The TrkB agonist, 7,8-dihydroxyflavone, impairs fracture healing in mice

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Introduction

Fractures are an extremely common injury of the skeletal system, with the residual lifetime risk of a minimal trauma fracture approximately 44% for women and 25% for men over the age of 60 in Australia¹. To date, there are very few effective non-surgical treatments to aid in the healing of fractures, and to prevent malunion and non-union of fractures, a complication that affects approximately 5-10% of patients worldwide²,³. Two non-surgical approaches that are being developed to clinically enhance fracture healing are biophysical enhancement e.g. electromagnetic field stimulation, and low-intensity pulsed ultrasonography⁴-⁷, and biological enhancement e.g. therapeutic use of vascular and osteogenic growth factors, stem-cells, and morphogenic molecules to aid bone regeneration⁸,⁹. Identifying novel molecules that promote some of the key biological events of fracture healing, including angiogenesis and innervation, required for proper fracture healing, appear to have the greatest potential to improve bony repair.

Circulating neurotrophic factors are a branch of osteogenic stimulating peptides that have such potential. Neurotrophins are up-regulated in a variety of repairing tissues with evidence to suggest that they have important roles during angiogenesis⁹-¹² and inflammation¹³-¹⁶, which are two key processes in fracture healing¹⁷. Specifically, the neurotrophin, nerve growth factor (NGF) and signalling via its receptor, TrkA, have been shown to stimulate osteoblastic mineralization and improve the mechanical properties of healing bone fractures¹⁸-²⁰. Another neurotrophic factor that has recently been shown to have a role in fracture healing is brain derived neurotrophic factor (BDNF). Since its discovery
in 1982, BDNF has been established as an important modulator of synaptic plasticity and pruning in the central nervous system (CNS) via two pathways; high affinity TrkB (pro-survival) and low affinity p75NTR (pro-apoptotic)31,24. Several studies in rodents and humans have identified high levels of BDNF and its receptor, TrkB, in and around the site of fracture healing25-27. Gene and protein expression of BDNF and TrkB were markedly elevated in chondrocytes and active osteoblasts in proliferating and mature zones of the endochondral ossification front in 7-week old rats38. Furthermore, BDNF gene expression was elevated in callus tissue 28 days post-osteotomy in rats, which is the transition period of cartilaginous callus being replaced by woven bone, as well as remodelling of woven bone to lamellar bone29,30. Location of BDNF and TrkB in the inflammatory phase of fracture healing and soft callus stages where endochondral ossification begins, may indicate that BDNF has an important, early role in bone healing via endochondral ossification and intramembranous ossification.

One of the important processes that drives the transition of cartilage to woven bone, and remodelling of callus is re-establishment of blood vessels. BDNF has been shown both in vitro and in vivo to stimulate endothelial cell migration, proliferation and formation of new blood vessels6,10,25,31 largely by increasing the production of vascular endothelial growth factor (VEGF)32. Another mechanism through which BDNF may also promote fracture healing is via the up-regulation of ossification proteins ALP and BMP-2 in osteoblasts, with BDNF treatment found to upregulate both ALP and BMP-2 in cementoblasts, a tooth root enamel mineralizing cell type similar to osteoblasts33. In vitro, BDNF has been identified in osteoblastic MC3T3-E1 cells24 however, an osteoblastic response to BDNF treatment has yet to be described in the literature. Taken together, these findings suggest that BDNF may have an active role in fracture callus and healing, possibly by mediating angiogenesis and promoting bone formation, however, there are no studies investigating topical or systemic administration of BDNF, or activation of its receptor TrkB during fracture healing. Since BDNF can stimulate both pro-survival and pro-apoptotic pathways, it is not an appropriate choice of molecule to investigate the sole signalling pathway of BDNF and TrkB. Small novel molecules have been identified to target and potently activate only TrkB including 7,8-dihydroxyflavone (7,8-DHF)35.

7,8-DHF is a flavonoid derivative with antioxidant and anti-inflammatory effects35-39 and has been shown to potently activate the TrkB receptor35,36,40. Doses of 5 mg/kg/day of 7,8-DHF strongly activated TrkB receptors in BDNF knockout mice35. Additionally, 7,8-DHF reduced neuronal apoptosis and inflammation in rodent models subjected to traumatic brain injury (TBI) at a dose of 5 mg/kg/day35,38,40,41. 7,8-DHF is well tolerated in rodent models of TBI36,38, depression37. Alzheimer’s disease42,43, aging44 and stress45, with no detrimental effects reported in mice, however, to our knowledge, there has been no data describing the effects of 7,8-DHF on the skeletal system. Therefore, on assessing all the previous literature, it is hypothesized that 7,8-DHF treatment should have a positive effect on fracture healing. In the current experiments, effects of 7,8-DHF on the structural and biomechanical features of healing fractures in mice, murine bone growth, and osteoblastic differentiation and mineralization in vitro were investigated.

Methods

Animals

Thirty C57BL/6 male mice were supplied by the Australian Animal Resource Centre (ARC, Western Australia). Mice were 12 weeks of age at the time of experimentation and were housed individually during the experiment under a 12 h light/dark cycle with access to water and food ad libitum. All experimental procedures were approved by the La Trobe Animal Ethics Committee (AEC 17-05), were within the guidelines of the Australian code of practice for the care and use of animals for scientific purposes by the Australian National Health and Medical Research Council, and in compliance with the ARRIVE guidelines for how to report animal experiments.

Experimental groups

All mice received unilateral tibial fractures. To assess the effects of TrkB agonist, 7,8-DHF on fracture healing, mice were randomly allocated to receive either vehicle (30% Kolliphor® HS 15 in 0.1M sodium phosphate buffer; n=15) or 7,8-DHF in vehicle (n=15). Kolliphor® HS 15 was used as vehicle due to the poor water solubility of 7,8-DHF. A microemulsion of 7,8-DHF was used made in 30% Kolliphor® HS 15 in 0.1M sodium phosphate buffer. Kolliphor® HS 15 is white, odourless emulsifying agent commonly used in human and veterinary injection formulations46-50. Intraperitoneal injections of 7,8-DHF (5 mg/kg/day) or vehicle were given daily for 14 days post-injury. Previous research has shown this regimen of 7,8-DHF was effective at activating TrkB receptors centrally and peripherally36,39,41-43. All mice were euthanised via carbon dioxide asphyxiation at 28 days post-fracture. Nine mice were excluded from analysis; 7 due to tibial/fibular fusions. 2 due to already broken at analysis.

Closed tibial fracture model

Tibial fractures were performed using previously described standard protocols31-35. The fracture model is a closed, internally fixated fracture of the right tibial mid-shaft. In brief, under isoflurane anaesthesia, a 5 mm incision was made in the skin superficial to the anteromedial tibial surface, distal to the knee joint. Periosteal tissue was removed and an entry point into the tibial shaft was made using a 26-gauge hypodermic needle. An intramedullary rod was inserted down into the medullary canal of the tibia, and a mid-tibial fracture was made using a pair of modified skin staple removers. An X-ray of the tibia was performed to confirm fracture using DEXCOWIN® portable X-ray device (DEXCOWIN Co., Ltd. Pasadena, CA, USA). The intramedullary rod was removed.
and replaced with a larger stabilising rod, and another X-ray was performed to confirm rod and fracture position. Incisions were closed using 5-0 synthetic surgical suture. Mice were given 5 mg/kg of Carprofen (RIMADYL®; Zoetis, Parsippany, NJ) and Buprenorphine (0.1 mg/kg) for pain relief.

Micro-computed tomography (µCT)

µCT was performed on fractured tibial calluses. Fractured tibiae were immersed in fixative (4% paraformaldehyde in 0.1M sodium cacodylate buffer) for 48 h then stored in 10% sucrose in 0.1M sodium cacodylate buffer at 4°C until use. Scanning of tibiae was performed using SKYSCAN 1076 in vivo X-ray micro-computed tomography (Bruker-microCT) in 70% ethanol with acquisition parameters of 9 μm voxel resolution, 0.5 mm aluminium filter, 48 kV voltage, 100 μA current, 2,400 ms exposure, rotation 0.5° across 180°, frame averaging of 1. Images were reconstructed using NRecon (V1.6.3.1) with the following parameters: smoothing factor, 1; ring artefacts, 6; beam-hardening corrections, 35%; pixel defect mask, 5%; C.S rotation, 0; and misalignment compensation, <3. Images were realigned and orientated using Dataviewer (V1.4.4) to obtain transaxial datasets for calluses. Analysis of the transaxial datasets was performed using CTAn (V1.11.8.0) and the region of interest (ROI) was identified as 300 slices (i.e. 150 slices proximal and distal to the fracture line of the callus); the border of the callus was manually traced. Thresholds used for parameter quantification were determined using the automatic “otsu” algorithm within CTAn and visual examination of un reconstructed X-ray images. A grayscale adaptive threshold of 41-255 was used for structural analysis of calluses 28-days post-fracture. 2D and 3D data, and 3D models were generated and the following parameters were used for structural analysis of callus: total callus volume (TV); new mineralized bone tissue volume (BV), bone fractional volume (BV/TV), mean polar moment of inertia (MMI), bone surface (BS) and mean cross sectional area (T.Ar).

Biomechanical assessment of fracture calluses

Following µCT analysis, a three-point mechanical bending test was performed on calluses 28-day post-fracture to assess the potential effect of 7,8-DHF treatment of the biomechanical properties of bone. Samples were equilibrated at room temperature on the day of assessment. Each tibia was mounted onto an 8 mm stabilising platform in a mediolateral position. A 200 N force transducer descended at a constant rate of 1.67 mm/sec and loaded each callus centrally. A load-displacement (x-y) graph was plotted and force (g) and deflection (mm) values were recorded. Biomechanically disrupted callus ends were imprinted onto dental wax. Magnified imprints were imaged using Leica DFC420 light microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland) connected to Leica IM50 imaging software (Leica). Cross sectional areas measured using Leica Qwin V3 Standard software (Leica). Differences in peak force to failure, load per unit area, stiffness and stiffness per unit area were calculated from the deflection data.

Cell culture

Cell culture was used to investigate the effects of 7,8-DHF on osteoblastic cell line, Kusa4b10. Kusa4b10 cells are a genetic sub-clone of multi-potential bone marrow stromal cells, Kusa O56, and have demonstrated a more osteoblastic phenotype than Kusa O cells, thus more suitable for investigations on osteoblastic differentiation56,57. Cells were cultured in α-MEM (Gibco® Life Technologies™, Auckland, NZ), supplemented with 10% Australian Premium Foetal Bovine Serum (FBS) (Australian Ethical Biologicals Pty. Ltd., Coburg, AU) and used between passages 10-16. All cultures were maintained in an incubator at 37°C in 5% CO2 and 95% O2. For these experiments, in order to gain an insight into the role BDNF has on fracture healing the influence of 7,8-DHF on osteoblastic marker expression, as well as mineralization was investigated. For studies that required Kusa4b10 cell differentiation, including RT-PCR and mineralisation studies, cells were sub-cultured at a density of 3000 cells/ml in α-MEM + 10% FBS for 3 days, after which medium was aspirated and cells were cultured in osteoblastic differentiation medium, containing α-MEM + 10% FBS supplemented with 50 μg/ml ascorbate and 10mM β-glycerophosphate48,56,57. Medium was replenished three times a week.

Real-time Polymerase Chain Reaction (RT-PCR)

Kusa4b10 cells were cultured in osteoblastic differentiation medium and treated with 50nM of 7,8-DHF (based on pilot research) or vehicle. The medium was replaced three times per week. Cells were isolated at three different timepoints; 3-, 7-, and 14-days. These three timepoints were chosen because they reflect three stages of Kusa4b10 cell differentiation, are similar timepoints used in the current studies investigating Kusa4b1056,59. Total RNA was prepared using PureZOL™ (Bio-Rad Laboratories Inc., Hercules, USA). RT-PCR was performed as previously described48,49. Briefly, reverse transcription was performed from 1 μg of total RNA

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Table 1. Oligonucleotide name and sequence (5’3’) used in Real-Time PCR.

| Oligonucleotide name | Sequence (5’3’) |
|----------------------|----------------|
| mGAPDH               | Sense - AATCTCCACTTTGCACTG |
| mRunx2               | Anti-sense - CCTCGTCCTGAGACADAAA |
| mAlkaline phospatase  | Sense – AAACCCAGACACAGATTC |
|                      | Anti-sense - TCCACGGCAGAGCC |

PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
using iScript™ cDNA Synthesis Kit (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control gene. RT-PCR was performed in triplicate using SsoFast™ EvaGreen® Supermix (Bio-Rad) and specific oligonucleotide primers (Table 1) on an IQ 96-well PCR system (Bio-Rad). Each amplification reaction contained 1 μl of cDNA and 300 nM of primer. Thermal cycling conditions included initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 55°C for 5 s. Melt-curve analysis was performed post-cycling to confirm specificity of the amplified products. Relative quantification of genes of interest mRNA expression normalised to the house-keeping gene and was determined using the $2^{\Delta\Delta C_t}$ method. Specific oligonucleotide primers (Table 1) were Runx2; a marker of

Figure 1. Effects of 7,8-DHF treatment on 28-day callus structural parameters using μCT. Longitudinal mid-point images representative of 300 slice reconstructed hemi-callus (a-b). 7,8-DHF treatment decreased total callus volume (TV, c; *$p=0.042$), mean polar moment of inertia (MMI, e; **$p=0.004$) and mean cross-sectional area of callus (CSA, f; *$p=0.047$) at 28 days post-fracture compared to vehicle treatment. 7,8-DHF: 7,8-dihydroxyflavone. BV/TV: bone fractional volume. Bars are mean ± SEM, n=9-12/group.
early osteoblasts⁶⁰, and alkaline phosphatase; a marker of mature osteoblasts⁵⁶.

**Mineralization Analysis**

For investigating the effects 7,8-DHF treatment has on osteoblastic cell mineralization, Kusa4b10 cells were cultured in osteoblastic differentiating medium treated with 0-, 10-, 50-, and 100nM of 7,8-DHF for 21 days (n=4/group). After 21 days, cells were stained with Alizarin red and inspected for mineralization nodules within the Kusa4b10 colonies. Kusa4b10 cells were washed three times in PBS, fixed in ice cold 70% ETOH for 30 min, and then stained with 0.5% Alizarin Red stain (pH 4.2) for 30 min. Cells were then washed five times in PBS and scanned images were taken. Mineralized areas were quantified using ImageJ software (National Institutes of Health, Bethesda, USA).

**Statistical analysis**

*In vitro* gene expression was analysed via two-way ANOVA, with Tukey’s multiple comparisons conducted where appropriate. All other outcomes were analysed with Mann-Whitney U tests. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., USA), with significance defined as $p<0.05$.

**Results**

7,8-DHF reduces callus size

Representative μCT reconstruction of longitudinal mid-point hemi-calluses are shown in Figure 1a-b. Bony union was reached in all calluses by 28 days post-fracture in both the 7,8-DHF-treated and control groups. Analysis revealed the 7,8-DHF-treated group had a significant reduction in total tissue volume (Figure 1c; $p=0.042$), no change in fractional bone volume (Figure 1d; $p=0.088$), decreased mean polar moment of inertia (Figure 1e; $p=0.004$), and reduced mean cross-sectional area (Figure 1f; $p=0.042$) of callus compared to controls.

7,8-DHF reduces peak force to failure in fracture calluses

A three-point bending test was used to assess the biomechanical properties of 28-day tibial calluses. As seen in Table 2, calluses of mice treated with 7,8-DHF following fracture had a significantly decreased peak force to failure ($p=0.011$) compared to controls. Conversely, stiffness was increased in calluses of mice treated with 7,8-DHF when compared to vehicle-treated mice ($p=0.012$). There was no significant difference in cross-sectional area, bending stress, or Young’s modulus between groups (Table 2).
7,8-DHF does not alter expression of osteoblastic markers in Kusa4b10 cells

Messenger RNA levels of osteoblastic markers Runx2 and alkaline phosphatase were quantified by RT-PCR in Kusa4b10 cells at 3-, 7-, and 14-days of differentiation. For Runx2 mRNA expression (Figure 2a), there was no main effect of treatment (F(1,29) = 0.613, p = 0.440), no main effect of time (F(3,29) = 1.603, p = 0.219), and no interaction of treatment and time (F(3,87) = 0.020, p = 0.981). For ALP mRNA expression (Figure 2b), there was no main effect of treatment (F(1,28) = 0.371, p = 0.547), a main effect of time (F(2,28) = 3.729, p = 0.037), and no interaction of treatment and time (F(2,84) = 0.112, p = 0.895). Post-hoc analysis revealed that ALP mRNA expression was increased in cells cultured for 14-days when compared cells cultured for 3-days (p = 0.041).

7,8-DHF does not influence mineralization in Kusa4b10 cells

Mineralization nodules were detected at 21 days in Kusa4b10 cells at all concentrations (0, 10, 50, 100 nm) of 7,8-DHF treatment (Figures 2c-d). There were no apparent changes in the number of nodules or mineralization between the treatment groups or controls, and there was no mineralization evident in the negative control cultures of undifferentiated Kusa4b10s.

Discussion

Although BDNF and its TrkB receptors have been localized in healing fractured of mice, rats, and humans, the role of TrkB signalling in fracture healing is not well understood. Here it was hypothesised that TrkB agonism using the flavonoid, 7,8-DHF, would have positive effects on fracture healing, as measured by structural and biomechanical analysis. To the contrary, it was found that systemic administration of 5 mg/kg/day of 7,8-DHF for 14 days in mice with healing tibial fracture, resulted in mechanically weaker calluses with reduced bone and tissue volume. Furthermore, in vitro experiments revealed no effect of 7,8-DHF on osteoblastic differentiation and mineralization. Together, these data show that fracture healing was impaired by the 7,8-DHF treatment paradigm used in this study, and that TrkB activation may not directly increase osteoblastic bone formation.

\[ \mu CT \] was used to investigate the influence of 7,8-DHF on the bone and callus content at 28 days post-fracture. Calluses of 7,8-DHF-treated animals were smaller than that of vehicle-treated animals, with reduced tissue volume and mean cross-sectional area. Additionally, \[ \mu CT \] analyses revealed that 7,8-DHF treatment decreased the theoretical mean polar moment of inertia: a measurement of inherent rotational stiffness of bone\(^{61}\) compared to controls. This finding was supported by three-point bending analysis, with peak-force to failure significantly reduced in the 7,8-DHF-treated mice compared to controls. Considered together, these findings indicate that 7,8-DHF negatively impacted healing of fractures, forming fractures that were both smaller in size and mechanically weaker.

Interestingly, the present findings are dissimilar to a study that investigated BDNF-functionalized cement treatment during femoral fracture healing in mice, which found BDNF treatment was beneficial to fracture healing\(^{62}\). Although \[ \mu CT \] bone formation was not directly assessed in the current study, that calluses were smaller and mechanically inferior likely indicates reduced callus bone formation in the 7,8-DHF-treated mice compared to controls. Considered together, these contrasting findings may be due to differences in the biodistribution of the two treatments used in the studies, with 7,8-DHF previously shown to readily cross the blood brain barrier (BBB)\(^{36,42,45}\), whereas exogenously applied BDNF is restricted to the peripheral circulation\(^{64}\). Previous experiments have shown peripheral BDNF has positive effects on bone\(^{28,30,33}\), and central depletion of BDNF increases bone mineral density\(^{65}\), highlighting the likely possibility BDNF has opposite, central and peripheral effects on bone, which are similar to those described for the hormone leptin\(^{66,67}\).

Therefore, it is proposed in this study that 7,8-DHF’s action on TrkB receptors occurred centrally in the brain and overrode possible peripheral effects on bone to negatively impact on fracture healing. A definitive study that investigated the selective deletion of BDNF in the brains of mice and its effect on bone phenotype\(^{65}\), demonstrated in these centrally-deleted BDNF mice, there was an increase in femoral lengths, and an overall increase in bone mineral density (BMD) compared to wild type mice\(^{65}\). Therefore, suggested in the current experiments is the central activation

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Table 2. Mechanical properties of vehicle and 7,8-DHF-treated calluses at 28-day post-fracture.

| Treatment          | Peak force (N) | Stiffness (x 10^4 Nm⁻²) | CSA (x 10^4 m²) | Bending stress (x 10^6 Nm⁻²) | YM (x 10^6 Nm⁻²) |
|--------------------|----------------|-------------------------|-----------------|-----------------------------|------------------|
| Vehicle (n=12)     | 13.48 ± 0.84   | 9.38 ± 0.50             | 4.56 ± 0.30     | 3.84 ± 0.39                 | 7.21 ± 1.18      |
| Mean ± SEM         |                |                         |                 |                             |                  |
| 7,8-DHF (n=9)      | 10.64 ± 0.51   | 13.01 ± 1.04            | 3.94 ± 0.29     | 3.75 ± 0.46                 | 13.79 ± 2.96     |
| Mean ± SEM         |                |                         |                 |                             |                  |
| p-value            | * 0.011        | * 0.012                 | 0.13            | 0.65                        | 0.06             |

7,8-DHF, 7,8-dihydroxyflavone; CSA, cross-sectional area; YM, Young’s modulus. Values are means ± SEM. *symbol indicates statistical significance (p<0.05) determined by a Mann-Whitney U tests.
of TrkB receptors via 7,8-DHF, which in turn negatively impacted bone metabolism and resulted in smaller fracture calluses and weaker fracture sites.

Additionally, in the present study, an in vitro model, Kusa4b10, was used to determine the effects of 7,8-DHF on osteoblasts and bone mineralization. Kusa4b10 cells are a more osteogenic sub-clone of murine multipotential bone marrow stromal cell line, Kusa O. Like Kusa O cells at day 0, Kusa4b10 resemble an osteoprogenitor phenotype and by day 14 resemble a mature osteoblastic phenotype and therefore are an ideal cell line to study regarding in vitro osteoblastic differentiation and mineralization. Our laboratory has previously localized TrkB receptors on parent line Kusa O, at day 14 of differentiation via Western blot (unpublished data). Likewise, TrkB receptors have also been localized in a murine osteoblastic precursor cells line, MC3T3-E1, which suggests a possible role for TrkB agonists on bone metabolism. This data is additionally supported in vivo rodent models and human fracture healing, which have localized TrkB receptors on chondrocytes and osteoblasts during bone growth and fracture healing. In the current study, it was found that 3-, 7-, or 14-days of 7,8-DHF treatment to Kusa4b10 cells did not alter gene expression of Runx2 and ALP, which are two markers osteoblastic differentiation. The current findings are similar to a study that showed that BDNF administration did not alter ALP gene expression in MC3T3-E1 after 5 days of treatment. However, in the same study, MC3T3-E1 had increased mineralization following BDNF treatment, whereas in the current study 7,8-DHF did not increase mineralization of Kusa4b10 cells at 21 days differentiation. Lack of difference in mineralization may be a result of the timepoint analysed. At 21 days, there was dense mineralization seen in controls and treated Kusa4b10, and had mineralization been analysed at an earlier timepoint, such as when the cells were just starting to form mineral there may have been a difference.

In summary, 5 mg/kg/day of 7,8-DHF treatment to mice with tibial fractures resulted in structurally smaller calluses and mechanically weaker fracture sites. It is proposed that 7,8-DHF acted centrally on TrkB receptors in the brain to negatively impact bone remodelling. The present findings suggest that BDNF has a role in bone remodelling, and we propose that there may be two opposing outcomes on bone remodelling depending if TrkB signalling pathways are activated in the central or peripheral nervous systems.

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