Wnt/β-catenin signalling promotes more effective fracture healing in aged mice than in adult mice by inducing angiogenesis and cell differentiation

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Abstract
To investigate whether activating the Wnt/β-catenin signalling pathway differentially promotes fracture healing in aged and adult individuals. Catnb\textsuperscript{Tm2Kem}, Catnb\textsuperscript{lox(ex3)} and wild-type adult and aged mice were used in this study. The femur was electroporated through a hole with a diameter of 0.6 mm. On the 7th, 14th and 21st days after fracture establishment, repair of the femoral diaphyseal bone was examined using X-ray and CT, the levels of mRNAs related to Wnt/β-catenin signalling were detected using real-time polymerase chain reaction (RT-PCR), and angiogenesis and cell differentiation were observed using immunohistochemistry. The numbers of osteoclasts were determined by TRAP staining. Wnt/β-catenin activation accelerated fracture healing in adult mice, with more pronounced effects on aged mice. Compared with wild-type mice at the corresponding ages, Wnt/β-catenin signalling activation induced higher levels of angiogenesis and cell differentiation in aged mice than in adult mice and promoted fracture healing. The administration of medications targeting Wnt/β-catenin signalling to aged patients may accelerate fracture healing to a greater extent.

Keywords
Wnt/β-catenin, fracture healing, ageing, osteoblasts, angiogenesis

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**Introduction**

Fractures are very common in aged individuals, and studies have reported fracture risks of 29% in males and up to 59% in females aged greater than 60 years. Although the incidence of cancellous bone fractures increases exponentially with age, the incidence of nonhip, nonvertebral (NHNV) fractures in aged patients is still higher, even in people aged greater than 80 years.\(^1,2\) Fracture healing mainly consists of three stages: inflammation, repair and remodelling. The fracture leads to a disruption of bone continuity and the vasculature, causing thrombosis and related biochemical signalling in the fracture area. This subsequently leads to the formation of granulation tissue, the release of inflammatory factors and aggregation of regional mesenchymal stem cells. In the repair phase, mesenchymal stem cells differentiate into chondrocytes or osteoblasts and ultimately form cortical or cancellous bone through remodelling. The process can last for several months to several years.\(^5\)

In the United States, the lifetime prevalence of fractures is 50%. While most fractures heal normally, approximately 5% to 10% of fractures do not heal each year, a rate that increases with advanced age.\(^4\) Nonhealing and delayed healing of fractures impose a significant burden on patients and society. Because patients do not achieve functional healing, they are unable to work normally, a situation that is more common among aged individuals. Age exerts an important effect on fracture healing, and a reduction in the incidence of nonunion and delayed recovery is an important clinical goal.\(^5\)

Several clinical strategies are available to promote fracture healing. Bone morphogenetic proteins (BMPs) assist with the healing of fractures, but the proteins must be surgically implanted into the fracture site to produce a therapeutic effect and may induce local bone resorption and heterotopic bone formation.\(^6\) Baht et al.\(^7\) reported an improvement in fracture repair in aged individuals following exposure to the bone marrow of young aged mice, but these samples are difficult to obtain for clinical applications.

Increased activation of Wnt signalling promotes tissue regeneration\(^8\) and the differentiation and proliferation of mesenchymal stem cells into osteoblasts, affecting fracture repair.\(^9\) Lithium preparations have been shown to promote fracture healing by activating Wnt signalling.\(^10\) In contrast, the suppression of Wnt signalling leads to delayed and nonunion fracture healing.\(^11\)

An increase in age is a disadvantage for fracture healing. Possible causes of delayed healing in aged patients include decreased differentiation of mesenchymal stem cells, impaired angiogenesis, and decreased levels of growth factors with ageing. Studies in some animal models have also confirmed that aged animals require longer periods to achieve fracture healing and recovery of physiological functions than younger animals.\(^12,13\)

The bones of adult and aged patients are known to differ, and the pathological processes of fracture healing in these two groups should be considered separately. However, researchers have not clearly determined whether the bone repair mediated by Wnt/β-catenin signalling differs between aged and adult individuals.
Methods and materials

Animals

Animal experimental procedures or research protocols were approved by the Ethics Committee of the Third Military Medical University of China. Catnb\textsuperscript{TM2Kem} mice and Catnb\textsuperscript{lox(ex3)} mice were obtained from Jianquan Feng (Department of Biomedical Sciences, Baylor College of Dentistry). Catnb\textsuperscript{TM2Kem} mice possess loxP sites in introns 1 and 6 of the gene encoding \( \beta \)-catenin, resulting in a null allele when treated with Cre recombinase. Catnb\textsuperscript{lox(ex3)} mice contain loxP sequences flanking exon 3, which results in the expression of a fully functional but stabilised \( \beta \)-catenin protein when subjected to Cre recombinase.

Sixty male adult (6-month-old) mice with an average weight of 308.3 \( \pm \) 21.5 g (261–348 g) and 60 male (12-month-old) mice with an average weight of 320.5 \( \pm \) 23.5 g (276–356 g) were used. The rats were exposed to a light/dark cycle of 10/14 h, kept at normal room temperature, and fed standard mouse diet and tap water.

Mice expressing the tamoxifen (TM; Sigma-Aldrich, St. Louis, MO, USA)-inducible Cre fusion protein were used. Genotyping was performed as previously reported\textsuperscript{14}. The 3.2-kb Col1-Cre ER\textsuperscript{TM}/\( \beta \)-catenin exon 3 fx +/+ was targeted in the mice in which \( \beta \)-catenin was constitutively activated in osteoblasts by TM injection•\textsuperscript{14,15}

Animal surgical procedures

Mice were anaesthetised using 1% sodium pentobarbital at a dose of 50 mg/kg. A 10 mm long incision was made on the anterolateral side of the femur, and the subcutaneous tissue and muscles were separated to expose the femur. Unilateral cortical defects were generated in the middle of the femur using a scalpel (Fine Science Tools #19007-07) with a diameter of 0.6 mm. The perforations disrupted the ipsilateral cortical wall, but not the contralateral cortical wall\textsuperscript{16,17}. The skin was then sutured, and the mice were allowed to recover from anaesthesia in a room with constant temperature.

Animal groups

Male Catnb\textsuperscript{lox(ex3)} and Catnb\textsuperscript{TM2Kem} mice and their wild-type (WT) male counterparts were divided into six groups after fracture: the WT adult group (6-month-old wild-type mice injected with saline, \( n = 28 \)), WT aged group (12-month-old wild-type mice injected with saline, \( n = 28 \)), CA adult group (6-month-old Catnb\textsuperscript{lox(ex3)} mice injected with TM, \( n = 16 \)), CA aged group (12-month-old Catnb\textsuperscript{lox(ex3)} mice injected with TM, \( n = 16 \)), KO adult group (6-month-old Catnb\textsuperscript{TM2Kem} mice injected with TM, \( n = 16 \)), and KO aged group (12-month-old Catnb\textsuperscript{TM2Kem} mice injected with TM, \( n = 16 \)). TM, saline and 5’-bromo-2-deoxyuridine were intraperitoneally injected using the methods described in a previous study\textsuperscript{18}. 
**Tissue preparation**

On days 0, 7, 14 and 21 after fracture establishment, four mice in the WT, CA and KO groups were sacrificed by administering an anaesthesia overdose, and total RNA was extracted from the fracture region. Four mice in each group were radiographed 7, 14 and 21 days after surgery; the mice were sacrificed, and the femur was removed and fixed overnight in a 1% formalin solution for X-ray examination and CT scans. Then, the femur was decalcified with ethylenediaminetetraacetic acid for 3 days, fixed, embedded, sectioned (4 μm thick), deparaffinised and hydrated.

**Radiological examination**

X-rays were captured with a Faxitron system (Faxitron X-ray, Wheeling, IL, USA) at the preset postoperative time points (days 7, 14 and 21 after fracture). The femur was placed in a tube filled with ethanol, and CT examination was performed by Viva CT 40 (Scanco Medical, Bassersdorf, Switzerland) (X-ray tube potential 45 kVp, voxel size 10 μm³). Images were analysed and 3D reconstructions created using the EVS Beam software with 1400 Hounsfield units as the threshold for observing bone. The regions of interest (ROI) and calculations were performed as described in a previous study.

Quantitative morphometric data were based on the region of interest as follows: a diameter of 0.8 mm was taken at the defect centre with a depth of 0.4 mm. The percentage of bone volume to total volume (BV/TV) was calculated for the bone injury sites.

**Histological staining**

The main process used for haematoxylin-eosin (H&E) staining is described below. The sections were dewaxed with xylene, immersed in water, immersed in haematoxylin staining solution for 5 min, transiently differentiated with ethanolic hydrochloric acid, counterstained with haematoxylin for 20 s, immersed in an eosin staining solution for 15 s, dehydrated with a gradient of alcohol solutions, cleared and finally sealed with neutral gum. Green/Safranin O staining and Masson’s trichrome staining were performed using the methods described in a previous study.

**Real-time polymerase chain reaction (RT-PCR)**

The femur was separated, and the soft tissue around the fracture was removed. Approximately 1 cm of the bone was removed from the centre of the fracture defect. The tissue was placed in a small EP tube and centrifuged at 5000 × g at 4°C to remove the bone marrow. Total RNA of the bone was extracted according to the manufacturer’s instructions. The instruments used for this procedure were treated with RNase-free DNase, and potential DNA contamination was avoided during the re-extraction process. RT-PCR was performed with the SYBR Green
assay to evaluate the mRNA levels of proteins involved in Wnt signalling.\textsuperscript{22} The primers are listed in Table 1.

| Gene       | Forward primer sequence (5'\textendash3') | Reverse primer sequence (3'\textendash5') |
|------------|---------------------------------|---------------------------------|
| β-catenin  | ACGGTGCCGCCGCCGCTTTATA           | TAGCCATTGTCCACGCAGCCG          |
| LEF-1      | AGAACACCCCCAGTGAGGGA             | GGCATCATTATGTACCGGAAT         |
| OSX        | TCTCAAGACACATGGACTCC             | GGTTAGCTATTTGCATACCCAGA       |
| GAPDH      | GAGAAGGTGGGGGGCTATT             | CCAATATGATTCCACCCCATG        |

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LEF-1: lymphoid enhancer-binding factor 1; OSX: osterix.

The details of the primer sequences used for RT-PCR are shown, including the forward (F) and reverse (R) sequences.

**Immunohistochemical (IHC) staining**

IHC staining was performed as previously described.\textsuperscript{23} The primary antibodies included goat anti-rabbit osteocalcin (OCN; 1:400), β-catenin (1:300), transcription factor 2 associated with runt (RUNX2; 1:200), anti-mouse matrix metallopeptidase 9 (MMP9; 1:200), vascular endothelial growth factor (VEGF; 1:200), and goat anti-rat BrdU (1:200) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Images of each slice were captured using an Olympus microscope to identify cells expressing the marker protein. Five regions within the observation range were randomly selected, and the number of positive cells was counted.

**Tartrate-resistant acid phosphatase (TRAP) staining**

After the slices were dewaxed with xylene, immersed in water, they were immersed in a TRAP incubation solution, placed in a 37°C incubator in the dark for 30 min, washed with water, immersed in methyl green staining solution for 5 min, dehydrated with ethanol, rendered transparent and then sealed.

Five regions within the observation range were randomly selected, and the number of positive cells was counted.

**Statistical analysis**

All experimental data are presented as the means ± standard deviations, and statistical analyses were performed using SPSS 19.0 software. Differences in the BV/TV ratio and mRNA expression levels between the adult and aged WT, CA and KO mice and between the adult and aged groups were analysed using one-way analysis of variance (ANOVA) and an LSD post hoc test. Differences in the BV/TV ratio, gene expression, angiogenesis and cell differentiation between CA and WT mice in
the aged group were compared with the adult group using a paired t test. $p < 0.05$ was considered statistically significant.

**Results**

*Fractures heal faster in adult WT mice than in aged WT mice*

X-ray images of the fracture site and 3D reconstructions of the adult and aged WT mice were examined at 7, 14 and 21 days after fracture. Significant defects were observed on the 7th day after surgery in the fracture sites of the two groups. On the 14th day after surgery, many blurred shadows were observed at the fracture site in adult mice, indicating callus formation. Aged mice showed no apparent blurring around the fracture site, indicating little or no callus formation. On the 21st day after surgery, the defects in the adult mice had almost completely disappeared, while the defects in the aged mice persisted (Figure 1(a) and (b)).

Images of H&E-stained bone were obtained from each group of mice at the corresponding postoperative time points (Figure 1(c)). On the 7th day after surgery, larger defects were observed at the fracture sites in both groups of mice. On the 14th day after surgery, adult mice displayed a large amount of soft tissue and new callus at the fracture site, while the aged mice had less soft tissue and little evidence of callus at the fracture site. On the 21st day after surgery, cortical connections were observed at the fracture sites of adult mice, and no significant cortical connections were observed at the fracture sites of aged mice.

The BV/TV ratio in bone defects at 14 and 21 days after surgery in adult mice was much higher than in aged mice (Figure 1(d)), indicating that adult mice would heal faster than aged mice. Changes in the mRNA expression levels during fracture healing were observed using RT-PCR. Higher levels of the β-catenin (Figure 1(e)) and lymphoid enhancer-binding factor 1 (LEF-1) mRNAs (Figure 1(f)) were detected in adult mice. Moreover, the peak of β-catenin expression occurred earlier during fracture healing in adult mice than in aged mice. Wnt/β-catenin signalling may be an important factor contributing to the earlier fracture healing observed in adult mice than aged mice.

*Wnt/β-catenin promotes fracture healing in both adult and aged mice, but these effects are more predominant in aged mice*

X-rays and three-dimensional reconstructions images revealed faster healing of the femoral defects in adult CA mice and slower healing in adult KO mice compared to adult WT mice, especially at 14 days after fracture (Figure 2(a) and (b)).

Images of H&E-stained bone were captured in each group of mice at the corresponding postoperative time points (Figure 2(c)). H&E staining showed earlier filling of the fracture site and broken connections by soft tissue in the adult CA mice than in the adult WT mice. Furthermore, at the same time point, worse fracture connections were observed in adult KO mice.
The healing of femoral defects was determined by analysing the images. At 14 and 21 days after fracture, a higher BV/TV ratio was observed in adult CA mice than in adult WT mice. At 7, 14 and 21 days after fracture, a lower BT/TV ratio was observed in adult KO mice than in adult WT mice (Figure 2(d)).

X-ray examinations and three-dimensional reconstructions revealed improved healing of femur defects in aged CA mice than in aged WT mice. The aged KO mice showed a lower BT/TV ratio compared to the aged WT mice (Figure 2(b)).

The mRNA expression levels detected in WT mice on different days after fracture served as an internal control. The results are presented as the means ± SD. n = 4 mice per group. **p < 0.01.

**Figure 1.** Healing of defects in the femoral bone of adult and aged WT mice: (a) X-ray, (b) 3D CT images, (c) H&E staining of femoral sections (× 100), (d) bone volume/tissue volume (BV/TV) ratio within the region of interest (ROI), and (e) β-catenin and (f) LEF-1 expression as detected by RT-PCR. The mRNA expression levels detected in WT mice on different days after fracture served as an internal control. The results are presented as the means ± SD. n = 4 mice per group. **p < 0.01.
Figure 2. Healing of defects in the adult and aged β-catenin active (CA), wild-type (WT) and β-catenin knockout (KO) mice. (a) X-rays, (b) 3D CT images and (c) images of H&E staining of femoral sections (×100). (d) Bone volume/tissue volume (BV/TV) ratios within the region of interest (ROI) were calculated in the CA adult mice and KO adult mice. (e) X-rays, (f) 3D CT images and (g) images of H&E staining of femoral sections (×100). (h) BV/TV ratios within the ROI were calculated in the CA aged mice and KO aged mice. (i) Comparison of BV/TV ratios between the CA and WT mice. (j) Expression of β-catenin and LEF-1 mRNAs detected using RT-PCR. The mRNA expression levels detected in WT mice on different days after fracture served as an internal control. The results are presented as the means ± SD. n = 4 mice per group.

*p < 0.05. **p < 0.01.
mice displayed much less healing than the aged WT mice, especially at 14 days after fracture (Figure 2(e) and (f)). The trend of H&E staining was similar. Aged WT mice displayed better fracture healing than aged KO mice but worse fracture healing than aged CA mice (Figure 2(g)).

Based on the results of the microscopic CT imaging examination, higher BV/TV ratios of ROIs were observed in aged CA mice on the 14th and 21st days after fracture, and lower values were observed in aged KO mice than in aged WT mice (Figure 2(h)).

Increased Wnt/β-catenin signalling accelerated fracture healing in both adult and aged mice, while the suppression of Wnt/β-catenin signalling was not conducive to fracture healing. Compared to the WT group, higher BV/TV ratios were observed in the aged CA mice at 14 and 21 days after fracture (Figure 2(i)). Compared to WT mice, CA mice displayed higher levels of β-catenin and LEF-1 mRNAs (Figure 2(j)).

**Wnt/β-catenin signalling exerts disparate effects on angiogenesis and cell differentiation**

On day 14 after fracture, the level of β-catenin mRNA in ROIs of aged CA mice was increased compared with aged WT mice (Figure 3(a)). The levels of LEF-1 (Figure 3(b)) and osterix (Osx) (Figure 3(c)) mRNAs in ROIs of adult and aged CA mice were increased compared with adult and aged WT mice. Compared to adult mice, the ratio of mRNA expression levels of β-catenin, LEF-1 and Osx in the CA group compared to the WT group in aged mice was higher (Figure 3(d)). Masson’s trichrome staining revealed the deposition of more collagen fibres in the fracture defect in adult CA mice than in adult WT mice, and more collagen fibres were also observed in aged CA mice than in aged WT mice (Figure 3(e) and (g)). Fast Green/Safranin O staining indicated that there was no cartilage formation in the fracture defect in the four groups of mice (Figure 3(f)).

On day 14 after surgery, a greater number of β-catenin-positive cells was observed in the femoral defect in adult WT mice than in adult CA mice (Figure 4(a) and (e)). However, compared to those in adult and aged WT mice, BrdU-positive cells of ROI in adult and aged CA mice were greater (Figure 4(b) and (f)).

At 14 days after surgery, compared to adult and aged WT mice, MMP9-positive cells (Figure 4(c) and (g)) and VEGF-positive cells (Figure 4(d) and (h)) of ROI in adult and aged CA mice were more numerous. Compared to the adult group, the ratio of BrdU-, MMP9- and VEGF-positive cells of ROI between CA mice and WT mice was higher (Figure 4(i)).

Mesenchymal stem cells and osteoblasts are the main cells that promote fracture healing. On day 14 after surgery, greater numbers of RUNX2-positive cells (Figure 5(a) and (d)) and OCN-positive cells (Figure 5(b) and (e)) were observed in the ROIs of adult CA mice than in adult WT mice. The numbers of these cells were also increased in aged CA mice compared with aged WT mice.
Figure 3. RT-PCR expression levels, Masson’s trichrome and Fast Green/Safranin O staining at 14 days after fracture. (a) Expression of β-catenin, (b) LEF-1 and (c) OSX mRNAs detected using RT-PCR (d) in the CA and WT mice. (e, g) Images of Masson’s trichrome staining (×200) and (f) Fast Green/Safranin O staining (×200) of the defect site 14 days after fracture. The β-catenin, LEF-1, and OSX expression levels on the fracture day were used as an internal control. n = 4 mice per group.

*p < 0.05. **p < 0.01 (gene expression in calluses of CA mice vs WT mice at 14 days after fracture and comparison of the mRNA expression levels in the aged and adult CA mice and WT mice).
Compared to the adult group, the ratio of RUNX2- and OCN-positive cells between the CA and WT mice was higher in the aged groups (Figure 5(g)).

Figure 4. β-Catenin, BrdU, MMP9 and VEGF levels at the defect site on day 14 after fracture. Immunostaining (×400) for (a) β-catenin, (b) BrdU, (c) MMP9 and (d) VEGF at the defect site in the femur on day 14 after fracture in adult and aged WT and CA mice. Quantification of the numbers of (e) β-catenin-, (f) BrdU-, (g) MMP9- and (h) VEGF-positive cells. (i) Comparison of the numbers of BrdU-, MMP9- and VEGF-positive cells detected in CA and WT mice. The results are presented as the means ± SD. n = 4 mice per group. *p < 0.05. **p < 0.01.
Compared to WT mice, there were fewer TRAP-positive cells found in the ROIs of adult and aged CA mice at 14 days after surgery (Figure 5(c) and (f)). However, compared to the adult groups 14 days after fracture, the ratio of TRAP-positive cells between the CA and WT mice in the aged groups was not different (Figure 5(g)).

Discussion

The ability to heal fractures decreases with ageing. The cellular and histological changes associated with tibia fracture healing in mice of different ages, including chondrocyte maturation, vascular invasion and bone formation at fractured sites, occur earlier in young mice than in aged mice.\(^\text{24}\) The results of our experiments show the same trend: fracture healing is slower in aged mice than in adult mice.

Endogenous Wnt signalling decreases with increasing age (Figure 1(e) and (f) and see Baht et al\(^\text{7}\) and Leucht et al.\(^\text{25}\)). Aged women show a significant decrease in skeletal LEF-1 transcription compared to young women, whereas a Wnt inhibitor increases SFRP1 levels, suggesting that a reduction in Wnt signalling may be one mechanism underlying the impaired bone formation observed during ageing.\(^\text{26}\) Endogenous Wnt signalling can be increased to baseline levels through ‘inhibitor inhibitor’ methods, such as the application of anti-sclerostin and anti-DKK1 antibodies, but these changes may not be sufficient to stimulate bone production.\(^\text{26,27}\) Lauing et al.\(^\text{28}\) used lithium chloride to promote fracture healing through Wnt/\(\beta\)-catenin signalling activation.

Upregulated expression of intermediates in the Wnt/\(\beta\)-catenin signalling pathway enhances the differentiation of mesenchymal stem cells into osteoblasts and increases cell proliferation. The rate of successful bone grafting after Wnt treatment has been increased by three-fold,\(^\text{27}\) and the suppression of Wnt signalling by DKK1 inhibits osteoblast proliferation and new bone formation.\(^\text{29}\) The administration of Scl-Ab to male giant monkeys of different ages increased Wnt/\(\beta\)-catenin signalling and improved bone formation and bone mass.\(^\text{6,30}\)

According to Lopas et al.,\(^\text{31}\) the healing process of fractures in aged mice is the same as in adult mice, but the rate of formation of a callus decreases. Upregulated expression of Wnt/\(\beta\)-catenin signalling promotes fracture healing in both aged and adult mice, while inhibition of Wnt/\(\beta\)-catenin signalling is a disadvantage. This promotion effect was found to be more pronounced in aged mice. The formation of micro-vessels is very important for bone repair. New blood vessels transport stem cells and inflammatory cells into the damaged area. VEGF promotes the formation of micro-vessels and stimulates osteogenic differentiation through its effects on endothelial cells.\(^\text{32}\) Hauser et al.\(^\text{33}\) observed increased expression of VEGF2, MMP9 and MMP2 mRNAs on both day 14 and 21 after fracture. The expression of MMP9 in bone marrow stromal cell cultures is stimulated by prostaglandin E2/EP4 agonists and promotes fracture healing.\(^\text{34}\)

On day 14 after fracture, MMP9- and VEGF-positive cells were more abundant in the femoral fracture region of CA mice than in WT mice in both adult and aged
Figure 5. RUNX2, OCN and TRAP expression at the defect site on day 14 after fracture. Immunostaining for (a) RUNX2 (×400), (b) OCN (×400) and (c) TRAP (×200) at the defect site in the femur on day 14 after fracture in adult and aged WT and CA mice. Quantification of the numbers of (d) RUNX2-, (e) OCN-, and (f) TRAP-positive cells and (g) comparison of the numbers of RUNX2- and OCN-positive cells in CA and WT mice. The results are presented as the means ± SD. n = 4 mice per group.
*p < 0.05. **p < 0.01.
groups. Compared to the adult group, the ratio of MMP9- and VEGF-positive cells between the CA and WT mice was higher in the aged groups. Thus, the activation of Wnt signalling induces higher levels of angiogenesis in the defect site in aged mice than in adult mice. This difference may be because the baseline level of angiogenesis in the fracture site of aged mice is lower than in adult mice. Therefore, when Wnt stimulates angiogenesis at the fracture site, a relatively larger amplitude of the effect is observed in aged mice.

The activation of Wnt signalling induces angiogenesis of mesenchymal stem cells, and Wnt inhibition blocks this process. As shown in the studies by Suen et al. and Cai et al., an increase in Wnt signalling improves angiogenesis and thus promotes fracture healing. Karaman et al. also found that kirenol can activate the Wnt/β-catenin pathway and promote fracture healing in vivo. Wnt signalling promotes bone formation by increasing the expression of proteins involved in osteogenic signalling, such RUNX2 and OCN. Jing et al. developed a Wnt3a protein therapy to activate the Wnt pathway, thereby promoting transcription of osteogenic genes and accelerating fracture healing, confirming these effects.

Kusumbe et al. observed substantial reductions in the numbers of vascular and osteoprogenitor cells in the bones of aged animals, and the proliferation of mesenchymal stem cells in aged mice was reduced compared with adult mice. According to clinical studies, when bone cells are implanted into aged donors, cell proliferation and osteogenic differentiation are impaired, leading to nonhealing. An increase in Wnt signalling increases the bone mineral density of aged people. Therefore, stimulation of Wnt signalling is sufficient to overcome these age-related defects and represents a potential treatment for osteonecrosis and fractures in aged patients.

In our study, higher levels of LEF-1 and Osx mRNAs and greater numbers of BrdU-, RUNX2- and OCN-positive cells in the ROIs were observed in adult and aged CA mice than in adult and aged WT mice on day 14 after fracture. Compared to the adult groups, the ratio of BrdU-, RUNX2- and OCN-positive cells between the CA and WT mice was higher in the aged groups. Therefore, active Wnt/β-catenin signalling might increase osteoblast differentiation, and this change was more apparent in aged mice. Active Wnt/β-catenin signalling also inhibits osteoclast formation in both adult and aged groups. However, compared to the adult groups 14 days after fracture, there was no significant difference in the number of TRAP-positive cells in the aged CA and WT mice. Osteoclasts may not be a particularly important factor contributing to differences in early fracture healing in adult and aged mice. On days 14 and 21 after fracture, the BV/TV ratios of the fracture sites in the adult and aged CA mice were higher than the ratios of the fracture sites in the adult and aged WT mice. Compared to the adult groups, the ratio of BV/TV values was higher in the aged CA and WT mice. We postulate that upregulated expression of intermediates in the Wnt/β-catenin signalling pathway promotes more effective bone repair in aged mice than in adult mice by inducing osteoblast differentiation and angiogenesis but not by inhibiting osteoclast function. The
effects of osteoblasts on the early stages of fracture healing are more important, although they include osteoclast-mediated osteogenesis. During fracture healing, the relevant cellular molecules from the soft tissue, endothelium and blood vessels need to reach the fracture site. The stem cells are relatively easy to obtain during healing and differentiate into osteoblasts and microvessels. In adult mice, there are sufficient microcirculation and stem cells in the cancellous bone for transformation, irrespective of the Wnt/β-catenin signalling pathway is activated to directly promote osteogenic differentiation and microcirculation in aged mice. But it still cannot promote the diaphyseal fracture healing of old mice reach the level of healing of adult mice. Wnt/β-catenin signalling accelerates fracture healing in both adult and aged mice by promoting angiogenesis and osteoblast differentiation in the fracture zone, and these effects are more pronounced in aged mice. Based on these findings, the administration of medications targeting Wnt/β-catenin signalling to aged patients will accelerate fracture healing.

Conclusion

The administration of medications targeting Wnt/β-catenin signalling to aged patients may accelerate fracture healing to a greater extent.

Author contributions

Data analysis was carried out by S-H H and S-X C, J-Z Y, L-Y, Q-W B and H-Q wrote the manuscript. All authors have read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics approval

The Ethics Committee of the Third Military Medical University of China approved the experiments (Ethics Committee License Number: SYXK(jun)20120031).

Animal welfare

The present study involved client-owned animals; it demonstrated a high standard (best practice) of veterinary care and involved informed client consent.
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