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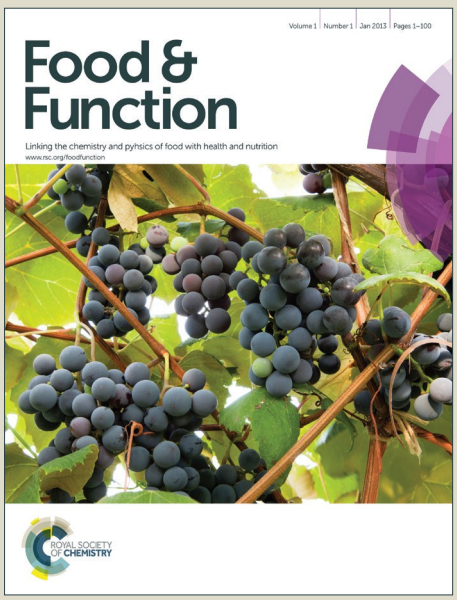

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ARTICLE

Comparison of black, green and rooibos tea on osteoblast activity

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Globally, tea is the second most consumed beverage after water. Habitual tea intake has been associated with higher bone mineral density, particularly in postmenopausal women. This association may be due to its polyphenols and resulting protective antioxidant effects. While in vivo studies have shown improved bone outcomes with consumption of individual purified tea polyphenols, it is unclear if a particular tea – due to its different profiles of polyphenols - is more beneficial than others. Therefore, we compared three different types of commercially available tea on osteoblasts: green, black and rooibos tea. Tea was normalized to 1 or 10 μg/mL gallic acid equivalents to assess differences in outcomes based on tea profiles rather than the quantity of polyphenol naturally present. The lower level of polyphenols (1 μg/mL gallic acid equivalents) – regardless of tea type and thus polyphenol profile - resulted in greater mineral content as well as cellular and alkaline phosphatase activity in Saos2 cells. Moreover, this was associated with higher markers of differentiation (osteopontin, sclerostin) and reduced cellular toxicity and pro-inflammatory markers (IL6, TNFα). Green, black and rooibos tea improved osteoblast activity at the low level and support epidemiological evidence suggesting tea consumption may benefit bone heath.

Introduction

Consuming a healthful diet in which recommended levels of macronutrients and micronutrients is achieved, supports bone health throughout the lifespan, and helps reduce an individuals risk of developing osteoporosis and experiencing a debilitating fragility fracture.[1,2,3] In addition to these essential nutrients, polyphenols such as flavonoids may also provide a dietary strategy that supports bone health, and potentially delay or prevent the onset of osteoporosis or work favourably with anti-osteoporosis medications. Polyphenols are present in a wide range of food and beverages with tea being a rich source. Tea flavonoids have a wide range of biological activities that include antioxidant mechanisms that may benefit bone health.[4,5,6]

Most studies in postmenopausal women have shown habitual tea consumption to be associated with higher BMD with equivocal data on the association with tea consumption and fracture (see review[6]). However, information is lacking on the association between a particular tea type and BMD or fragility fracture. Mechanisms are also not well understood. A study in a Scottish population identified a positive association between BMD and flavonoid intake, and tea consumption represented more than 50% of total flavonoid intake.[7] Unlike green and black tea that is produced from *Camellia sinensis*, rooibos is a herbal tea made from *Aspalanthus linearis* that has been cultivated and consumed for centuries in Africa. The difference in plant allows rooibos to have a very different polyphenol profile[8] in comparison to green and black tea (Figure 1), which may lead to differing effects on bone cell metabolism.

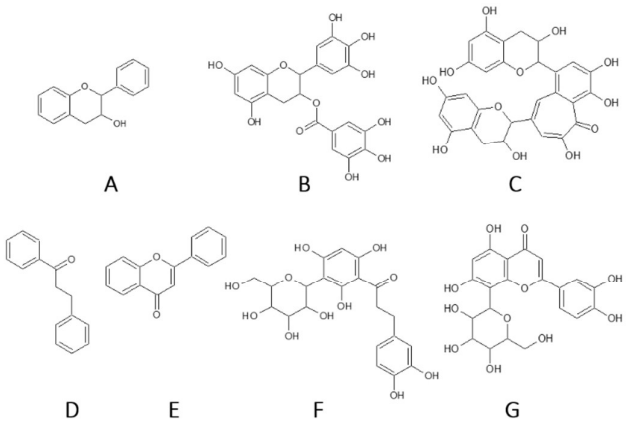

Figure 1. Common polyphenols in green, black and rooibos herbal tea. The structure of (a) the general flavon-3-ols backbone (b) EGCG from green tea, (c) theaflavin from black tea, (d) the general dihydrochalcone backbone, (e) the general flavone backbone, (f) aspalathin and (g) orientin from rooibos. While the majority of green tea catechins and black tea theaflavins stem from flavon-3-ols (a-c), rooibos contains a more diverse set of polyphenol structures such as dihydrochalcones and flavones (d-g).

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Polyphenols from green, black and rooibos herbal tea have been shown to promote bone development both *in vivo* and/or *in* vitro.[9-17] Consumption of purified green tea polyphenols improved bone microarchitecture[9] and improved antioxidant status[10] in ovariectomized (OVX) rats and attenuated inflammatory markers in a model of inflammation-mediated bone loss.[10] Also in the OVX rat model, consumption of a predominant polyphenol in green tea, epigallocatechin gallate (EGCG, Figure 1), improved both BMD and bone microarchitecture.[11] Consumption of black tea extract was also shown to improve BMD and preserve levels of calcium and phosphate in the bone of OVX rats[12] and reduce inflammatory markers such as TNFα.[13] Unlike green and black tea, there is currently no information regarding *in vivo* effects of rooibos on bone health.

Catechins make up the largest fraction of polyphenols in green tea, with EGCG and epigallo catechin (EGC) present in the largest concentrations of the individual phenolics.[14,15] *In vitro* studies have demonstrated that EGCG increases mineralization and markers of osteoblast activity (i.e. alkaline phosphatase) and differentiation.[16] While black tea makes up over 80% of world production [www.teausa.com], its effect on bone cell metabolism has not been studied. Theaflavins (Figure 1) and thearubigins dominate the black tea polyphenol profile, with low levels of catechins.[14,15] Recent studies have shown that two different flavonoids that are present in rooibos, hyperoside[17] and orientin[18], promote mineralization by osteoblasts without promoting cellular death (Figure 1).

The objective of this study was to compare and contrast the effect of commercially-available green tea (GT), two different black teas (English breakfast, EB and Golden Monkey, GM) and rooibos tea (RT) on human osteoblast activity as a function of their polyphenol profiles. Two types of black tea were chosen to assess differences in osteoblast outcomes based on quality. English Breakfast tea was chosen as it represents an inexpensive and commonly consumed black tea, while Golden Monkey black tea represents a more expensive, higher quality black tea.

Experimental

Saos2 cells were purchased from Cedarlane (Burlington, Ontario, supplier ATCC). HAM-F12, 10X Trypsin, PBS, fetal bovine serum (FBS), antibiotic-antimycotic and flasks were purchased from Lonza (Mississauga, Ontario). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Alfa Aesar (Reston, VA). The Cytotoxicity Detection Kit Plus (LDH) was purchased from Roche (Laval, Quebec). Dragonwell green tea, Golden Monkey (GM) black tea and English Breakfast (EB) black tea, and rooibos herbal (RT) were purchased from local tea shops. Human Milliplex bone magnetic bead panels for bone metabolism were purchased from EMD Millipore (Billerica, MA) and read by xPONENT software on Magpix Luminex (Austin, TX). Optical density (OD) measurements were analyzed using a BIO-TEK Synergy HT Multi-Detection Microplate Reader (Winooski, VT). Other chemicals, including Folin-Ciocalteu's reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma-Aldrich (Oakville, Ontario).

Cells were maintained and differentiated as previously described in which cells were maintained in 10% FBS supplemented HAM's F12 media with 1% antibiotic-antimycotic, 2.0 mM L-gluatmine, 28mM HEPES, 1 mM $CaCl_2$ (Media 1). Cells were passaged weekly, and the media replaced every other day.[18] Differentiation ensued using Media 2, which consisted of the addition of 10 nM dexamethasone and 50 µg/mL of ascorbic acid to Media 1, with the media replaced every other day. Following one week of differentiation in plates, Media 3 was used to initiate mineralization, which consisted of Media 2 in addition to 0.01 M β-glycerophosphate until day 15, as well as vehicle (distilled water, dH_2O) or aqueous tea.

For all experiments, a dH_2O control was used for comparison with cells treated with different teas. For each media change, a new batch of tea was brewed and its polyphenol content calculated. Tea was supplemented into media to create a final concentration of 1 or 10 µg/mL of gallic acid equivalence of polyphenols. Trypan blue staining was used for cell counting in all experiments. Plates or flasks used are listed under each experiment.

2.1 Polyphenol Extraction

Polyphenols were extracted from tea using dH_2O (denoted as aqueous tea) or 70% methanol.

Methanol Extraction[19]: 200 mg of tea was added to 5 mL of 70% methanol. The sample was mixed and heated to 70°C for 10 minutes. Upon cooling, the mixture was centrifuged at 200 x g for 10 minutes and filtered through 0.2 µM filter. The same tea was extracted a second time with another 5 mL of methanol for a total volume of 10 mL.

Aqueous tea extraction: To mimic how individuals brew tea, tea (200 mg) was also prepared using dH_2O, following the manufacturer's instructions for temperature and time (temperature and steep times were the following: GT: 79°C, 3 min; EB and GM: 96°C, 4 min; RT: 96°C, 5 min). Extractions occurred twice using 5 mL of dH_2O on the same 200 mg of tea and pooled together at the end for a total of 10 mL dH_2O to mimic extraction with methanol. Final solutions were also centrifuged at 200 x g and filtered using 0.2 µM filters to remove debris.

2.2 Total Polyphenol Content

Total polyphenol content was determined using Folin-Ciocalteu's method (ISO 14502-1) with gallic acid as the standard. Tea samples were diluted to 1:100 using dH_2O. 1 mL of the diluted tea was added to 5 mL of a 1:10 dilution of Folin-Ciocalteu's reagent in dH_2O. Following, 4 mL of 7.5% sodium carbonate solution was added and the mixture sat for 1 hour at room temperature. The OD of the mixture was measured at 765 nm.[19] Tea samples were added to cell culture to create a final concentration of either 1 or 10 µg/mL of GAE so that any observed effects could be attributed to the different polyphenol profiles among the tea types rather than the different levels of total polyphenol that naturally exist.

2.2 Cell activity

Cell activity was determined by examining mitochondrial reductase activity through its ability to reduce MTT. Cells were plated in 96 well plates at $1x10^4$ cells/well, and allowed to adhere over 24 hours. Media was replaced at 24 hours. MTT reduction was measured at 24 and 48 hours following the addition of the tea. Cells were washed three times with PBS and then supplemented with media without tea, with 5 mg/ml MTT for 4 hours. Media was removed and crystals were dissolved in 0.04 M HCl-isopropanol. OD was measured at 570 nm with a reference measurement at 610nm.

2.3 Cell Toxicity

Lactate dehydrogenase (LDH) activity was measured in cell media to quantify toxicity. Cells were plated following the MTT protocol. LDH was measured by an *in vitro* toxicology assay kit from Roche. Cells were plated, and media with tea was added following 24 hours. Media with tea was replaced after an additional 24 hours, and cells were harvested at either 24 or 48 hours to be analyzed by Tox7 kits. Control consisted of cells with dH_2O.

Cellular cytotoxicity was expressed as a percent using the following equation:

$$Cytotoxicity\ (\%) = \frac{(exp. - low\ control)}{(high\ control - low\ control)} * 100\% \quad [1]$$

The low control consisted of cells with dH_2O, while the high control represented cells completely lysed open for maximum LDH release.

2.4 ALP Activity

Cells were grown in 12 well plates at $1x10^4$ cells/well, with media replaced every other day. Tea addition started at day 8 with the use of Media 3. Cells were lysed after day 3 or 7 into mineralization by mechanically homogenizing cells using ice cold 0.05% Triton X-100 solution dissolved in 0.05 M Tris-HCl. Cells were scraped, and centrifuged at 10,000 rpm at 4°C for 10 minutes. Cell lysates were introduced to p-nitrophenyl phosphate. P-nitrophenol production was monitored over 5 minutes and was stopped by the addition of 0.5 M NaOH. The OD was measured at 410 nm and normalized to protein concentration using the Bradford assay.[20,21] Cells were tested on days 3 and 7 days after the addition of media 3.

2.5 Mineralization

Mineral content was quantitatively assessed using the alizarin red assay[22]. Cells plated at $5x10^3$ cells/well in 24 well plates. On Day 8, mineralization media with β-glycerophosphate and treatment (tea, using dH_2O or vehicle, only dH_2O) was added to promote mineralization. For controls, β-glycerophosphate and dH_2O was used to directly compare tea samples; cells with only β-glycerophosphate were used as a negative control. Media with tea was replaced every other day from day 8 to 15. On day 15, cells were fixed in 4% paraformaldehyde and kept at 4°C for 24 hours. Following, cells were washed with PBS and replaced with 0.04 M alizarin red S stain for 20 minutes on a plate shaker at room temperature. Cells were washed again. Extraction was accomplished with a 10% cetylpyridinium chloride solution for 1 hour at room temperature. Mineral was quantified by measuring OD at 550 nm. Mineral content of cells exposed to tea were directly compared to their plate control and represented as % control.

2.6 Antioxidant Activity

Antioxidant activity of aqueous tea samples was determined using DPPH radical scavenging.[23] Briefly, 50 µl of diluted tea that was normalized to 1 µg/ml, was added to 1.95 mL of 60 µM DPPH solution for 1 hour at room temperature, in the dark. Optical density was then measured at 517 nm and compared to a control of dH_2O. Antioxidant activity is represented as inhibition of DPPH radicals.

$$Antioxidant\ Activity\ (\%) = \frac{(control - sample)}{(control)} * 100\% \quad [2]$$

2.7 Osteoblast Regulatory Proteins

Tea polyphenols were normalized to 1 µg/mL GAE and added to media 3. Cells were plated at $5x10^5$ per 75 cm^2 flask and normalized to cell count using typan blue. Cells were harvested at days 3 and 7 after the addition of Media 3. Cells with tea were compared to cells with media 3 plus dH_2O. On days 3 and 7, media was removed from plates and centrifuged at 3000 x g for 15 minutes to remove remaining cells and equivalent volumes of supernatant was analyzed for inflammatory markers (TNFα, IL6) and bone markers (SOST, OPG, OPN) (Millipore, HBNMAG-51K).

2.8 Statistical Analysis

Statistical analysis was performed using a 1- or 2-way ANOVA using IBM SPSS Statistics 21. Dependent variables for 1-way ANOVA included antioxidant activity with an independent variable of tea type. Dependent variables for 2-way ANOVAs included total polyphenol content, mineral content, cellular activity, ALP activity, TNFα, IL6, SOST, OPG and OPN (with tea type and extraction method, tea type and dose, or tea type and time as independent variables). For all statistical analyses, homogeneity of variance was tested using Levene's t-test. If Levene's test failed ($p > 0.05$), Bonferroni t-tests were used as the post-HOC analysis; in the event Levene's test passed ($p < 0.05$), Games-Howell t-tests were used for post-HOC analysis. Outliers were determined using Grubb's test with a critical $\alpha = 0.05$.

Each individual sample (n) uses its own batch of polyphenols brewed from the tea leaves, therefore an $n = 3$ represents 3 separate experiments using 3 separate batches of polyphenols brewed for each tea type. Each single experiment is repeated in biological replicates, i.e. adding the same batch of polyphenols to multiple wells during an experiment and then reading the output measurement, such as absorbance, of each of those wells as duplicates or triplicates.

Results

3.1 Cellular Activity

Due to the lack of mineralization by higher (10 µg/mL GAE) doses of black teas (EB, GM), mineralization assays were followed up by cellular activity assessments after 24 and 48 hours of tea addition, to make sure the addition of the tea was not negatively affecting osteoblast growth. No interaction was found between tea type and time ($p > 0.05$) nor was there a main effect for time ($p > 0.05$). There was a main effect for tea type ($p < 0.001$, Figure 2).

Post Hoc analysis revealed that similar to the mineralization results, at 24 hours, both doses of GT ($p < 0.05$, $p < 0.05$) and RT ($p > 0.001$, $p = 0.003$) had greater levels of cellular activity than control, while only the low dose of EB ($p < 0.05$) had greater activity. Neither dose of GM was different from control. Tea that resulted in greater cellular activity compared to control was not different from each other.

Similar to 24 hours, at 48 hours, both levels of GT ($p = 0.001$, $p < 0.05$) and RT ($p < 0.05$, $p < 0.05$) were greater than control. However, the low dose of EB was at significance ($p = 0.05$), while its higher dose and both doses of GM were not different from control.

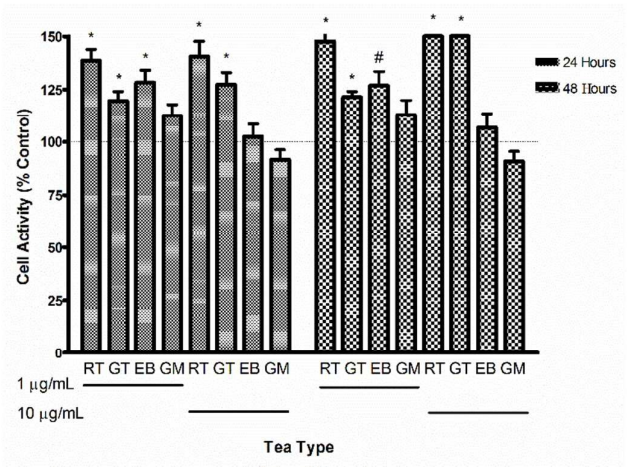

Figure 2. Cellular activity. Cells were exposed to 1 or 10 μg/mL GAE of tea, each day for 48 hours. Cellular activity was measured using the MTT assay and calculated as % relative to control. Bars indicate mean ± SEM, n = 12, with each sample representing an individual well and absorbance was determined in triplicate. * indicate significant differences from control (p < 0.05).; # indicates p = 0.05 compared to control.

3.2 Cellular Toxicity

Although measurements for cell activity were completed at 1 μg/mL GAE, the MTT assay is not an absolute marker of cell viability as it strictly measures mitochondrial reductase activity and does not measure cell death. Since lactate dehydrogenase (LDH) is an enzyme released into the media upon cell death, measurement of LDH activity was examined after 24 and 48 hours of tea addition as a measure of cell toxicity (Figure 3).

No interaction was found between tea type and time (p < 0.05). Main effects existed for both tea type (p < 0.001) and time (p < 0.001). Treatment with tea extracts demonstrated to be additive from Day 1 to Day 2.

At Day 1, all tea types, EB (p = 0.004), GT (p = 0.009), GM (p < 0.05) and RT (p < 0.05) were able to reduce LDH levels in comparison to control (dH_2O). This suggests the teas are providing a protective element to the health of the cell. Lower LDH activity indicates less cell death in comparison to the control. EB was more effective than GM (p < 0.05) and RT (p = 0.006) but not GT (p > 0.05). Therefore, at low levels (1 μg/mL) the inexpensive black tea (EB) and GT were able to exhibit positive effects quicker than the expensive black tea (GM) and RT by reducing toxicity in Saos2 cells.

By Day 2, EB (p = 0.002), GT (p < 0.05), GM (p < 0.05) but not RT (p = 0.06) reduced LDH levels in comparison to control. There were no differences across tea types.

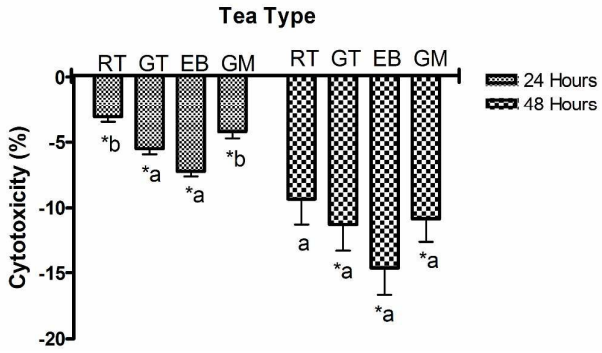

Figure 3. Cellular toxicity. Cells were exposed to 1 μg/mL GAE of tea, each day for 48 hours. Cellular toxicity was measured using LDH activity and calculated as % of cytotoxicity, with reference to control cells and total possible LDH release. Bars indicate mean ± SEM, n = 12, with each sample representing an individual well, with absorbance determined in triplicate. Bars with different letters represent significant differences between tea types (p < 0.05) within that time point. * indicates significant differences from control (p < 0.05).

3.3 Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured to determine osteoblast activity. An interaction was found between tea type and time (p < 0.001), as well as a main effect for tea type (p < 0.001) and time (p < 0.001). In general, greater ALP activity was seen at day 3 into mineralization, in comparison to control (Figure 4). At Day 3, RT (p = 0.003), GT (p = 0.002), EB (p = 0.005) and GM (p = 0.005) had greater ALP activity in comparison to control. EB, GT and GM all had greater ALP activity than RT (p < 0.05, p = 0.007 and p < 0.05, respectively).

At day 7, RT (p < 0.05), GT (p = 0.008), EB (p < 0.05) and GM (p = 0.003) maintained greater ALP activity in comparison to control. GT had greater ALP activity than GM (p < 0.05).

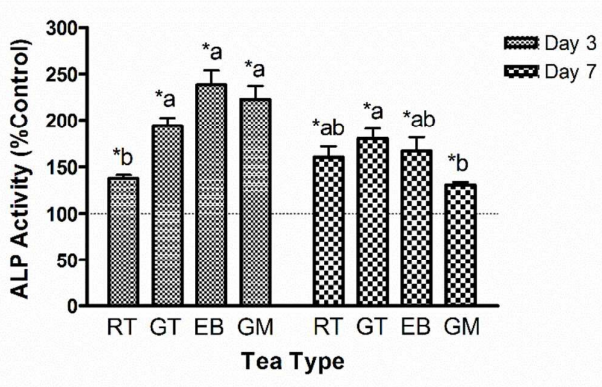

Figure 4. ALP activity. Cells were exposed to 1 μg/mL GAE of tea every other day for 3 or 7 days. ALP activity was determined through the conversion of p-nitrophenyl phosphate to p-nitrophenol over 5 minutes, and calculated as % relative to control. Bars indicate mean ± SEM, n = 5, with each sample representing 4 wells, with absorbance determined in duplicate. Bars with different letters represent significant differences between groups (p < 0.05) within that time point, asterisks indicate significant differences from control (p < 0.05).

3.4 Mineral Content

There was an interaction between dose and tea type ($p < 0.001$) as well as a main effect for dose ($p = 0.001$) in which lower doses of tea were more effective at inducing mineralization than the higher dose of tea. A main effect for tea type also exists ($p < 0.001$, Figure 5). Post Hoc analysis revealed that both doses of GT (1 µg/ml GAE and 10 µg/mL GAE, $p < 0.01$, $p < 0.01$) and RT (1 µg/ml GAE and 10µg/mL GAE, $p < 0.05$, $p < 0.01$) increased mineralization relative to control. However, only the low dose of both black teas, EB (1 µg/mL GAE, $p < 0.001$) and GM (1 µg/mL GAE, $p < 0.001$), increased mineralization relative to control. GT doses (1 versus 10 µg/mL GAE) were not different from each other or different from either dose of RT, or low dose (1 µg/mL GAE) of EB and GM. Low dose of (1 µg/mL GAE) GM produced more mineral than 1 µg/mL GAE of RT but not the 10 µg/mL GAE dose of RT. The negative control was similar ($p = 0.982$) to the dH2O control (data not shown).

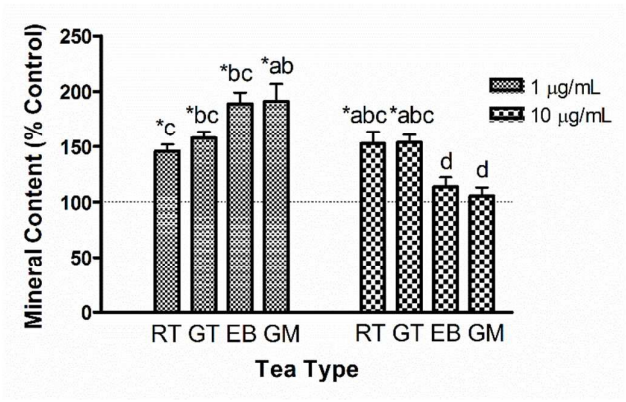

Figure 5. Mineral content. Cells were exposed to 1 or 10 µg/mL GAE of tea every other day for 7 days. Mineral was quantified using the alizarin red staining and calculated as % relative to control. Bars indicate mean ± SEM, $n = 5$, with each sample representing 4 individual wells with absorbance determined in triplicate. Bars with different letters represent significant differences between tea types ($p < 0.05$). * indicates significant differences from control ($p < 0.05$).

3.5 Total Polyphenol Content

There was an interaction ($p < 0.001$) between tea type and extraction method, as well as main effects for tea type ($p < 0.001$) and extraction method ($p < 0.001$). Extractions utilizing methanol produced more polyphenols than water (Figure 6).

Using 70% methanol or water for extraction, GT and EB have more polyphenols than GM ($p < 0.001$, $p < 0.001$) and RT ($p < 0.001$, $p < 0.001$).

On average, water extraction yielded approximately 50% of the polyphenols compared to methanol extraction. In order, from highest to lowest total polyphenol content, the tea ranked as follows: EB ~ GT > RT ~ GM. From here, total polyphenol profile was normalized to 1 or 10 µg/mL GAE to assess differences in osteoblast activity by tea addition based on polyphenol profile rather than by total polyphenol content.

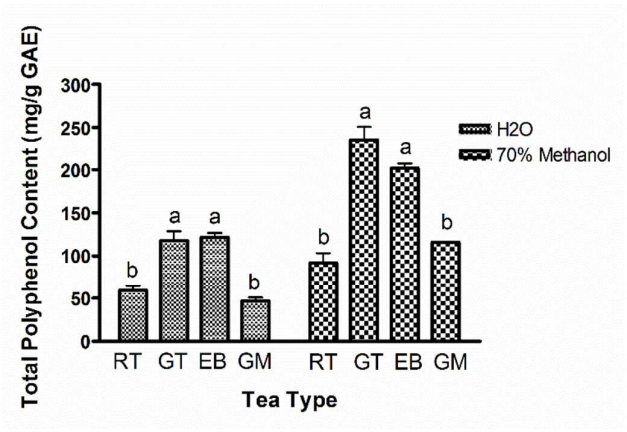

Figure 6. Total polyphenol content. Tea was prepared using either 70% methanol or dH_2O. Polyphenol content was determined using Folin-Ciocalteu's method with gallic acid as the standard. $n = 3$ for 70% methanol and $n = 6$ for dH_2O, with each sample representing an individual portion of tea tested three times for total polyphenol content with absorbance determined in triplicate. Bars with different letters represent significant differences among tea types for an extraction method ($p < 0.05$).

3.6 Antioxidant Activity

Due to the lack of consistent benefit seen at 10 µg/mL GAE tea, further studies proceeded with assessment of 1 µg/mL GAE of tea. With normalizing tea to 1 µg/mL GAE, antioxidant activity was examined by the ability of a specific tea to scavenge the DPPH radical over 1 hour (Figure 7). EB ($p < 0.001$), GT ($p < 0.001$), GM ($p < 0.001$) and RT ($p < 0.001$) had greater antioxidant activity in comparison to control (dH_2O). GT had greater antioxidant activity in comparison to EB ($p = 0.006$), GM ($p = 0.001$) and RT ($p = 0.008$). GM, EB and RT were not different from each other.

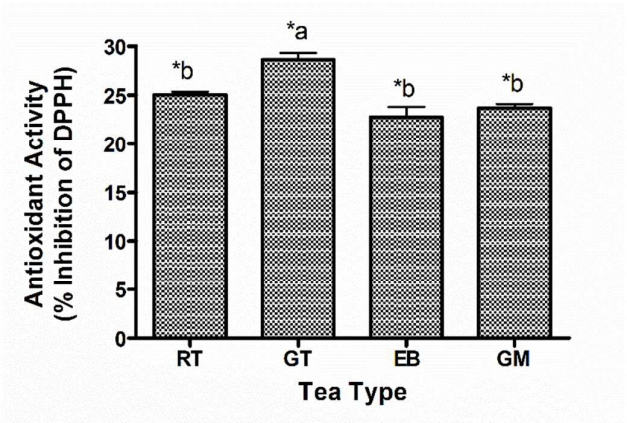

Figure 7. Antioxidant activity. Tea was produced using dH2O, and normalized to 1 µg/mL. Antioxidant activity was measured in terms of scavenging the DPPH free radical. Bars represent mean ± SEM, $n = 7$, with each sample representing an individual sample of tea, with absorbance determined in triplicate. Bars with different letters represent significant differences between tea types ($p < 0.05$). * indicates significant differences from control ($p < 0.05$).

3.7 Osteoblast Regulatory Proteins

Protein markers specific to osteoblast metabolism were assessed at days 3 and 7 after the addition of mineralization media. Multiple time points were chosen to assess how proteins secreted changes as mineralization progresses and in response to the addition of different tea that contained 1 μg/mL GAE.

3.7.1 Pro-inflammatory Markers: IL6 and TNFα

An interaction exists between tea type and time ($p < 0.001$), as well as main effects for tea type ($p < 0.001$) and time ($p < 0.001$) (Table 1). Day 7 levels of IL6 were higher than Day 3. Analysis of tea type at Day 3 into mineralization showed that RT ($p < 0.001$) and GM ($p < 0.05$) had lower IL6 levels than control. EB ($p > 0.05$) and GT ($p > 0.05$) were not different from control at Day 3. By Day 7, all tea types, EB ($p < 0.001$), GT ($p < 0.001$), GM ($p < 0.001$) and RT ($p = 0.001$), had lower levels of IL6 in comparison to control with no differences amongst tea types.

Assessment of TNFα revealed an interaction between tea type and time ($p < 0.001$), as well as main effects for tea type ($p < 0.001$) and time ($p < 0.001$) (Table 1). Similar to IL6, day 7 levels of TNFα were higher than Day 3. At Day 3, RT ($p < 0.05$) and GM ($p < 0.05$) had lower levels of TNFα than control. Levels of EB ($p = 0.054$) and GT ($p = 0.086$) did not reach significance. By Day 7, EB ($p < 0.001$), GT ($p < 0.001$), GM ($p < 0.001$) and RT ($p < 0.001$) had lower levels of TNFα in comparison to control. EB and GT had lower levels of TNFα in comparison to RT ($p = 0.002$, $p = 0.007$).

3.7.2 Osteoblast Regulatory Markers: OPN, OPG, SOST

Analysis of OPN indicated an interaction exists between tea type and time ($p < 0.001$), as well as main effects for tea type ($p < 0.001$) and time ($p < 0.001$) (Table 2). Levels of OPN were higher at Day 7 in comparison to Day 3. At Day 3, EB ($p = 0.001$), GT ($p < 0.001$) and GM ($p < 0.001$), but not RT ($p > 0.05$) had higher OPN levels than control. There were no differences amongst EB, GT and GM. At Day 7, EB ($p < 0.001$), GT ($p < 0.001$), GM ($p < 0.001$) and RT ($p = 0.003$) had higher levels of OPN in comparison to control. EB, GT and GM had greater levels of OPN than RT ($p = 0.003$, $p < 0.001$, $p < 0.001$). GM resulted in greater levels of OPN than GT ($p < 0.05$).

Analysis of OPG indicated an interaction exists between tea type and time ($p < 0.001$), as well as main effects for tea type ($p < 0.001$) and time ($p < 0.001$) (Table 2). Levels of OPG were greater at Day 3 in comparison to Day 7. At Day 3, EB ($p = 0.002$), GT ($p = 0.004$), and GM ($p = 0.002$) had OPG levels lower than control. RT ($p > 0.05$) did not reach significance. No differences were seen amongst the three tea types. By Day 7, EB ($p < 0.001$), GT ($p = 0.001$), and GM ($p = 0.001$) had OPG levels lower than control.

Analysis of SOST indicated an interaction exists between tea type and time ($p < 0.001$), as well as main effects for tea type ($p < 0.001$) and time ($p < 0.001$) (Table 2). Levels of SOST were overall greater at Day 3 in comparison to Day 7. Analysis of tea types at Day 3 indicate all tea types, EB ($p < 0.001$), GT ($p < 0.001$), GM ($p < 0.001$) and RT ($p = 0.005$) had higher levels of SOST in comparison to control. EB and GT had greater levels of SOST than GM ($p < 0.001$, $p < 0.001$, respectively) and RT ($p < 0.001$, $p < 0.001$, respectively). GM had higher levels of SOST than RT ($p < 0.001$). At Day 7, EB ($p < 0.001$), GT ($p < 0.001$) and GM ($p < 0.001$), but not RT ($p > 0.05$) had higher SOST levels than control. No differences were seen amongst tea types.

Discussion

This is the first study to directly compare and show that green, black and rooibos herbal tea all have beneficial effects in human osteoblasts. Greater mineral content with each tea type was accompanied by a higher cell and ALP activity, reduced cell toxicity as well as greater antioxidant activity – with GT having the highest level of antioxidant activity. Coinciding with the greater antioxidant activity, inflammatory markers were reduced over the longer term to a greater extent with addition of GT and EB. This suggests that GT and EB are more effective antioxidants over long durations, potentially through additive effects with repeated stimulation. OPN secretion was greater than control when GT, EB and GM were added to cells by day 3, whereas RT only reached significance at day 7. Initially, all tea types increased levels of SOST, but by day 7 RT demonstrated similar SOST levels to control. This suggests that while not to the extent of GT, EB or GM; RT is effective at stimulating mineralization for longer periods, while GT, EB and GM stimulate mineralization to a much greater degree in a short period and thus show signs of differentiation earlier on than RT. Lower levels of OPG were demonstrated in our previous study, as an artefact of the cell line[18], and this was further confirmed with the addition of tea.

In this study, we tested 1 and 10 μg/mL GAE of tea on mineralization in human osteoblasts. These doses were chosen to represent a level likely attainable in the diet (1 μg/mL GAE) and a level that could be present with supplementation (10 μg/mL GAE). With the higher dose of black tea (10 μg/mL) not providing any benefits to mineralization, potentially due to toxic pro-oxidant effects, the rest of the study focused on the use of tea diluted to a final concentration of 1 μg/mL GAE. Normalizing tea to 1 μg/mL GAE required a dilution of 10,000 that equated to a level of approximately 0.01 % of initial polyphenols produced. Previous work has shown consumption of 2-3 cups of green tea per day leads to 4.8-22 μg/mL, 11.7-43.9 μg/mL and 1.8-7.5 μg/mL of EGCG, EGC and EC, respectively, in saliva; while plasma levels reached 326 ng/mL, 550 ng/mL and 190 ng/mL for EGCG, EGC and EC.[24]

The addition of tea at low concentrations (1 μg/mL) demonstrated positive effects on osteoblast behavior. This was seen with increased levels of cellular activity and lower levels of cell toxicity, as measured by mitochondrial reductase activity and LDH release, respectively. This suggests that tea polyphenols are indeed protective at low concentrations, regardless of tea type. While the GT and RT also demonstrated positive effects on cellular activity at higher levels (10 μg/mL), neither black teas (GM or EB) promoted cellular activity at this higher dose. Because the amount of polyphenol was similar and only the polyphenol profiles differ between the tea types, we speculate that the polyphenols within the black teas (i.e. theaflavins) are acting, to a small extent, as pro-oxidants, and causing cellular stress. This is also evident as the higher levels of black tea do not promote mineralization relative to controls. While black tea is less studied, other work examining extreme polyphenol consumption in the form of green tea leaves or as a solution, resulted in significant increases of hydrogen peroxide within their saliva, a sign of oxidative stress.[25] Other research has shown that tea (green and black) or their individual catechins EGC and EGCG will promote oxidative stress when added to glucose-6-phosphate dehydrogenase deficient erythrocytes or when consumed by glucose-6-phosphate dehydrogenase deficient patients. The addition or consumption of the tea or its individual catechins was shown to reduce GSH levels and increase GSSG and

methemoglobin levels, suggesting a shift to greater oxidative stress on the system.[26] GT and black teas contain very similar polyphenol profiles, with GT having higher levels of catechins and black tea containing higher levels of theaflavins. Therefore future research should caution the use of high levels of polyphenol extracts for mouse and human studies.

Reducing inflammatory cytokines benefits bone health.[27] Several studies exist that examine the benefits of green tea polyphenols during oxidative stress conditions,[9] but the majority of these interventions involve tea polyphenols that were initially extracted with solvents such as alcohol and later re-dissolved in water. We show that on average, tea produced with water results in half the polyphenols in comparison to tea produced with 70% methanol tea, a typical extraction solvent. Therefore when extracts are used, they contain highly concentrated polyphenols and do not mimic tea consumption. Nonetheless, these studies provide information regarding potential interactions at the molecular level. For example, rats given 0.5% of purified green tea extract in which the polyphenols were re-dissolved in their drinking water, had less bone loss, and similar to our study, this was accompanied by lower pro-inflammatory cytokines TNFα.[9] Another study with 0.5% green tea polyphenols supplemented into the drinking water of OVX rats also demonstrated higher antioxidant levels and higher femur BMD than OVX models.[10] Similar to these studies, we show whole GT to have high antioxidant activity even when normalized to 1 µg/mL GAE. Furthermore, the addition of GT to osteoblasts resulted in higher mineral content with lower pro-inflammatory markers IL6 and TNFα. Decreases in inflammatory markers were also accompanied by lower toxicity and higher cellular activity. Previous work with purified GT polyphenols demonstrated that incubation in human osteoblast cell culture prior to the addition of H_2O_2 could prevent the production of reactive oxygen species and improve cell viability.[28] Anti-oxidant and anti-inflammatory effects were also demonstrated with both black tea and rooibos. Theaflavins found in black tea have previously been shown to have anti-inflammatory effects in murine macrophages through blocking the activation of NF-kβ.[29] While the addition of 2.5% aqueous black tea extract to ovariectomized rats also demonstrated significant decreases in oxidative stress, pro-inflammatory cytokines IL6 and TNFα and bone resorption.[13] Orientin and luteolin, two flavonoids found in rooibos were also shown previously to lower pro-inflammatory cytokines in osteoblasts.[18]

In addition to the anti-inflammatory effects of green, black and rooibos tea, there was higher levels of OPN and SOST secreted, osteoblast differentiation markers. OPN is an organic component integrated into bone matrix by osteoblasts to inhibit further nucleation of hydroxyapatite crystals.[30] OPN anchors osteoclasts to the surface of hydroxyapatite crystals and thus regulates bone turnover.[30] Knock out models of OPN have demonstrated a 30% reduction in fracture toughness[31] due to the inability to repair micro-cracks. OPN secretion is increased during osteoblast differentiation[32] and during bone formation under mechanical loading.[33] As OPN was remarkably higher in tea treated osteoblasts compared to control, this suggests that tea addition leads to increases in osteoblast mineralization and differentiation. Similar to our work, hyperoside, a unique flavonoid found in rooibos tea was shown to enhance differentiation in osteoblasts.[17] This was further confirmed by the greater levels of SOST, which is another marker of osteoblast differentiation. SOST is an inhibitor of the Wnt pathway and is released by osteocytes.[34] Loss of function mutations that occur in the gene SOST gene result in extreme levels of bone formation such as Van Buchem's disease.[35] However, over-expression of SOST results in low bone mass and a loss of bone strength.[34] Release of SOST is indicative of osteoblast maturation into osteocytes, which work to control bone turnover and microarchitecture.[36] RT stimulates greater mineralization than control, but less than that of GT, EB and GM, and therefore exhibits higher differentiation than control early on into mineralization but this effect does not persist with time. With RT, the OPN and SOST levels are also substantially lower than GT, EB and GM, indicating slower maturation of osteoblasts, but perhaps longer, sustained periods of mineralization.

Our results demonstrate that low levels of polyphenols, in their original food matrix, are beneficial to osteoblast activity. It is likely the combination as opposed to individual polyphenols that contributes to the overall benefit seen in osteoblasts, as previous work with individual polyphenols require much greater concentrations to identify positive outcomes[16,18]. Therefore it is likely a synergistic, or at the very least an additive effect that promotes greater mineralization. While these studies are the first to examine whole tea that is not concentrated, at such low doses, it is difficult to say whether these concentrations are achievable in humans. Bioavailability of compounds differ from one person to the next based on their metabolic activity, whether the tea is consumed alone or with other food, etc. Consumption of green tea (20mg/kg bodyweight in 200mL of water) in humans lead to peak plasma concentrations of 77.9 ng/mL, 223ng/mL, 124ng/mL, for EGCG, EGC and EC. This is the equivalent of consuming approximately 2 cups of tea, in which the EGCG, EGC, and EC equated to approximately 424 ng/mL.[37] This suggests our levels of 1 µg/mL or 1000 ng/mL, is very relevant to plasma levels, especially considering catechins represent approximately 30% of tea's dry weight.[38]

Conclusions

In conclusion, our results demonstrate that all three types of tea studied (green, black and rooibos) promote greater mineral formation in Saos2 cells at a low concentration. Higher levels of mineral were associated with greater ALP and cellular activity, and increased levels of differentiation markers, in addition to lower levels of cellular toxicity and pro-inflammatory markers. Future work should address osteoclast activity in the presence of tea. Promising data with individual tea polyphenols from both green (EGCG) and black tea (theaflavin-3,3'-digallate) added to rat osteoclast precursor cells shows reduced matrix metalloproteinase activity, and lower osteoclast differentiation and migration.[39] With whole tea containing multiple polyphenols, the addition of tea could potentially lead to additive or synergistic effects to further inhibit osteoclast activity and maturation. Interestingly, EB not only had higher total polyphenol content, but also had similar or greater activity measurements compared to GM, suggesting

that tea quality may not be directly linked to potential health benefits. This is another area for future study.

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Table 1. Pro-inflammatory markers. Saos2 cells were grown in the presence of tea at 1 µg/mL GAE or control, and assessed for their inflammatory markers at day 3 and day 7 using an ELISA-based multiplex kit. Data represents mean ± SEM, $n = 4$, with each sample representing 4 individual 75cm^2 flasks, read in triplicates. * indicates significant differences from control at the same day.

	Control		*RT*		*GT*		*EB*		*GM*		p-values		
	Day 3	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Tea Type**	**Time**	**Tea Type x Time**
IL6 (pg/mL)	2.82 ± 0.10	36.42 ± 1.03	1.69 ± 0.21*	24.93 ± 0.72*	2.35 ± 0.14	23.02 ± 0.72*	2.42 ± 0.08	22.97 ± 2.82*	2.14 ± 0.06*	21.52 ± 1.40*	$p < 0.001$	$p < 0.001$	$p < 0.001$
TNFα (pg/mL)	0.38 ± 0.03	2.91 ± 0.06	0.21 ± 0.01*	2.10 ± 0.09*	0.25 ± 002	1.71 ± 0.07*	0.22 ± 0.01	1.66 ± 0.05*	0.19 ± 0.01*	1.81 ± 0.04*	$p < 0.001$	$p < 0.001$	$p < 0.001$

Table 2. Osteoblast regulatory markers. Saos2 cells were grown in the presence of tea at 1 μg/mL GAE or control, and assessed for changes in OPN, OPG and SOST at days 3 and 7 into mineralization, using an ELISA-based multiplex kit. Data represents mean ± SEM, $n = 4$, with each sample representing 4 individual 75cm^2 flasks, read in triplicates. * indicates significant differences from control at the same day.

	Control		*RT*		*GT*		*EB*		*GM*		p-values		
	Day 3	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Tea Type**	**Time**	**Tea Type x Time**
OPN (pg/mL)	202.00 ± 2.86	12.15 ± 0.01	258.00 ± 66.46	258.00 ± 16.17*	583.25 ± 9.84*	909.00 ± 26.69*	697.50 ± 25.07*	972.75 ± 55.61*	648.25 ± 15.40*	1098.25 ± 36.34*	$p < 0.001$	$p < 0.001$	$p < 0.001$
OPG (pg/mL)	8120.25 ± 372.89	1920.50 ± 38.71	8297.25 ± 1226.57	1734.25 ± 75.81	3611.75 ± 57.65*	1511.50 ± 71.57*	3932.50 ± 194.44*	1499.75 ± 27.15*	3309.50 ± 120.67*	1506.00 ± 30.57*	$p < 0.001$	$p < 0.001$	$p < 0.001$
SOST (pg/mL)	749.50 ± 12.55	418.75 ± 10.47	992.67 ± 24.55*	495.50 ± 40.23	1952.75 ± 32.83*	938.00 ± 32.11*	1944.25 ± 56.98*	887.75 ± 23.00*	1641.25 ± 33.06*	903.50 ± 33.60*	$p < 0.001$	$p < 0.001$	$p < 0.001$