Thymosin β_4 Administration Enhances Fracture Healing in Mice

Rhys D. Brady,[1] Brian L. Grills,[1] Johannes A. Schuijers,[1] Alex R. Ward,[1] Brett A. Tonkin,[2] Nicole C. Walsh,[2] Stuart J. McDonald[1]*

[1]Department of Human Biosciences, La Trobe University, Victoria, Australia, [2]St Vincent's Institute of Medical Research, Department of Medicine, St Vincent's Hospital, University of Melbourne, Fitzroy, Victoria, Australia

Received 3 February 2014; accepted 10 June 2014
Published online 8 July 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22686

ABSTRACT: Thymosin β_4 ($T\beta_4$) is a regenerative peptide that we hypothesized would promote healing of fractured bone. Mice received a bilateral fibular osteotomy and were given i.p. injections of either $T\beta_4$ (6 mg/kg) or saline. Calluses from saline- and $T\beta_4$-treated mice were analyzed for: (1) biomechanical properties and (2) composition using micro-computed tomography (μCT) and histomorphometry. Biomechanical analysis showed that $T\beta_4$-treated calluses had a 41% increase in peak force to failure ($p < 0.01$) and were approximately 25% stiffer ($p < 0.05$) than saline-treated controls. μCT analysis at 21 days post-fracture showed that the fractional volume of new mineralized tissue and new highly mineralized tissue were respectively 18% and 26% greater in calluses from $T\beta_4$-treated mice compared to controls ($p < 0.01$; $p < 0.05$, respectively). Histomorphometry complemented the μCT data; at 21 days post-fracture, $T\beta_4$-treated calluses were almost 23% smaller ($p < 0.05$), had nearly 47% less old cortical bone ($p < 0.05$) and had a 31% increase in new trabecular bone area/total callus area fraction compared with controls ($p < 0.05$). Our finding of enhanced biomechanical properties of fractures in mice treated with $T\beta_4$ provides novel evidence of the therapeutic potential of this peptide for treating bone fractures. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1277–1282, 2014.

Keywords: thymosin β_4; fracture healing; bone regeneration; mice

Thymosin β_4 ($T\beta_4$) is a small, naturally occurring peptide that is expressed by most cells. $T\beta_4$ is found in particularly high concentrations at the site of tissue injury, which suggests that endogenous $T\beta_4$ plays a role in tissue repair and regeneration.[1] Recently, several studies have demonstrated the therapeutic potential of $T\beta_4$ administration for stimulating healing of dermal, corneal, and cardiac injuries.[2–4] Such $T\beta_4$-induced promotion of tissue repair has been attributed to several regenerative properties of the peptide that includes: enhancement of stem cell recruitment and differentiation,[5,6] stimulation of cell survival,[7] modulation of wound site inflammation,[7,8] prevention of bacterial infection,[9] and promotion of angiogenesis.[10,11] Further adding to the therapeutic potential of $T\beta_4$ is that the peptide is quite small (4,964 Da) and does not bind to heparin, which permits $T\beta_4$ to freely diffuse into tissues and exert its regenerative effects.[12] Furthermore, $T\beta_4$ is widely considered a safe molecule for therapeutic use, with several animal studies and human clinical trials reporting no adverse effects due to $T\beta_4$ treatment.[12] Despite the therapeutic potential of $T\beta_4$ stimulating tissue regeneration, no studies have analyzed whether $T\beta_4$ can also promote healing of long bone fractures.

We hypothesized that $T\beta_4$ treatment is likely to be beneficial to bone fracture healing, largely due to the well-documented cellular and molecular similarities in the healing processes of bone fractures and the aforementioned soft tissue injuries shown to benefit from $T\beta_4$ treatment. Firstly, the mitogenic and chemotactic properties of $T\beta_4$ may enhance osteoprogenitor cell differentiation and recruitment, thereby enhancing the initiation of the repair process. $T\beta_4$ could also stimulate osteoblastic or chondrocytic differentiation and by this means increase intramembranous or endochondral ossification. Furthermore, the well-documented pro-angiogenic properties of $T\beta_4$ may importantly enhance re-vascularization of the fracture site. Finally, $T\beta_4$ has also been shown to promote wound matrix metalloproteinase (MMP) expression,[13] suggesting $T\beta_4$ may stimulate matrix remodeling in healing fractures. We hypothesized that if $T\beta_4$ is able to stimulate any of the aforementioned processes it will likely accelerate bone fracture healing.

Though no studies have reported the effects of $T\beta_4$ on bone fracture healing, recent preliminary studies suggest $T\beta_4$ may have potential to enhance bone formation following injury.[14,15] Intraperitoneal injections of $T\beta_4$ (partial sequence) in rats with either a calvarial defect or an extracted tooth increased both the percentage cells possessing the osteoblastic transcription factor, osterix, and the fractional bone volume of the healing sites.[14,15] Additionally, recent studies on the influence of $T\beta_4$ on cultured odontoblasts provide preliminary evidence of the peptide's ability to promote odontogenic differentiation and formation of mineralized nodules.[16]

In the present study, we aimed to determine whether a series of $T\beta_4$ injections could accelerate the healing of mid-shaft fibular fractures in mice. We hypothesized that the well-documented regenerative properties of $T\beta_4$ would enhance both fracture callus formation and remodeling, thereby enhancing the restoration of mechanical integrity of healing fractures.

METHODS

Animal Surgery and $T\beta_4$ Treatment

This project was approved by the La Trobe University Animal Ethics Committee (AEC 11–18). At the time of surgery, 16-week-old C57Bl/6 male mice ($n = 40$) received a

Brian L. Grills and Stuart J. McDonald contributed equally to this paper.

Grant sponsor: La Trobe University, Faculty of Health Sciences Research Grant.

Correspondence to: Dr. Brian Grills (T: +61-3-9479-5705; F: +61-3-9479-5784; E-mail: brian.grills@latrobe.edu.au)

bilateral fibular osteotomy whilst under isoflurane-induced anesthesia. Transverse fractures were created 12 mm proximal to the calcaneal tuberosity with microtenotomy scissors as previously described.[17] Half the mice received a 6 mg/kg i.p. injection of $T\beta_4$ diluted in 100 μl of sterile saline at the time of fracture and at 3, 6, 9, and 12 days post-fracture. This injection regimen has been previously shown to increase recovery of neurological function in mice.[18] The remaining mice received an i.p. injection of 100 μl of sterile saline only (control) at the same time points as the $T\beta_4$-injected animals. Animals were randomly assigned to control and $T\beta_4$-treated groups. Fractures from controls and treated mice were harvested at 14 and 21 days post-fracture (6 mice per group) for analysis by micro-computed tomography (μCT) and histology, with the remaining 16 animals used to generate fractures that were analyzed at 42 days post-injury by three-point bending (left fibula, 7–8 fractures per group) and μCT and histological analyses (right fibula, 4–5 fractures per group).

Biomechanical Assessment

The effect of $T\beta_4$ treatment on the mechanical properties of healing fibular fractures was assessed using a three-point bending test on calluses at 42 days post-fracture. Fibular fractures were dissected from the animals at autopsy, immersed in silicone oil and stored at −20°C until testing. Samples were allowed to equilibrate for 1 h at room temperature then mounted in the testing apparatus with the fulcrum directly overlying the previous fracture site. Each fibula was loaded at a constant rate of 1.67 mm/s, with load and deflection data recorded continuously using transducers connected to an *x*–*y* plotter by preamplifiers. After testing, the ends of each mechanically fractured callus were imprinted into dental wax. Using images of each imprint, cross-sectional (CS) areas (mm^2) were measured using a Leica DMRBE microscope linked to a PC with Leica Qwin software (Leica Microsystems, Wetzlar, Germany). The overall cross-sectional area of each callus at its breaking point was then calculated by averaging the measured areas of each mechanically fractured end. Biomechanical properties of peak force to failure, stiffness, ultimate tensile stress, and Young's modulus were calculated from the load deflection data. Mann–Whitney tests were used to compare biomechanical properties between samples of control and $T\beta_4$-treated mice.

μCT

Fractured fibulae were fixed overnight in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and stored at 4°C in 0.1 M cacodylate buffer containing 10% sucrose (pH 7.4). Images were acquired using a Skyscan 1076 scanner (Bruker-microCT) in 70% ethanol at 9 μm voxel resolution, 0.5 mm aluminum 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 (version 1.6.3.1) and the following parameters: CS to image conversion, 0.0–0.11; ring artifact, 6; pixel defect mask, 5%; and beam hardening correction, 35%. Following reconstruction, the region of interest (ROI) for each bone was determined using CTAN (version 1.11.8.0, Bruker MicroCT) as being a 3 mm region longitudinally centered on the callus (i.e., 1.5 mm either side of the fracture line of the callus); the border of the callus was manually traced. Old cortical bone was excluded using threshold delineation (global threshold >100) to allow quantification of new bone formation. Quantification of structural parameters was performed using two grayscale thresholds in order to distinguish between new mineralized tissue and new highly mineralized tissue. Thresholds were determined using the automatic "otsu" algorithm within CTAn, visual inspection of images and qualitative comparison with histological sections. The algorithms used for structural analysis were as follows: new mineralized tissue (MT)—global threshold of 53–100 and new highly mineralized tissue (HMT)—global threshold of 77–100. 2D and 3D data were generated for all analyses and 3D models were generated using the "marching cubes" algorithm from thresholded data (in CTAn) and colored according to threshold (in ParaView version 4.1.0, Kitware, Inc. Clifton Park, NY). MicroCT image reconstructions of longitudinal, mid-point hemi-calluses were color-coded according to the degree of mineralization; red—new mineralized tissue, green—new highly mineralized tissue, and blue—original cortical bone. For publication, color-coded μCT images were imported into Adobe Photoshop Elements 5.0 (Adobe Systems, Inc., San Jose, CA) and the original gray background of the μCT images was converted to a white background to highlight these above three colors, that is, red, green, and blue. Following imaging, calluses were prepared for histology.

Mann–Whitney tests were used to compare differences in total volume of the callus (TVc), new mineralized tissue volume of callus (MTVc), new highly mineralized tissue volume of callus (HMTVc), MTVc/TVc (fractional volume of new mineralized tissue), and HMTVc/TVc (fractional volume of new highly mineralized tissue) between samples of control and $T\beta_4$-treated mice.

Histological Processing, Staining, and Histomorphometry

Scanned specimens were dehydrated using a graded series of ethanols (70%, 90%, and 100% for 1 h in each solution) and then stored overnight in 100% ethanol at room temperature. Samples were then immersed in chloroform for 2 h and changed every hour, to defat the marrow and this was followed by immersion in 100% ethanol for 2 h (changed every hour) before plastic embedding.

Samples were placed in a solution of equal parts 100% ethanol and LR White resin (London Resin Company Limited, Reading, England) for 3 h prior to being placed under vacuum for 24 h in LR White resin. Following this, samples were polymerized in LR White resin at 60°C for 24 h in glass moulds. Moulds were broken to release blocks.

Three-micron thick longitudinal sections were cut at the midpoint of undecalcified callus on a Leica RM 2155 Rotary Microtome (Leica Microsystems) with a tungsten carbide blade. Sections were stained using Goldner's modification of Masson's trichrome stain. Four sections per group were examined and photographed on a Leica DMBRE microscope. Sections of calluses were assessed both qualitatively and quantitatively. To identify whether $T\beta_4$ treatment influenced the amount of bone in callus, the area of each callus that contained old cortical bone (identified via its compact/lamellar appearance), new trabecular bone (new non-lamellar bony spicules forming on old cortical bone as well as within callus) and marrow was measured using Leica Qwin software. Composition of calluses from control and $T\beta_4$-treated mice were compared using Mann–Whitney tests.

RESULTS

Mechanical Testing

Biomechanical data for 42-day fractures in control and treated mice are shown in Table 1. Peak force to failure of fracture samples increased around 41% in

Table 1. Mechanical Characteristics of Control and Tβ_4-Treated Samples at 42 Days Post-Fracture

Treatment	Peak Force (N)	Stiffness ($\times 10^4\,Nm^2$)	CSA ($\times 10^{-7}\,m^2$)	UTS ($\times 10^7\,Nm^{-2}$)	YM ($\times 10^9\,Nm^{-2}$)
Saline ($n=7$)					
Mean ± SEM	2.95 ± 0.31	3.77 ± 0.20	3.65 ± 0.31	1.80 ± 0.26	5.33 ± 0.82
Tβ_4 ($n=8$)					
Mean ± SEM	4.17 ± 0.33	4.76 ± 0.40	4.05 ± 0.19	2.13 ± 0.23	4.98 ± 0.48
p-Value	<0.01	<0.05	0.25	0.22	0.87

CSA, cross sectional area; UTS, ultimate tensile stress; YM, Young's modulus.Values are means ± SEM.

Tβ_4-treated mice compared with control equivalents ($p < 0.01$). Treatment with Tβ_4 also increased stiffness by approximately 26% when compared with control samples ($p < 0.05$). There were no significant differences in cross sectional area, ultimate tensile stress or Young's modulus.

μCT Analysis

Analysis of calluses from 14- to 21-day treated and control mice is shown in Table 2. Analysis of 14-day calluses showed no differences in any of the measured parameters between the two groups. At 21 days post-fracture, the fractional volume of new mineralized tissue (MTVc/TV_C) was approximately 18% greater in calluses from Tβ_4-treated mice compared to controls ($p < 0.01$). At this time-point, the fractional volume of new highly mineralized tissue (HMTVc/TV_C) was approximately 26% larger in Tβ_4-treated mice compared to controls ($p < 0.05$). These changes are depicted in longitudinal, midpoint 3D reconstructions of representative hemi-calluses in Figure 1A and B. There were no significant differences in TVc, MTVc, or HMTVc at 21 days post-fracture between the two groups.

MicroCT analysis of 42-day calluses was not possible using the threshold settings appropriate to analyze 14- and 21-day samples. Specifically, though our threshold settings allowed for accurate differentiation between old cortical bone (global threshold >100) and newly formed mineralized tissue (global threshold of 53–100) in calluses at 14- and 21 days post-fracture; such differentiation was not possible in 42-day calluses as the degree of mineralization of new bone was approaching that of existing cortical bone, thus precluding discreet delineation of these two tissues. There were no differences in the volume of mineralized tissue within calluses between control and treated groups at 42 days post-fracture (results not shown).

Histology and Histomorphometric Analysis

Qualitative histological assessment of calluses at 14-day post-fracture showed no discernable difference between control and treated groups (results not shown). At 21 days post-fracture, however, there was an obvious increase in the proportion of new trabecular bone in Tβ_4-treated calluses compared to controls (Fig. 1C and D). By 42 days there was no obvious qualitative difference in the histological appearance of calluses between control and treated mice (results not shown). It was noted that cartilage was minimally present or absent in all control and Tβ_4-treated calluses at all three time points. Data obtained from histomorphometric analysis are shown in Figure 2. The histomorphometric data reflected the qualitative histological assessment of calluses. At 14 days post-fracture, there were no differences in callus area, total bone area, old cortical bone area, new trabecular bone area, percentage of new trabecular bone, or percentage of total bone between the treated and control groups. At 21 days post-fracture, however, calluses from Tβ_4-treated mice were almost 23% smaller (Fig. 2A, $p < 0.05$), had nearly 47% less old cortical bone

Table 2. μCT Analysis of Control and Tβ_4-Treated Calluses at 14 and 21 Days Post-Fracture

	TV_C (mm^3)	MTV_C (mm^3)	$HMTV_C$ (mm^3)	MTV_C/TV_C (%)	$HMTV_C/TV_C$ (%)
14d callus					
Saline ($n=6$)	1.70 (0.36)	0.49 (0.08)	0.16 (0.01)	30.73 (2.96)	11.46 (2.38)
Tβ_4 ($n=5$)	1.51 (0.24)	0.50 (0.07)	0.18 (0.03)	33.65 (3.37)	11.71 (0.95)
p-Value	0.56	1.00	0.79	0.66	0.43
21d callus					
Saline ($n=5$)	1.74 (0.10)	0.69 (0.06)	0.28 (0.02)	39.57 (2.00)	16.18 (1.07)
Tβ_4 ($n=6$)	1.38 (0.20)	0.65 (0.10)	0.29 (0.05)	46.79 (1.40)	20.39 (1.10)
p-Value	0.13	0.54	1	<0.01	<0.05

TV_C, tissue volume of callus; MTV_C, new mineralized tissue volume of callus; $HMTV_C$, new highly mineralized tissue volume of callus; MTV_C/TV_C, new mineralized tissue fraction of callus; $HMTV_C/TV_C$, new highly mineralized tissue fraction of callus.Values are means ± SEM.

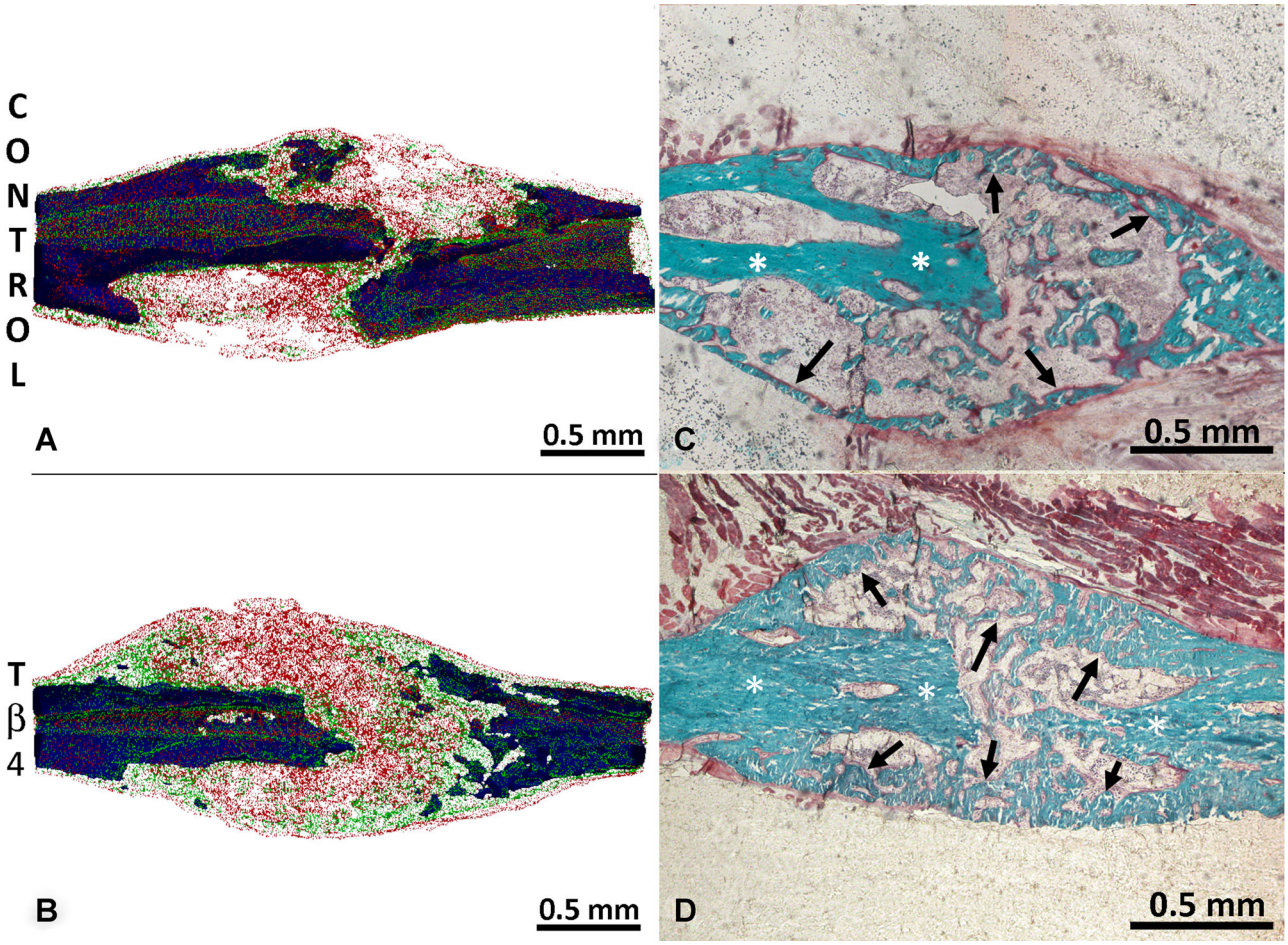

Figure 1. Longitudinal, mid-point views of representative μCT 3D reconstructions of 21-day fracture hemi-calluses from both control (A) and $T\beta_4$-treated mice (B) and corresponding representative histological sections of the same calluses for controls (C) and $T\beta_4$-treated animals (D). For μCT images, reconstructions are color-coded according to the degree of mineralization; red—new mineralized tissue (global threshold 53–100), green—new highly mineralized tissue (global threshold 77–100), blue—original cortical bone (global threshold >100). Note the increased proportion of both new mineralized tissue (red) and new highly mineralized tissue (green) in $T\beta_4$-treated callus (B) compared with control callus (A). Histological assessment (C and D) shows that $T\beta_4$-treated callus (D) had a larger fraction of new trabecular bone (arrows) compared to control callus (C) (* old cortical bone, Goldner's trichrome stain; histology original magnifications 50×).

(Fig. 2C, $p < 0.05$) and had approximately a 31% increase in new trabecular bone area/total callus area fraction compared with controls (Fig. 2F, $p < 0.05$). There were no differences in area of total bone, area of new trabecular bone or percentage of total bone between treated and control calluses at 21 days. There were no histomorphometric differences in 42-day calluses between the two groups and all bone in calluses of both groups at this time was cortical (lamellar) in appearance (results not shown).

DISCUSSION

This study demonstrates for the first time the therapeutic potential of the regenerative peptide $T\beta_4$ in treating bone fractures. When compared with healing fibular fractures from saline-treated mice, fractures from animals treated with $T\beta_4$ had higher fractional bone volume at 21 days post-fracture and increased mechanical integrity at 42 days post-fracture.

Overall, results from our study support the recent findings demonstrating the potential of $T\beta_4$ to induce bone formation.[14,15] Three-point bending analysis revealed that calluses from $T\beta_4$-treated mice had increased peak force to failure when compared with calluses from control mice, which demonstrates that $T\beta_4$ treatment may have the potential to reduce re-fracture risk, particularly given that long bones such as the fibula often experience significant bending moments *in vivo*.[19] Furthermore, fractures from $T\beta_4$-treated mice also displayed greater stiffness, which indicates that $T\beta_4$ treatment is likely to reduce the risk of callus deformation upon loading. Degree of bone stiffness is recognized to strongly correlate to the extent of mineralization,[20,21] therefore our finding of superior callus stiffness in $T\beta_4$-treated mice may indicate that treatment enhanced callus formation and mineralization. This rationale was supported by the μCT results, with $T\beta_4$-treated calluses featuring increased fractional

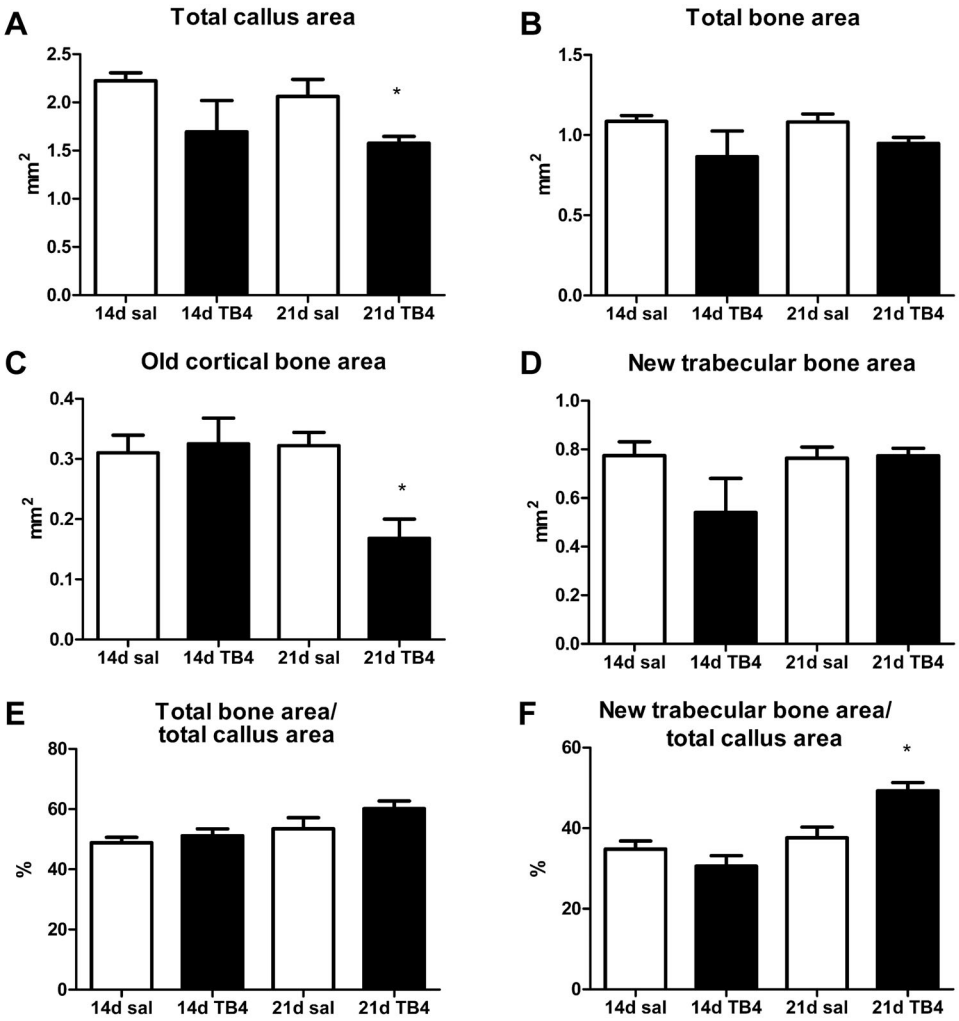

Figure 2. Histomorphometric analysis of bone parameters at 14 and 21 days post-fracture. Total callus area and old cortical bone area was less at 21 days in $T\beta_4$-treated mice compared to controls (A and C, $^*p < 0.05$). Percentage of new trabecular bone area/total callus area was greater in $T\beta_4$-treated mice 21 days post-fracture (F, $^*p < 0.05$) ($n = 5$ per group). No difference between treated and control groups was observed in any parameter at 14 days post-fracture (A–F) ($n = 4$ per group).

volume of new mineralized tissue and new highly mineralized tissue. Comparison of μCT and histological images revealed that calluses did not contain any cartilage or calcified cartilage and that it can therefore be assumed that all mineralized and highly mineralized tissue present was new bone formed by intramembranous ossification. Histological results confirmed the notion that $T\beta_4$-treatment enhanced formation of bone during fracture healing; there were no differences in bone fractions in 14-day callus, however, by 21 days post-fracture, calluses from $T\beta_4$-treated animals contained significantly higher new trabecular bone fractions than those from controls.

Though these findings provide strong evidence that $T\beta_4$ promotes bone fracture healing, the precise cellular and molecular mechanisms through which this occur remains to be determined. mRNA analysis of granulation tissue found in sockets 3 days post-tooth extraction found $T\beta_4$ treatment had increased expression of genes associated with angiogenesis and cell proliferation whilst also reducing expression of inflammatory cytokines.[15] These initial findings indicate that $T\beta_4$ may also promote healing of bone fractures via mechanisms similar to that reported in the healing of other tissues.[22] In addition to promoting these non-specific regenerative processes, recent *in vitro* studies on the influence of $T\beta_4$ on odontoblasts suggest the peptide has the potential to increase expression of bone specific growth factors BMP2 and BMP4, as well as osteoblastic transcription factors runx2 and osterix.[16]

Interestingly, results from this study also provide preliminary evidence that $T\beta_4$ may accelerate bone remodeling during fracture healing, with calluses from treated animals tending to be smaller in area and containing reduced old cortical volumes at 21 days post-fracture. $T\beta_4$ has been shown to increase expression of several MMPs during soft tissue wound healing, with

researchers concluding that the peptide may promote remodeling of extracellular matrix following injury. Several studies have demonstrated the important role of MMPs in regulating the remodeling of callus at various stages post-fracture.[23] Therefore it is possible $T\beta_4$ may have increased callus MMP expression and thereby promoted remodeling during healing. Future studies will look at the expression of MMPs in callus post-$T\beta_4$ treatment and analyze the potential for $T\beta_4$-induced promotion of remodeling during fracture healing.

Studies are underway to assess the possible mechanism by which TB_4 enhances fracture healing and the effectiveness of this peptide in other fracture models. We hypothesize the multiple tissue regenerating properties of $T\beta_4$ may also promote healing of bone fractures in humans and this peptide may prove to be an effective therapeutic treatment to accelerate fracture healing.

ACKNOWLEDGMENTS

This project was supported by a La Trobe University, Faculty of Health Sciences Research Grant. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the content of this article.

REFERENCES

1. Goldstein AL, Hannappel E, Kleinman HK. 2005. Thymosin beta4: actin-sequestering protein moonlights to repair injured tissues. Trends Mol Med 11:421–429.
2. Malinda KM, Sidhu GS, Mani H, et al. 1999. Thymosin beta4 accelerates wound healing. J Invest Derm 113:364–368.
3. Sosne G, Chan CC, Thai K, et al. 2001. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. Exp Eye Res 72:605–608.
4. Cavasin MA. 2006. Therapeutic potential of thymosin-beta4 and its derivative N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after infarction. Am J Cardiovasc Drugs 6:305–311.
5. Philp D, St-Surin S, Cha HJ, et al. 2007. Thymosin beta 4 induces hair growth via stem cell migration and differentiation. Ann N Y Acad Sci 1112:95–103.
6. Smart N, Bollini S, Dube KN, et al. 2011. De novo cardiomyocytes from within the activated adult heart after injury. Nature 474:640–644.
7. Bock-Marquette I, Saxena A, White MD, et al. 2004. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature 432:466–472.
8. Sosne G, Qiu P, Christopherson PL, et al. 2007. Thymosin beta 4 suppression of corneal NFkappaB: a potential anti-inflammatory pathway. Exp Eye Res 84:663–669.
9. Huang LC, Jean D, Proske RJ, et al. 2007. Ocular surface expression and in vitro activity of antimicrobial peptides. Curr Eye Res 32:595–609.
10. Philp D, Huff T, Gho YS, et al. 2003. The actin binding site on thymosin beta4 promotes angiogenesis. FASEB J 17:2103–2105.
11. Smart N, Risebro CA, Melville AA, et al. 2007. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature 445:177–182.
12. Goldstein AL, Hannappel E, Sosne G, et al. 2012. Thymosin beta(4): a multi-functional regenerative peptide. Basic properties and clinical applications. Expert Opin Biol Ther 12:37–51.
13. Philp D, Scheremeta B, Sibliss K, et al. 2006. Thymosin beta4 promotes matrix metalloproteinase expression during wound repair. J Cell Physiol 208:195–200.
14. Adachi K, Matsuo K, Akasaki Y, et al. 2013. Effects of thymosin beta4 on the bone formation of calvarial defects in rats. J Prosthodont Res 57:162–168.
15. Matsuo K, Akasaki Y, Adachi K, et al. 2012. Promoting effects of thymosin beta4 on granulation tissue and new bone formation after tooth extraction in rats. Oral Surg Oral Med Oral Pathol Oral Radiol 114:17–26.
16. Lee SI, Kim DS, Lee HJ, et al. 2013. The role of thymosin beta 4 on odontogenic differentiation in human dental pulp cells. PLoS ONE 8:e61960.
17. Kellum E, Starr H, Arounleut P, et al. 2009. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. Bone 44:17–23.
18. Zhang J, Zhang ZG, Morris D, et al. 2009. Neurological functional recovery after thymosin beta4 treatment in mice with experimental auto encephalomyelitis. Neuroscience 164:1887–1893.
19. Raftopoulos DD, Qassem W. 1987. Three-dimensional curved beam stress analysis of the human femur. J Biomed Eng 9:356–366.
20. Mulder L, Koolstra JH, den Toonder JM, et al. 2008. Relationship between tissue stiffness and degree of mineralization of developing trabecular bone. J Biomed Mater Res A 84:508–515.
21. Follet H, Boivin G, Rumelhart C, et al. 2004. The degree of mineralization is a determinant of bone strength: a study on human calcanei. Bone 34:783–789.
22. Goldstein AL, Hannappel E, Sosne G, et al. 2012. Thymosin beta4: a multi-functional regenerative peptide. Basic properties and clinical applications. Expert Opin Biol Ther 12:37–51.
23. Schindeler A, McDonald MM, Bokko P, et al. 2008. Bone remodeling during fracture repair: the cellular picture. Semin Cell Dev Biol 19:459–466.