained within each of the kits were within normal ranges.

C-reactive protein (CRP): CRP levels were measured at months 0 and 3 by sandwich ELISA using the high sensitivity human CRP ELISA kit (CAT# ab108826, Abcam, Cambridge, MA, USA) per kit instructions. Following the assessment of absorbance (OD) values, mean (± SEM) concentrations of CRP (in pg/mL) were calculated for each time point and compared between groups.

*Melatonin***:** Nocturnal melatonin levels were measured by sandwich ELISA using the Melatonin-Sulfate Urine ELISA kit (CAT# RE54031, IBL International, Germany) per kit instructions and then mean (± SEM) concentrations were compared among groups. A standard curve was generated for this assay according to the manufacturer's instructions, and melatonin levels (in ng/mL) were calculated.

Cortisol: Cortisol levels taken right upon awakening (AM) or evening (PM) were measured by ELISA using the human cortisol enzyme immunoassay kit (CAT# 1-3002, Salimetrics, State College, PA, USA), according to kit instructions. Following the assessment of absorbance (OD) values, mean (± SEM) concentrations of cortisol (in μg/dL) were calculated for each time point and then compared within and between groups.

2.6. Psychometric analyses.

The effect of herbal teas on subjective measures was assessed using four validated questionnaires: assessing Quality of Life in osteoporosis (QUALIOST) (54-56), Spielberger's State-Trait Anxiety Inventory (STAI) (57, 58), Cohen's Perceived Stress Scale (PSS) (58-60) and the Center for Epidemiologic Studies Depression (CES-D) (61). At baseline and then at month 3, these questionnaires were administered to the study cohort. Participants were asked to complete all questions in a quiet and isolated environment. PSS, STAI, QUALIOST, and CES-D scores were calculated for each time point, reported as mean ± SEM and then compared within and between groups.

A modified version of the daily diary used in our previous MOPS clinical trial (19) and MOTS clinical trial (52) was utilized in this study to capture each participant's daily experiences while in the OsTea trial. Participants were instructed to record daily information about tea consumption, use of prescription and nonprescription medications, the number of hours slept each night, exercise, and feelings of well-being. New diary pages were given to the participants at their monthly visits after collecting the prior month's diary pages. At the end of the study, an analysis of the diary comments was performed. General well-being was assessed by stratifying the comments into categories based on the participants' comments, which included those related to: aches and pains; mood; gastrointestinal (GI) symptoms; neutral comments; temporary illness; and sleep. These comments were then sub-stratified into positive, negative, or neutral comments based on a general health rating of each of the comment where +1= positive comment, 0= neutral comment and -1= negative comment. Tea effects on sleep duration and exercise intensity were analyzed from the participants' diary logs. Through the use of the U.S. Centers for Disease Control and Prevention (CDC) guidelines (62), exercise intensity was given a rating where no exercise = 0; light exercise = 1; moderate exercise = 2; and high intensity or vigorous exercise = 3. Total

participation (in days participated), cups of tea consumed (per day and over the course of the study), sleeping duration, and exercise intensity were also calculated and reported as mean ± SEM. These data were then compared within and between groups. Use of multivitamins/herbal/OTC supplements were also documented using the diary logs and compared between groups.

2.7. Microbiome analysis from the stool sample.

For the collection of the stool sample, participants were given a gut kit (Explorer^{TM} microbiome sampling kit, uBiome, CA, USA) at the initial visit and at the month 2 visit along with detailed instructions on how to collect and store their stool samples in sampling vials. Participants were asked to collect a stool sample the day prior to initiating tea consumption and the night preceding their last visit. After collection of the stool samples using the kit's ingredients, the sample containing vial was directly sent to UBiome, Inc, where they quantified (using qPCR) gut bacteria's paired end reads of partial 16S rRNA gene sequences after extracting DNA from fecal samples. The final data were stratified and presented based on the common functionality of different major genera of bacteria such as carbohydrate metabolizing bacteria, amino acid metabolizing bacteria, lipid metabolizing bacteria, micronutrient (Vitamin-K, vitamin-B9) producing bacteria, multi-functioning bacteria (Lactobacillus, Bifidobacterium), and diet and lifestyle-related bacteria (artificial sweeteners microbes, weight influencing microbes). The abundance (reported either in percentage or in number 1x) of these microbes was compared to individuals (uBiome database control: 100% or 1) who reported having no ailments and high levels of wellness. Those who had a percentage over 100 or value higher than 1 were considered to have higher levels of these microbes compared to the database control. Microbiome analyses were then conducted for everyone at baseline and month 3 of tea consumption.

2.8. Preparation of tea extracts for *in vitro* culture and treatments.

Tea polyphenols were extracted according to the International Organization of Standardization (ISO 14502-1) with slight modification. Teas (0.1g) were steeped with 10 mL of dH_2O for 2–4 min according to the manufacturer's recommended temperature (80-85°C). Following the prescribed steeping time, samples were filtered with a 0.2 μm filter to remove any debris from tea leaves (Figure 1A). These herbal teas were brought from different reputable sources. Tea was prepared fresh for each treatment time point described below.

Human adult mesenchymal stem cells (MSCs) (Lonza, Walkersville, MD, USA), very immature states of osteoblasts, were used experimentally because the goals of the study were to assess herbal tea effects on osteoblastogenesis. Briefly, MSCs were maintained, grown, and passaged at 80% confluence in mesenchymal stem basal cell growth medium (Os-) (Lonza, MD, USA) at 37°C, 5% CO_2 and 90% humidity. Cells were seeded (at passage 3-5) at an initial density of 3×10^3 cells/cm^2 per well of a 48-well transwell plate (Corning, NY, USA) and allowed to settle for 24h. Cells were then exposed to either basal growth medium (Os-) or osteogenic medium (Os+) (Lonza, USA) containing vehicle (dH_2O), coriander, oolong, rooibos, or tulsi (1/100 dilutions of steeped tea). For the melatonin studies performed on MSCs, cells were treated with or without melatonin (50 nM)(Sigma, USA), or the non-selective melatonin receptor antagonist, luzindole (1μM) (Tocris, USA), or the MT2 melatonin receptor antagonist, 4P-PDOT (1μM) (Tocris, USA). Ethanol (0.001%) was used as the vehicle control. For the oxidative stress studies, cells were exposed to the same treatments described above except that the medium contained final

concentrations of 0.3mM L-buthionine-(S, R)-sulfoximine (Acros) and 16mM of the L-glutamic acid monosodium salt hydrate (Sigma) and the treatments were added on days 1-2, days 10-11 or days 20-21. Full media changes continued once every day until day 21. The 21-day period was chosen based on the previous studies (63) demonstrating differentiation of MSCs into osteoblasts required this period (Figure 1B).

2.9. Morphological assessment, Cellular viability, Osteoblast differentiation, and mineralization.

Cell images for morphological analysis were taken using the EVOS^{TM} inverted light microscope (ThermoFisher Scientific, Waltham, MA, USA) at 10X magnification. The effect of the teas on cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay per manufacturer's instructions following the same treatment conditions described above. On day 21 of the treatment period, 25 ml MTT (dissolved in sterile water; 5 mg/ml; Sigma-Aldrich) was added to each well (0.5 mg/ml final concentration) and allowed to incubate for 3h (5% CO_2 and 37°C). Next, the plates were centrifuged at 50 g (37°C for 5 mins) to settle any floating cells and formazan crystals. Next, the medium was aspirated and 250 ml DMSO (Fisher Scientific, Pittsburgh, PA) was added to each well to stop the reaction. The plates were then wrapped in aluminum foil and incubated at room temperature for 15 mins to dissolve the MTT-formazan crystals. Absorbance was measured at 570 nm (VICTOR3 1420 multilabel counter; PerkinElmer).

Calcium mineralization (quantitative and qualitative) was assessed by alizarin red staining according to kit instructions (EMD Millipore, Billerica, MA) on day 21 of the treatments. Qualitative assessments of osteogenesis occurred using a Vistavision microscope (VWR International, Allison Park, PA) with a progress C3 camera (Jenoptik). These images were then analyzed using NIH "ImageJ" analysis. Alizarin red levels were calculated from the optical density (OD) value and then normalized against (Os+/Veh_No oxidative stress)-treated cells. Osteoblastic mineralization activity was then compared within and between groups where average and SEM values were calculated and then statistically analyzed (See Section 2.10).

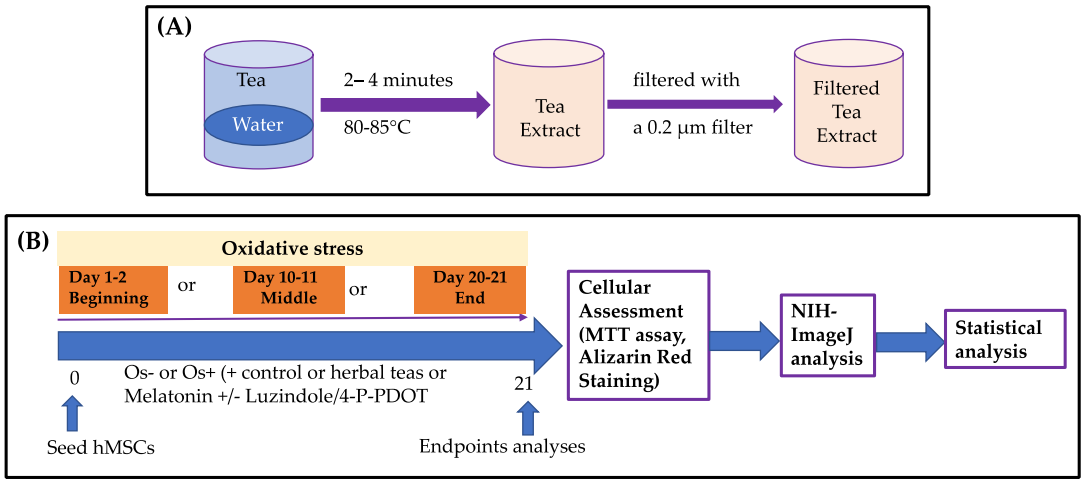

Fig. 1. (A)Tea extract preparation for *in vitro*, and (B) *in vitro* treatment paradigm.

2.10. Statistical interpretations.

To analyze the effectiveness of the randomization of OsTea participants, a comparison between the baseline characteristics among the groups were performed using analysis of variance (ANOVA) followed by Tukey multiple comparison test and Student's two-tailed t-test separately with Welch's correction for unequal variances (continuous data). Mean changes from baseline to month 3 in continuous variables were compared between treatment groups using ANOVA followed by Tukey multiple comparison test. Further Student's one-tailed t- test for all endpoints with Welch's correction was performed. Outcome variables with repeated measures were analyzed using mixed effects analysis of variance. In this analysis, groups and times were considered as fixed effects while subjects nested within the treatment groups were considered random. Pearson correlation was performed to analyze the correlation between bone markers, melatonin, cortisol ratios and CRP levels with the morphometric changes. Dairy comments were analyzed using one-way ANOVA followed by Tukey post hoc analysis. All statistical testing was carried out using JMP versions 14.2 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) for Macintosh. Primary and secondary endpoints analysis were performed using the intention-to-treat principle. Results were considered significant at $P < 0.05$.

For the *in vitro* analyses, statistical differences among groups were analyzed using three-way analysis of variance (ANOVA) followed by Tukey post hoc analysis. Results were considered statistically significant at $P < 0.05$. All studies were individual experiments performed in triplicate (N=3).

3. RESULTS

3.1. Recruitment and enrollment.

The recruitment strategies employed in the OsTea study resulted in 146 inquiries from different areas of Pittsburgh as well as its nearest cities.

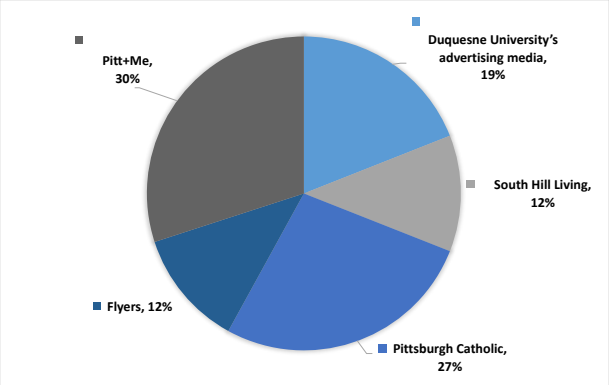

Fig. 2. Responses to recruitment strategies employed in the study (n=146).

A summary of participants' screening and enrollment process is illustrated in Figure 3. All 146 respondents were reached for a phone screening. Of those completing the phone interview, 111 (76%) did not meet the inclusion or exclusion criteria. Individuals were excluded due to having normal bone density T-score or not having a DXA scan performed to verify the diagnosis of

osteopenia (52%); having osteoporosis with or without taking medications (10%); having osteopenia taking prescription medications for preventing bone loss (10%); osteopenia due to other reasons (1%); unwilling to commute to study location (4%); or deciding not to participate (did not share their reasons) (23%). Among the 35 participants invited to enroll in the OsTea study, all accepted our invitation and were then randomized to receive either coriander (placebo; n=9) or tulsi (n=8), rooibos (9) or oolong (9). Although three subjects withdrew from the study (two from placebo (month 1 and month 2) and one from rooibos (month 1) all data collected up to that point were included in the analysis. The OsTea cohort consisted of 33 self-identified Caucasian women, 1 self-identified African American woman, and 1 self-identified Caucasian man.

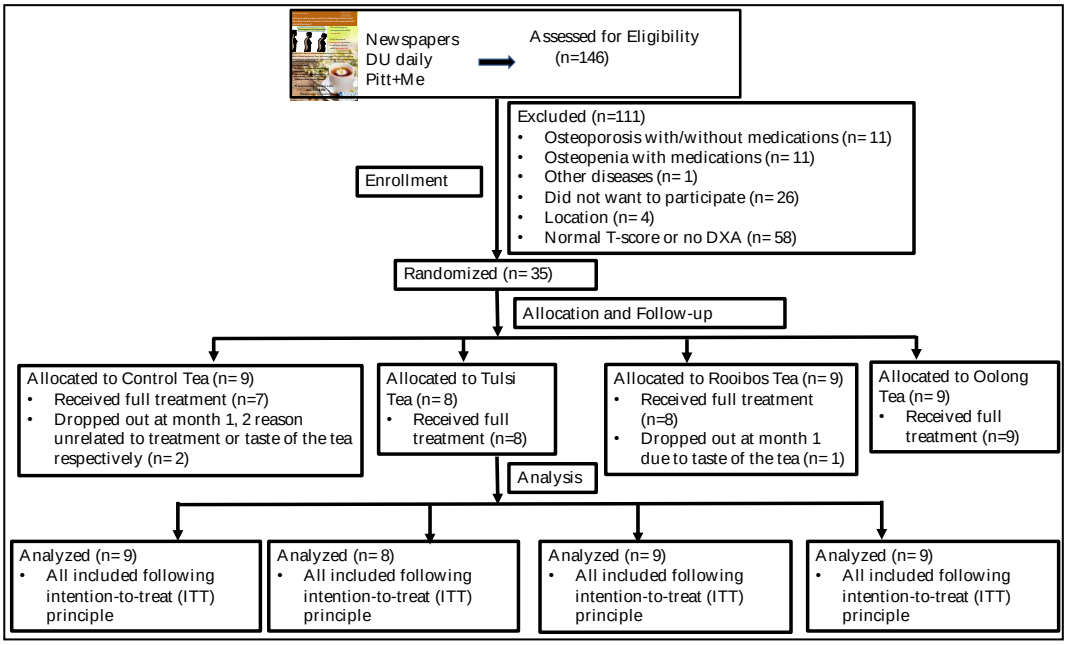

Fig. 3. Flow diagram of study subjects' recruitment and enrollment.

3.2. Baseline characteristics of cohort.

Our recruitment strategies resulted in a diverse population with an average age of 63.45 and a range of 36-83 years of age as reported in Table 1. Each participant presented with osteopenia albeit in different parts of the body (e.g., lumbar spine, total hip, and femoral neck). Psychological evaluation suggested that all subjects had normal mental health with no significant anxiety, stress, or depression. Nearly 90% of subjects were taking either calcium/vitamin D3, multivitamins and/or other dietary supplements (i.e., folic acid, zinc, magnesium, grape seed oil, pomegranate oil, fish oil etc.) not shown to interfere with study endpoints. Almost all subjects were recognized as healthy sleepers with an active lifestyle. Despite using a computer-generated block randomization scheme for stratification, significant differences in urine CTX and nocturnal melatonin levels were found between rooibos and placebo groups. Specifically, baseline CTX levels were higher and nocturnal melatonin levels were lower in the rooibos group compared to placebo. All other parameters did not differ between cohorts suggesting an overall and well-adjusted randomization. Baseline characteristics of the cohort, both total and stratified are illustrated in Table 1.

Table 1. Baseline characteristics of the study cohort un-stratified and stratified by treatment.

	Un-stratified	Stratified			
		Placebo	**Tulsi**	**Rooibos**	**Oolong**
Age	63.45 ± 2.28 (36-83)	57.88 ± 4.44 (36-71)	61.87 ± 3.01 (47-72)	68.37 ± 2.41 (60-78)	65.66 ± 3.17 (56-83)
Blood pressure (BP)					
Systolic (mmHg)	135 ± 5.19 (108-171)	130.786 ± 4.49 (111.00-147.50)	134.42 ± 5.15 (108-149)	150.18 ± 4.60 (129-171)	126.27 ± 3.29 (115-141)
Diastolic (mmHg)	80.45 ± 1.20 (67-98)	80.14 ± 2.53 (71-91)	80.00 ± 3.94 (67-95)	83.72 ± 2.91 (75-98)	77.94 ± 2.76 (67-94)
Bone markers					
P1NP (*ng/mL)*	16.76 ± 5.72 (2-209.9)	3.90 ± 0.54 (2.44-6.77)	21.73 ± 16.44 (4-136.79)	29.98 ± 25.67 (3-209.9)	11.43 ± 8.25 (2-77.39)
CTX (ng/mL)	71.64 ± 19.84 (2.15-312.74)	**39.15 ± 14.81 (2.15-106.39)**	56.98 ± 75.67 (2.15-111.35)	**129.46 ± 33.31* (54.47-312.74)**	61.00 ± 9.92 (12.33-94.89)
CRP (*pg/mL)*	0.97 ± 0.18 (0-5.04)	0.7 ± 0.32 (0-2.48)	1.174 ± 0.35 (0-3.02)	1.38 ± 0.61 (0-5.04)	0.62 ± 0.18 (0-1.6)
Melatonin (ng/mL)	87.8 ± 16.60 (5-187.70)	**109.46 ± 21.21 (30.55-182.88)**	84.00 ± 24.69 (14-185.18)	**42.37 ± 19.40* (5-173.68)**	115.36 ± 24.65 (19-187.70)
Cortisol: Morning (µg/*dL)*	0.373 ± 0.015 (0.00-1.04)	0.39 ± 0.11 (0.13-1.04)	0.344 ± 0.01 (0.00-0.426)	0.351 ± 0.06 (0.00-0.637)	0.40 ± 0.06 (0-0.723)
Cortisol: Evening (µg/*dL)*	0.083 ± 0.001 (0.00-0.171)	0.08 ± 0.01 (0.00-0.171)	0.08 ± 0.01 (0.00-0.139)	0.08 ± 0.01(0.00-0.144)	0.08 ± 0.01 (0.00-0.149)
PSS	9.10 ± 0.96 (1-23)	10.7 ± 1.7 (7-20)	10.5 ± 2.6 (1-23)	6.5 ± 1.9 (1-18)	8.6 ± 2.2 (1-19)
CES-D	6.25 ± 0.50 (1-29)	7.0± 1.8 (1-16)	7.1 ± 3.4 (1-29)	5.8 ± 2.1 (1-15)	5.0 ± 1.8 (1-18)
STAI: State	28.06 ± 1.16 (20-53)	27.5±2.0 (20-38)	31.5 ± 3.6 (20-53)	26.7 ± 2.1 (20-35)	26.4 ± 3.1 (20-46)
STAI: Trait	34.11 ± 1.26 (20-54)	35.5 ± 4.0 (22-55)	36.7 ± 4.8 (20-53)	31.1 ± 2.8 (24-47)	33.0 ± 4.1 (20-54)
QUALIOST					
Physical	23.89 ± 0.47 (21-33)	22.7 ± 0.3 (21-24)	25.0 ± 1.1 (21-30)	23.7 ± 0.9 (21-30)	24.1 ± 1.6 (20-33)
Psychological	36.76 ± 0.41 (32-47)	37.1 ± 0.8 (35-41)	36.6 ± 1.6 (32-47)	37.6 ± 1.7 (32-45)	35.6 ± 0.8 (32-39)
Total/Osteopenia	31.27 ± 1.24 (23-53)	30.7 ± 2.0 (23-38)	34.2 ± 3.7 (25-53)	28.2 ± 1.5 (23-37)	31.8 ± 2.6 (23-48)

*Each group represents an n=8-9. Data represent are reported as mean ± SEM. QUALIOST= Quality of Life in Osteoporosis (osteopenia), STAI= state and trait anxiety inventory, PSS= perceived stress scale, CES-D= center for epidemiologic studies- depression. *p < 0.05 vs. placebo and bold & italic = p < 0.1 vs. placebo.*

3.3. Herbal tea effects on bone markers.

To assess the effect and underlying mechanism of herbal teas on bone health in the OsTea cohort, bone markers, P1NP and CTX were analyzed at baseline (month 0) and month 3. Within and between groups assessments demonstrated no effect of 3-month tea consumption on P1NP

levels or the change in P1NP levels from baseline to month 3 suggesting that coriander, tulsi, rooibos or oolong did not modulate osteoblast activity (Figure 4A, 4D and Table 2). For CTX, within group measurements for placebo (coriander), oolong and tulsi did not demonstrate any significant differences. However, between group measurements demonstrated that baseline CTX levels were higher in the rooibos group compared to placebo (coriander) (Figure 4B). Also, for rooibos, the change in levels (from month 3 to baseline) resulted in CTX levels being significantly lower when compared to placebo (coriander) (Figure 4E) suggesting that rooibos may be decreasing osteoclast activity following 3 months of tea consumption (Table 2). Since osteoclast activity is tightly coupled to osteoblast activity, bone marker turnover was assessed by calculating the ratio (P1NP: CTX) of bone formation to bone resorption. No change in P1NP: CTX levels occurred within or between groups suggesting that the tea did not modulate bone marker turnover (Figure 4C, 4F and Table 2).

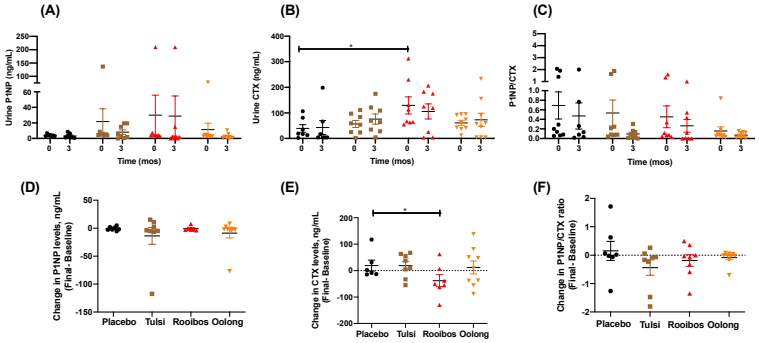

Fig. 4. Herbal teas effects on urinary bone marker turnover in all groups.

*Scatter plots represent bone formation markers (**A**) total procollagen type 1 amino-terminal propeptide (P1NP), (**B**) Collagen Type I C-Telopeptide (CTX), and (**C**) the ratio of CTX: P1NP that were measured at months 0 (baseline) and 3 (final). Changes from baseline to final in **(D)** P1NP, **(E)** CTX, and **(F)** P1NP/CTX ratio after herbal tea consumption (placebo: black, tulsi: brown, rooibos: red, oolong: orange) were calculated and compared among groups; n=8/9 per group). Mean changes from baseline to month 3 were compared among treatment groups using ANOVA followed by Tukey multiple comparison test. Further Student's one-tailed t- test for all endpoints with Welch's correction were performed. The solid lines indicate the mean (± SEM) value for each group. *p ≤ 0.05 versus placebo at similar time point.*

3.4. Herbal tea effects on inflammatory markers.

C-reactive protein (CRP) was assessed to determine if the teas modulated inflammation, which could also impact on bone health over time. CRP levels, which were found to be exceptionally low at baseline (month 0) (64) did not significantly change within or between groups following 3-month consumption of tea (Figure S1, Table 2).

3.5. Herbal tea effects on hormonal markers.

Melatonin levels were measured because melatonin has demonstrated modulatory effects on bone markers (19). Within group assessments demonstrated no significant difference of the teas on nocturnal melatonin levels following 3 months consumption. However, between group measurements demonstrated significant differences between rooibos and placebo where month 0

melatonin levels were lower in the rooibos group compared to coriander (Figure 5A). Also, trends (p=0.06) towards an increase in the change in nocturnal melatonin levels (month 3 vs. month 0) was observed following rooibos consumption vs placebo demonstrating that rooibos may be impacting on nocturnal melatonin levels (Figure 5B, Table 2).

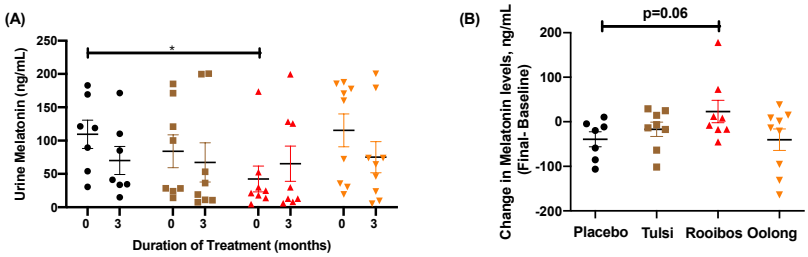

Fig. 5. Herbal teas effects on urinary nocturnal melatonin levels.

*Scatter plots represent **(A)** nocturnal melatonin levels at months 0 (baseline) and 3 (final) and **(B)** Changes from baseline to final after herbal tea consumption (placebo: black, tulsi: brown, rooibos: red, oolong: orange; n=8/9 per group). Mean changes from baseline to month 3 were compared among treatment groups using ANOVA followed by Tukey multiple comparison test. Further Student's one-tailed t- test for all endpoints with Welch's correction were performed. The solid lines indicate the mean (± SEM) value for each group. *p ≤ 0.05 versus placebo at similar time point.*

For cortisol, morning and evening levels did not differ within and between groups at baseline and month 3 following tea consumption (Figure S2A). Normal cortisol rhythms were observed where levels were highest in the morning and lowest in the evening (Figure S2C). Mean changes in cortisol levels over 3 months per group are shown in Table 2 and Figure S2B.

3.6. Herbal teas effects on blood pressure.

Monthly assessments of blood pressure, as shown in Figure S3, demonstrated that three months consumption of herbal teas or placebo did not significantly modulate systolic (Figure S3A) or diastolic (Figure S3B) blood pressure within groups or between groups (Table 2).

3.7. Herbal tea effects on psychometric analysis.

To determine if and how herbal teas impacted on subjective measures, the Perceived Stress Scale (PSS), Center of the Epidemiological Study of Depression (CES-D), and Spielberger's State-Trait Anxiety Inventory (STAI) questionnaires were administered, respectively, at baseline and month 3 of the study. Average CES-D scores (score <16) in all groups suggested the absence of depressive symptomatology (Figure 6A). Similarly, average PSS scores (score < 20) at baseline reflected a low stress level (Figure 6C), while average STAI scores reflected low state and trait anxiety levels (range: 20-39) in almost all participants. Out of all the teas tested, tulsi demonstrated the most significant effects, particularly on measures of stress and anxiety. Specifically, downward trends (p=0.08 and p=0.05) were observed for measures of perceived stress (Figure 6D) and trait anxiety (Figure 6H), respectively. No other changes in measures of depression (Figures 6A, B) perceived stress (Figures 6C, D) or state anxiety (Figure 6E, F) occurred when compared within groups or between groups.

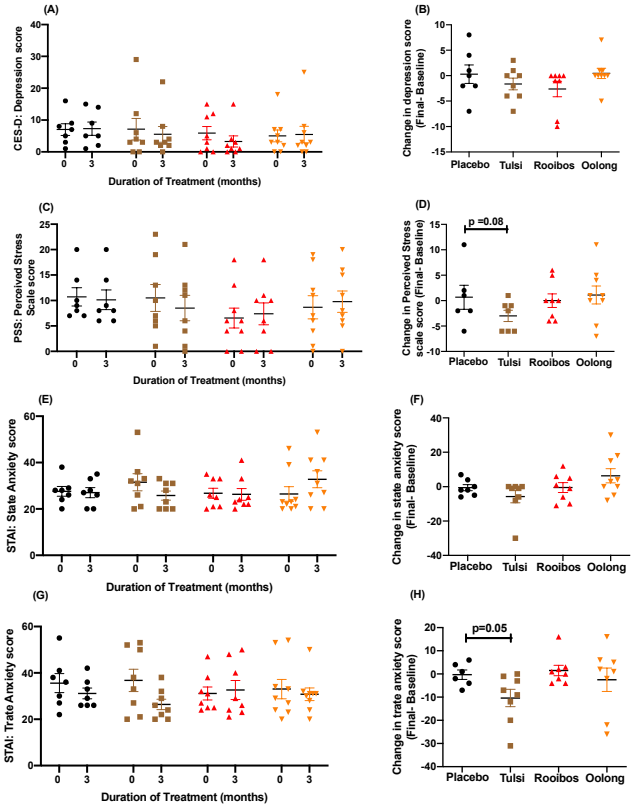

Fig. 6. Herbal teas effects on depression score, perceived stress scale and anxiety scores from CES-D, PSS and STAI questionnaires, respectively.

Scatter plots represent ***(A)*** *depression scale* ***(C)*** *Stress* ***(E)*** *state anxiety* ***(G)*** *trait anxiety scores at months 0 (baseline) and 3 (final),* ***(B)*** *change in depression score and* ***(D)*** *change in stress scale score* ***(F)*** *change in state anxiety score and* ***(H)*** *change in trait anxiety score from baseline to final after herbal tea consumption (placebo: black, tulsi: brown, rooibos: red, oolong: orange). Mean changes from baseline to month 3 were compared among treatment groups using ANOVA followed Tukey multiple comparison test. Further Student's one-tailed t- test for all endpoints with Welch's correction were performed. The solid lines indicate the mean (± SEM) value for each group (n=8/9). *p ≤ 0.05 versus placebo at similar time point.*

To determine if tea consumption impacted on measures of quality of life, the QUALIOST questionnaire was administered at baseline and month 3. As illustrated in Figure 7A and 7D, consumption of the teas was without effect on the physical domain scores (impact because of physical problem) compared to placebo. However, with respect to the emotional/psychological domain scores (impact because of emotional problem), when compared to placebo (coriander group), rooibos demonstrated a downward trend (p=0.09) towards improvement while tulsi and oolong were without effect (Figure 7B, E). With respect to the total osteoporosis/osteopenia-related domain scores (impact because of osteoporosis/osteopenia problem) and when compared to placebo, tulsi tea consumption demonstrated significant improvements (Figure 7C, F, Table 2).

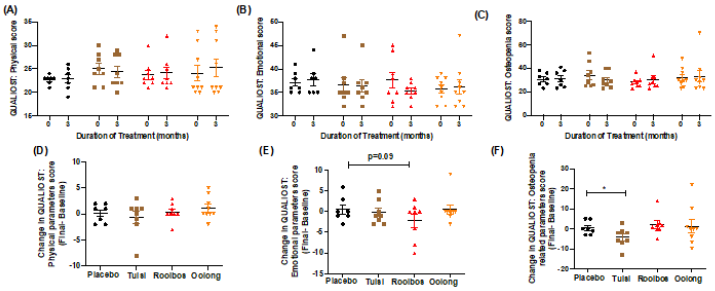

Fig. 7. Herbal teas effects on quality of life in osteopenia from QUALIOST questionnaire.

*Scatter plots represent scores of QUALIOST parameters **(A)** physical **(B)** emotional **(C)** osteopenia at months 0 (baseline) and 3 (final), changes in the scores of QUALIOST parameters: **(D)** physical **(E)** emotional **(F)** osteopenia from baseline to final after herbal tea consumption (placebo: black, tulsi: brown, rooibos: red, oolong: orange). Mean changes in scores from baseline to month 3 were compared among treatment groups using ANOVA followed by Tukey multiple comparison test. Further Student's one-tailed t- test for all endpoints with Welch's correction were performed. The solid lines indicate the mean (± SEM) value for each group (n=8/9). *p ≤ 0.05 versus placebo at similar time point.*

Table 2. Treatment effects on bone marker and psychometric parameters.

	Placebo			***Tulsi***			***Rooibos***			***Oolong***		
	Baseline	**Final**	**Change**	**Baseline**	**Final**	**Change**	**Baseline**	**Final**	**Change**	**Baseline**	**Final**	**Change**
Blood pressure (BP)												
Systolic (mmHg)	130.786 (± 4.49)	128.21 (± 4.48)	-2.57 (± 7.52)	134.42 (± 5.15)	131.0 (± 7.24)	-0.25 (± 5.07)	150.18 (± 4.60)	142.43 (± 5.65)	-7.75 (± 3.66)	126.27 (± 3.29)	126.22 (± 5.13)	-0.056 (± 4.98)
Diastolic (mmHg)	80.14 (± 2.53)	77.50 (± 3.1)	-2.64 (± 4.12)	80.00 (± 3.94)	76.42 (± 3.49)	-1.18 (± 3.73)	83.72 (± 2.91)	81.81 (± 3.59)	-1.91 (± 1.80)	77.94 (± 2.76)	77.61 (± 2.6)	-0.33 (± 3.17)
Bone markers												
P1NP *(ng/mL)*	3.90 (± 0.54)	3.2 (± 1.18)	-0.68 (± 1.28)	21.73 (±16.4)	10.62 (± 3.4)	-13.69 (± 15.07)	29.98 (± 25.67)	38.46 (±34.3)	-0.76 (± 1.72)	11.43 (± 8.25)	2.53 (± 1.14)	-8.89 (± 8.57)
CTX (ng/mL)	39.15 (±14.8)	42.74 (±27.3)	**19.08 (± 20.89)**	56.98 (± 75.6)	75.67 (±19.8)	18.69 (± 16.02)	129.46 (± 33.31)	105.49 (±29.42)	**-38.23 (± 22.63) ***	61.00 (± 9.92)	72.91 (± 25.8)	11.91 (± 24.4)
CRP (*pg/mL*)	0.7 (± 0.32)	1.66 (± 0.3)	0.95 (± 0.27)	1.174 (± 0.35)	1.93 (± 0.2)	0.76 (± 0.39)	1.38 (± 0.61)	1.19 (± 0.2)	-0.19 (± 0.75)	0.62 (± 0.18)	1.47 (± 0.14)	0.85 (± 0.23)
Melatonin (ng/mL)	109.46 (± 21.2)	70.15 (±21.0)	***-39.30 (±16.81)***	84.00 (±24.6)	67.24 (±29.4)	-16.76 (±16.01)	42.37 (±19.40)	65.34 (±26.4)	***22.97 (±25.26)***	115.36 (±24.65)	75.09 (±23.34)	-40.26 (± 23.9)
Cortisol (µg/dL): *Morning*	0.39 (± 0.11)	0.45 (± 0.09)	0.06 (± 0.07)	0.344 (± 0.01)	0.342 (±0.04)	-0.002 (± 0.038)	0.351 (± 0.06)	0.37 (± 0.04)	0.019 (± 0.06)	0.40 (± 0.06)	0.40 (± 0.07)	-0.002 (± 0.04)
Cortisol *(µg/dL): Evening*	0.08 (± 0.01)	0.04 (±0.0)	-0.04 (± 0.01)	0.08 (± 0.01)	0.04 (± 0.0)	-0.04 (± 0.016)	0.08 (± 0.01)	0.06 (± 0.01)	-0.02 (± 0.01)	0.08 (± 0.01)	0.04 (± 0.08)	-0.35 (± 0.00)
PSS: Perceived stress	10.7 (±1.7)	10.1 (±1.9)	***0.6 (±2.36)***	10.5 (±2.6)	8.5 (±2.5)	***-3.0 (±1.1)***	6.5 (±1.9)	7.3 (±2.1)	0.0 (±1.3)	8.6 (±2.2)	9.7 (±2.0)	1.1 (±1.7)
CES-D: Depression	7.0 (±1.8)	7.2 (±2.1)	0.2 (±1.8)	7.1 (±3.4)	5.5 (±2.4)	-1.6 (±1.1)	5.8 (±2.1)	3.2 (±1.7)	-2.6 (±1.5)	5.0 (±1.8)	5.4 (±2.5)	0.4 (±1.0)
STAI: State anxiety	27.5 (±2.0)	27.0 (±2.1)	-0.57 (±1.77)	31.5 (±3.6)	25.7 (±1.9)	-5.75 (±3.62)	26.7 (±2.1)	26.2 (±2.5)	-0.50 (±2.87)	26.4 (±3.1)	32.7 (±3.6)	6.33 (±4.08)
STAI: Trait anxiety	35.5 (±4.0)	31.1 (±2.3)	***-0.33 (±2.01)***	36.7 (±4.8)	26.3 (±2.2)	***-10.37 (±3.74)***	31.1 (±2.8)	32.6 (±4.1)	1.50 (±2.26)	33.0 (±4.1)	30.7 (±2.7)	-2.50 (±5.06)
QUALIOST												
Physical	22.7 (±0.3)	22.8 (±0.9)	0.14 (±0.6)	25.0 (±1.1)	24.3 (±1.2)	-0.62 (±1.17)	23.7 (±0.9)	24.1 (±1.2)	0.37 (±0.62)	24.1 (±1.6)	25.2 (±1.9)	1.11 (±0.7)
Psychological	37.1 (±0.8)	37.7 (±1.2)	***0.57 (±1.1)***	36.6 (±1.6)	36.3 (±1.3)	-0.25 (±1.01)	37.6 (±1.7)	35.3 (±0.6)	***-2.25 (±1.63)***	35.6 (±0.8)	36.2 (±1.5)	0.55 (±1.1)
Total/ Osteopenia	30.7 (±2.0)	31.1 (±2.7)	**0.42 (±1.27)**	34.2 (±3.7)	30.0 (±2.3)	**-4.25 (±1.7) ***	28.2 (±1.5)	30.3 (±3.1)	2.12 (±1.90)	31.8 (±2.6)	33.2 (±4.9)	1.33 (±3.19)

*n=8-9 per group and represented as mean ± SEM. QUALIOST= Quality of Life in Osteoporosis (osteopenia), STAI= state and trait anxiety inventory, PSS= perceived stress scale, CES-D= center for epidemiologic studies- depression. *p < 0.05 vs. placebo, and bold & italic = p < 0.1 vs. placebo.*

3.8. General well-being and compliance.

Based on diary data, there was no significant variation among groups in terms of total number of days participated in the study, compliance with drinking tea, the number of cups of tea consumed per day, sleeping duration, exercise intensity, or supplement intake as shown in Table 3.

Table 3. Treatment effects on general well-being and compliance from diary data.

	Placebo	***Tulsi***	***Rooibos***	***Oolong***
Days of participation	79.87 (± 23.19)	79.62 (± 21.85)	72 (± 23)	89.55 (± 5.19)
Compliance	96%	94%	98%	95%
Cups /Day	2.82 (± 0.18)	2.84 (± 0.23)	2.9 (± 0.16)	2.8 (± 0.28)
Sleep *duration (hs)/night*	7.02 (± 0.46)	6.84 (± 0.89)	6.53 (± 1.15)	7.0 (± 0.66)
Exercise intensity *(CDC score:0-3)*	0.94 (± 0.26)	0.78 (± 0.31)	1.12 (± 0.55)	1.15 (± 0.34)
Multivitamins/ OTC supplements	75%	100%	100%	88.89%
Calcium or *vitamin D or both*	75%	100%	100%	88.89%

*n=8-9 per group and represented as mean ± SEM. *p < 0.05 vs. placebo, and bold & italic = p < 0.1 vs. placebo.*

The daily diary logs contained an open-ended question asking the participant to write down anything they felt was noteworthy regarding overall health in general. These comments were then categorized into themes and given a rating (negative= -1, neutral = 0 or positive= +1). Diary comments were analyzed to assess if the consumption of the tea affected general well-being. As illustrated in Figure S4, an average of 30.57 comments were made per participant over the course of the study (46.13 comments in placebo, 21.76 comments in tulsi, 14 comments in rooibos, 24.143 comments in oolong). Although each tea group resulted in different proportions by type of question (i.e., aches and pains; mood; gastrointestinal (GI) symptoms; neutral; temporary illness; and sleep) (Figure S4A), no significant differences in the health ratings were observed between tea groups (Figure S4B).

3.9. Herbal teas effect on gut microbiome.

Several factors influence the efficiency of digestion of the three main macronutrients (carbohydrates, proteins, fats) of the human diet which, in turn, modulate the substrates available to the gut microbiota for consumption. Based on the data parameters received from UBiome, the abundance of serotonin-producing bacteria was found to be significantly decreased following rooibos consumption compared to placebo (coriander) (Table S1 and Figure 8D).

No other parameters were found to be statistically significant within and between groups although important qualitative observations were found as detailed below. Although not statistically significant, tulsi tea consumption compared to baseline decreased the abundance of all types of carbohydrate-, amino acids- and lipid-metabolizing bacteria as well as their metabolite-producing gut bacteria. Tulsi tea also decreased (not significantly) the abundance of vitamin-B9 producing microbes, and increased vitamin-K producing microbes (Table S1 and Figure S5, Figure S6, Figure S7 and Figure 8).

Consumption of rooibos tea increased (not significantly) the abundance of carbohydrate-, amino acids- and lipid- metabolizing microbes, but decreased the abundance of most of their

metabolite-producing microbes (i.e., butyrate, propionate, acetaldehyde, polyamine, serotonin, trimethylamine-TMA). Like tulsi, rooibos tea consumption decreased (not significantly) the abundance of vitamin-B9 producing microbes, but increased vitamin-K producing microbes. Unlike all other teas, rooibos increased (not significantly) the overall abundance of lactobacillus bacteria, which play a large role in digestion (66) as shown in Table S1 and Figure S5, Figure S6, Figure S7 and Figure 8.

For oolong tea, a reduction (not significantly) in the abundance of almost all groups and subgroups of the microbiome that were tested was observed, except for carbohydrate-, amino acids-, and lipid- metabolizing bacteria as listed in Table S1 and Figure S5, Figure S6, Figure S7 and Figure 8.

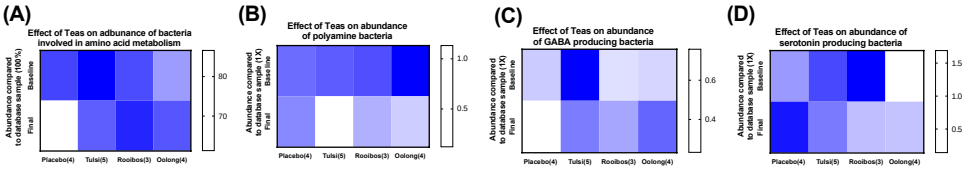

Fig. 8. Herbal teas effects on (A) amino acid metabolizing bacteria, (B) polyamine (decarboxylation product of basic amino acids (65)) producing bacteria, (C) GABA (products of arginine metabolism (65)) producing bacteria, and (D) serotonin (facilitated by tryptophan degradation (65)) producing bacteria.

The Y-axis of each graph represents the abundance (reported either in percentage or in number 1x) of these microbes, compared to uBiome database control. Each column represents mean value of each treatment group (placebo, tulsi, rooibos and oolong). Along with the treatment types the X-axis in the bracket also contain number of participants analyzed for each group. The intensity of the color in the heatmap indicates the abundance of the respective microbiome.

3.10. Tea effect on MSC differentiation into osteoblasts and cellular viability under normal and oxidative stress conditions.

Tulsi tea: During osteoblastogenesis, tulsi tea protected differentiating (Os+) osteoblasts against oxidative stress when given at days 10-11 (middle time point when stem cells are in the pre-osteoblastic stage) reflected by attenuation in both oxidative stress-mediated decreases in cellular viability (Figure 9A) and ALZ staining (Figure 9B) vs coriander controls. Three-way ANOVA analysis demonstrated that tulsi, through effects on cellular viability, prevented loss of Alizarin red (ALZ) staining when stem cells are differentiating into osteoblasts and exposed to oxidative stress given at days 10-11.

Rooibos tea: Although rooibos tea did not have any effect on cellular viability under both growth (basal) and osteogenic medium conditions (Figure 10A), it did increase ALZ staining when oxidative stress was given at middle (Days 10-11 when the stems cells were in the stage of pre-osteoblast) and end (Days 20-21 when the stems cells were in the stage of mature osteoblast) timepoints (Figure 10B) vs coriander controls. Three-way ANOVA analysis demonstrated that the protective action of rooibos observed in ALZ staining was dependent more on differentiation status rather than oxidative stress alone or combined.

Oolong tea: Oolong tea, like tulsi, prevented against loss of cell viability (Figure 11A) and ALZ staining (Figure 11B) induced by oxidative stress delivered at Days 10-11 (middle timepoint when stem cells are in the pre-osteoblastic stage) in hMSCs grown in growth (Os-) or osteogenic

(Os+) medium vs coriander controls. Three-way ANOVA analysis demonstrated that oolong protected cell viability under oxidative stress irrespective of time or media condition (Os- or Os+).

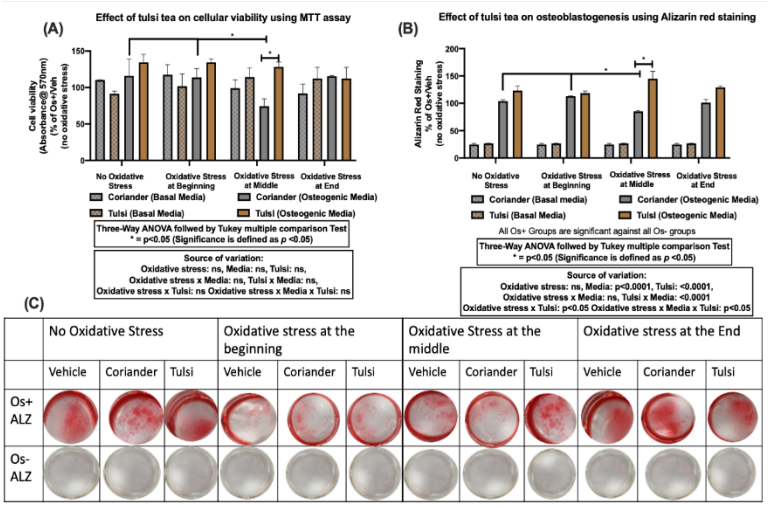

Fig. 9. Effect of tulsi tea on hMSCs.

MSCs were exposed to tulsi tea for 21 days and under normal and oxidative stress condition. Tulsi tea's effect on ***(A)*** *cellular viability by MTT assay,* ***(B)*** *osteoblast differentiation via alizarin red staining were assessed and compared to control (coriander),* ***(C)*** *well picture after alizarin red staining. Each bar represents the mean (± SEM) absorbance of MTT at 570 nm or related OD of alizarin red staining for respective group performed in triplicate. Both MTT and ALZ staining values from all groups were normalized by vehicle treated group at no oxidative stress (Os+/Veh_No oxidative stress) for respective assay. Data were analyzed using Three-way analysis of variance (ANOVA) followed by Tukey post hoc analysis (n=3 per group).*

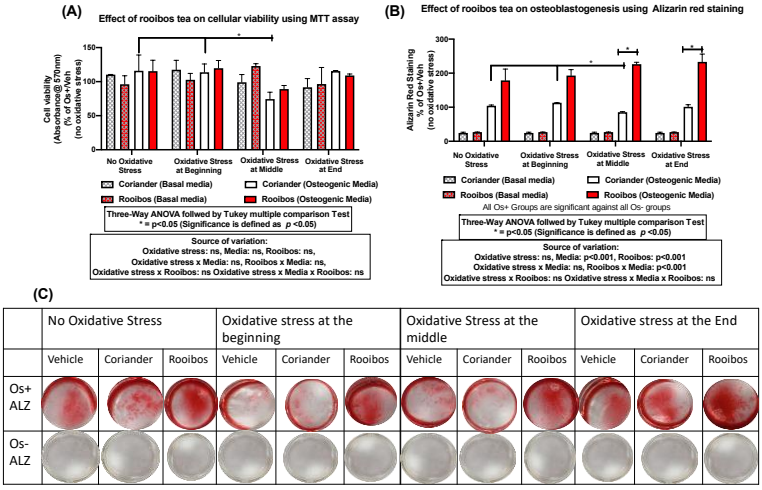

Fig. 10. Effect of rooibos tea on hMSCs.

MSCs were exposed to rooibos tea for 21 days and under normal and oxidative stress condition. Rooibos tea's effect on ***(A)*** *cellular viability by MTT assay,* ***(B)*** *osteoblast differentiation via alizarin red staining were assessed and compared to control (coriander),* ***(C)*** *well picture after alizarin red staining. Each bar represents the mean (± SEM) absorbance*

of MTT at 570 nm or related OD of alizarin red staining for respective group performed in triplicate. Both MTT and ALZ staining values from all groups were normalized by vehicle treated group at no oxidative stress (Os+/Veh_No oxidative stress) for respective assay. Data were analyzed using Three-way analysis of variance (ANOVA) followed by Tukey post hoc analysis (n=3 per group).

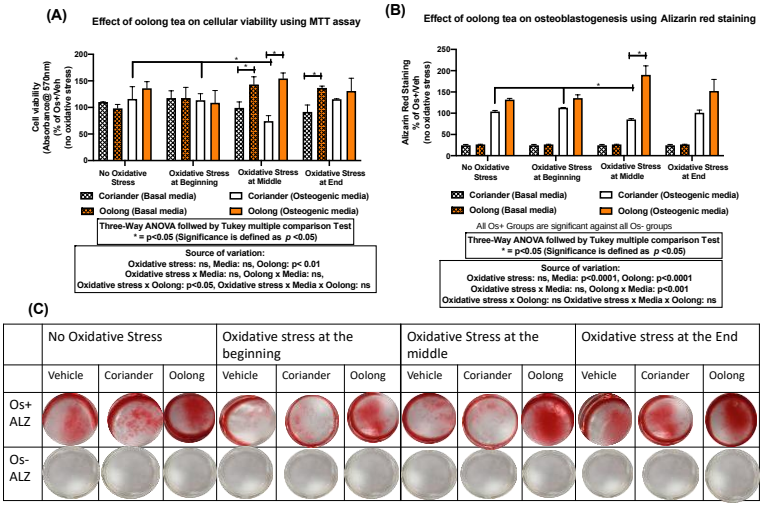

Fig. 11. Effect of oolong tea on hMSCs.

MSCs were exposed to oolong tea for 21 days and under normal and oxidative stress condition. Tulsi tea's effect on ***(A)*** *cellular viability by MTT assay,* ***(B)*** *osteoblast differentiation via alizarin red staining were assessed and compared to control (coriander),* ***(C)*** *well picture after alizarin red staining. Each bar represents the mean (± SEM) absorbance of MTT at 570 nm or related OD of alizarin red staining for respective group performed in triplicate. Both MTT and ALZ staining values from all groups were normalized by vehicle treated group at no oxidative stress (Os+/Veh_No oxidative stress) for respective assay. Data were analyzed using Three-way analysis of variance (ANOVA) followed by Tukey post hoc analysis (n=3 per group).*

3.11. Melatonin on MSC differentiation into osteoblasts and cellular viability under normal and oxidative stress conditions.

As a significant rooibos effect was observed on nocturnal melatonin levels in the urine and on serotonin-containing gut microbiota (precursors to melatonin synthesis) in the clinical component of the OsTea study, melatonin effects on MSC differentiation into osteoblasts under oxidative stress conditions was performed. Under normal conditions, melatonin increased cellular viability of MSCs (Figure 12A) and osteoblastogenesis (Figure 12B) using the MTT assay and ALZ staining, respectively. During osteoblastogenesis, melatonin protected differentiating (Os+) osteoblasts against oxidative stress when given at days 10-11 (middle time point when stem cells are in the pre-osteoblastic stage) reflected by attenuation in both oxidative stress-mediated decreases in cellular viability (Figure 12A) and ALZ staining (Figure 12B) vs vehicle controls. Three-way ANOVA analysis demonstrated that melatonin, through effects on cellular viability, prevented loss of Alizarin red (ALZ) staining when stem cells are differentiating into osteoblasts and exposed to oxidative stress given at days 10-11.

Melatonin mediated increases in osteoblastogenesis were inhibited in the presence of melatonin (MT1 and MT2) receptor antagonist, luzindole (Figure 12D) and MT2 melatonin receptor antagonist, 4-P-PDOT (Figure 12F) under normal (no oxidative stress) conditions or when oxidative stress was given at days 10-11. These changes in osteoblastogenesis mediated by luzindole and 4-P-PDOT were not accompanied by a loss in cellular viability (Figure 12C, 12E). No significant luzindole and 4-P-PDOT effects were observed on MSCs in basal media in MTT assay and ALZ staining (Figure S9).

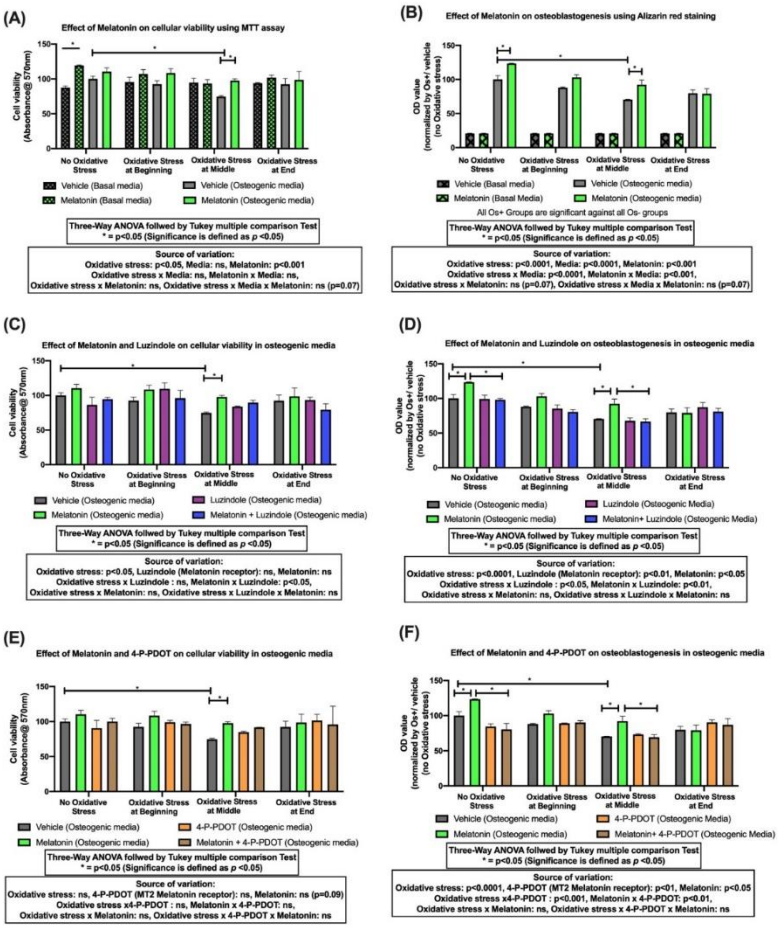

Fig. 12. Effect of Melatonin on hMSCs.

MSCs were exposed to melatonin in presence or absence of luzindole 4-P-PDOT for 21 days and under normal and oxidative stress condition. Melatonin's effect on ***(A)*** *cellular viability by MTT assay,* ***(B)*** *osteoblast differentiation via ALZ staining were assessed and compared to vehicle control. In osteogenic media* ***(C)*** *cellular viability by MTT assay in presence of luzindole,* ***(D)*** *osteoblast differentiation via alizarin red staining in presence of luzindole* ***(E)*** *cellular viability by MTT assay in presence of 4-P-PDOT,* ***(F)*** *osteoblast differentiation via alizarin red staining in presence of 4-P-PDOT were assessed. Each bar represents the mean (± SEM) absorbance of MTT at 570 nm or related OD of alizarin red staining for respective group performed in triplicate. Both MTT and ALZ staining values from all groups were normalized by vehicle treated group at no oxidative stress (Os+/Veh_No oxidative stress) for respective assay. Data were analyzed using Three-way analysis of variance (ANOVA) followed by Tukey post hoc analysis (n=3 per group).*

4. DISCUSSION

In the OsTea translational study, the effect of tulsi, rooibos, and oolong teas on bone health, quality of life, and osteoblastogenesis using human mesenchymal stem cells were investigated and compared against coriander tea, the placebo. Tea effects on bone markers, hormones involved in bone health and inflammation, and the microbiome were assessed along with the subjective markers related to quality of life. Overall, it was observed that out of the four groups, rooibos and tulsi demonstrated clinically significant effects on both objective and subjective markers of health even though all teas positively impacted human osteoblast differentiation from bone marrow-derived stem cells under oxidative stress—an *in vitro* condition that can easily translate to those same conditions clinically that can lead to osteopenia in humans.

Specifically, rooibos consumption significantly decreased CTX levels compared to placebo (coriander) consistent with past studies demonstrating a rooibos-mediated inhibitory action on osteoclast activity through attenuation of NF-κB activity (39-41). Osteoclastogenesis leads to large, multinucleated cells following binding of RANKL to RANK on preosteoclasts triggering the NF-κB signaling pathway (40) and so an inhibition of osteoclast activity through daily consumption of rooibos tea may offer a safe intervention to prevent bone loss (39-41). The osteoclast-inhibiting actions of rooibos tea may be attributed to aspalathin, one of its chemical constituents, which has demonstrated osteoclast-inhibiting activity *in vitro* (39). Although no significant changes were observed in P1NP levels or urinary bone turnover marker activity (i.e., P1NP: CTX ratio) in our OsTea cohort, rooibos significantly increased osteoblast-mediated calcium deposition under oxidative stress conditions that was not associated with increases in cell viability. This could be explained by *in vitro* studies demonstrating that rooibos flavonoids, orientin and luteolin, stimulate calcium deposition in human osteoblasts through the Wnt pathway (36-39).

In the OsTea study, those in the rooibos tea group also demonstrated trends towards an increase ($p=0.06$) in nocturnal melatonin levels compared to placebo (coriander). Although no studies were found demonstrating any interaction between rooibos and melatonin, some possible explanations could be due to a synergy between rooibos and melatonin on anti-inflammatory and antioxidant mechanisms. This is supported in the in vitro studies where rooibos and melatonin protected MSCs against oxidative stress through different mechanisms—melatonin was similar to tulsi and oolong where the greatest protection against oxidative stress occurred at days 10-11 of exposure while rooibos demonstrated protection when oxidative stress was given at days 10-11 and 20-21. The protection against oxidative stress-induced MSC loss by melatonin, tulsi and oolong occurred primarily through their actions on MSC viability. This is supported by the fact that MSCs grown under these conditions are in a highly proliferative state (63) and that the increases in alizarin red staining in the presence of melatonin, tulsi and oolong were accompanied by increases in cell viability.

For rooibos, its protective actions on osteoblast differentiation occurred when oxidative stress was given at days 10-11 and 20-21 and rooibos' effects were not accompanied by effects on MSC viability suggesting that it was working through signaling mechanisms involved in osteoblast differentiation and not viability. These unique actions of rooibos and melatonin may have provided enough protection on MSCs/osteoblasts to produce the clinical actions observed for rooibos on decreasing CTX levels since melatonin-mediated increases in osteoblastogenesis are associated with decreases in osteoclastogenesis (22). Rooibos tea, rich in polyphenols, possesses strong anti-inflammatory and antioxidant effects (33, 67). Although rooibos did not impact on cortisol or CRP

levels and no correlations were found between these markers of inflammation on any endpoints measured (Table S2), perhaps these properties of rooibos increased the synthesis and secretion of melatonin, which is also a strong free radical scavenger and inducer of antioxidant enzymes (68). Another connection between rooibos and melatonin was observed in the microbiome where rooibos decreased the abundance in serotonin-producing bacteria. Serotonin, a precursor to melatonin, is converted to melatonin through the actions of acetyl serotonin methyl transferase and hydroxy-indole-O-methyl transferase (69). Both enzymes are expressed in the gut (70) and gut melatonin levels surpasses blood levels by 10–100 times and pineal gland levels by 400 times (70). Perhaps, rooibos is impacting on gut and circulating melatonin levels through its actions on serotonin-producing bacteria as well as other gut microbiota (e.g., Lactobacillus, Bifidobacterium) through the flavonoids shown to be present in rooibos from other published reports (33, 71-73). Interestingly, in diabetic rats, melatonin demonstrated similar protective actions like rooibos where both exerted their protective actions by attenuating malondialdehyde (MDA) levels in a tissue-specific manner (74) and melatonin demonstrated a protective effect on cellular viability in gingival cells damaged by the cytotoxic effect of glutamate and DL-buthionine sulfoximine (75). Regarding rooibos's effects on subjective parameters, a trend ($p=0.09$) towards an improvement in emotional parameters in QUALIOST was observed, which appears to be the first study to report on rooibos tea's effect on quality-of-life status in a population with osteopenia.

Regarding the gut microbiome, confounders like microbial diversity, diet, and lifestyle (65), and the sampling process (72), made it difficult to draw concrete conclusions. However, it is important to point out that rooibos produced most of effects on microbiome. Specifically, rooibos tea consumption increased lactobacillus and GABA producing bacteria, both of which are found to increase GABA production in the gut (76). GABA-mediated stimulation of osteoblastogenesis has been demonstrated via upregulation of osteogenic gene expression and inhibition of inflammatory cytokines and reactive oxygen species (77). Vitamin K-producing bacteria also increased after rooibos tea consumption. Because consumption of dietary vitamin K has been associated with lower risk of hip fracture (78, 79), perhaps rooibos tea consumption over time may lower fracture risk. For serotonin producing bacteria, rooibos consumption was found to decrease their levels. The relationship between gut-derived serotonin and bone quality is not clear as some studies report a benefit while others negative actions on bone formation perhaps due to the targets that serotonin interacts with (80, 81).

Although no clinical effects of oolong were observed, oolong tea was found to protect hMSCs under oxidative stress conditions reflected by an increase in hMSC viability and osteoblast differentiation, like tulsi. Oolong tea contains several low molecular weight antioxidants, which may have scavenging activities to prevent DNA damage (82), which may have contributed to its protective effects on osteoblastogenesis under oxidative stress conditions.

For tulsi, consumption primarily affected subjective measures related to stress, anxiety, and bone health. Specifically, consumption of tulsi over 3 months significantly improved scores of PSS, STAI- trait anxiety and osteoporosis/osteopenia-related parameters in QUALIOST suggesting that tulsi tea reduced anxiety, stress and emotional parameters associated with having osteopenia. The psychotherapeutic properties of tulsi have been previously explored in preclinical and clinical models. *In vivo* experiments revealed its anti-anxiety and anti-depressant properties (83, 84), enhancement of cognitive function, and protection against aging-induced memory deficits (42). Furthermore, in humans, tulsi has been observed to reduce stress, anxiety, and depression in a controlled programmed trial (85). Another 6-week, randomized, double-blind, placebo-controlled study reported that tulsi significantly improved general stress scores, sexual and sleep

problems, and symptoms such as forgetfulness and exhaustion (86). Reducing mental stress and anxiety is important, as mental stress may compound the toxic effects of physical stress (i.e., physical restraint, physical exertion, exposure to cold and excessive noise) leading to even further stress and anxiety (42). This aspect of tulsi may also explain why a significant improvement in the QUALIOST as it relates to osteoporosis/osteopenia-related parameters occurred. Although, clinically, tulsi primarily impacted on subjective and not objective measures, tulsi did demonstrate direct protective actions on osteoblast differentiation and viability under oxidative stress conditions. The compounds (i.e., BSO, glutamic acid) used in the in vitro studies increase reactive oxygen species (ROS) by inhibiting intracellular GSH biosynthesis (87). Studies have shown that increases in ROS can lead to rapid increases in intracellular calcium followed by cell death (75, 87). Tulsi flavonoids, orientin and vicenin, have been shown to prevent bone marrow stem cell death in adult Swiss mice following radiation exposure (88). These findings may explain, in part, tulsi's protective effects on hMSCs viability only under oxidative stress conditions.

The OsTea study is the first translational and pilot study investigating the effect of 3 herbal teas (tulsi, rooibos and oolong) on both bone health and quality of life in a population with osteopenia coupled with *in vitro* assessments in human mesenchymal stem cells under oxidative stress conditions to mimic the *in vivo* condition. The blood pressure readings and diary data revealed no significant increases or differences in compliance, comments, and rating scales among the groups, indicating all teas were well-tolerated. Strengths of this study include a diverse population with respect to age, gender (1 male, 34 female) and ethnicity consuming tea in a natural setting; a broad range of endpoints—both objective measurements (bone marker levels, inflammatory markers, melatonin) and subjective measurements (psychometric, diary logs)—coupled with a translational component to provide potential yet important mechanistic data on the teas' actions on bone-forming osteoblasts.

Limitations of the study include using coriander as a placebo providing positive effects on anxiety and insomnia in some pre-clinical models (53), self-reported tea consumption, short duration, no BMD measurements, not controlling for diet, and a low "n" for the microbiome samples due to the company conducting the analyses going out of business. Future studies are needed to investigate whether similar effects of these teas are observed in a larger (n>20) and more diverse population and stratified by different age groups following long-term consumption and to ascertain tea effects on bone mineral density and fracture risk. Moreover, future mechanistic studies targeting osteoblasts, osteoclasts and melatonin are now warranted based on the novel findings of rooibos-mediated actions on CTX and melatonin levels in an osteopenic population; and rooibos-, tulsi- and oolong-mediated actions on osteoblast differentiation in vitro. Finally, identifying the phytochemicals within the teas responsible for these positive actions on bone cells opens new areas of research specifically in the development of novel osteopenia/osteoporosis therapies.

Overall, these findings provide both clinical and mechanistic support for the use of herbal teas, particularly rooibos and tulsi, for improving quality of life and bone health in a population with osteopenia. This study also demonstrated clinically significant and potentially unique interactions between rooibos and melatonin on oxidative stress conditions in the body. The OsTea—a translational and pilot RCT—determined that normal and regular consumption of tea, particularly rooibos and tulsi, over a 3-month period produced positive effects on both objective and subjective markers of bone health in a population with osteopenia. Considering that tea is relatively safe, it can be used in an aging population without untoward side effects. Moreover, this study

demonstrated that tea could be used to improve bone and mental health "early on" to prevent loss of bone later in life.

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AUTHORSHIP

Study design: FM and PWE; Study conduct: FM and PWE; Data collection: FM and PWE; Data analysis: FM, AL, HH, HE and PWE; Data interpretation: FM, AL and PWE; Drafting manuscript: FM and PWE; Revising manuscript content: FM and PWE; Approving final version of manuscript: FM, AL, HH, HE and PWE.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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