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Comprehensive validation of a custom three-point bending system for standardized diabetic fracture models in rats

Qidong Guo[1], Weijie Wang[2] and Zheng Guo[3*]

Abstract

Background Diabetes mellitus (DM) significantly impairs fracture healing, necessitating reliable animal models to study diabetic fracture repair mechanisms and therapeutic interventions. This study aimed to develop and comprehensively validate a standardized three-point bending system for inducing precise, reproducible mid-shaft transverse femoral fractures in rats, addressing existing methodological gaps of insufficient reproducibility and detailed calibration in previous models.

Methods A custom-designed three-point bending fracture induction system was developed using AutoCAD software based on the original principle introduced by Einhorn et al. (1984). After manufacturing and calibration, the system was validated first using 22 cadaveric rat femurs and subsequently applied to live rats ($n=44$), including diabetic (streptozotocin-induced, $n=22$) and non-diabetic animals ($n=22$). Fracture induction reproducibility was assessed through radiographic, histologic and mechanic analysis. Additionally, statistical analysis was conducted using GraphPad Prism 9 software. Coefficients of variation (CV) for fracture-healing parameters (callus diameter, calcification ratio, maximum bending force at re-fracture) were calculated and compared statistically with similar parameters from previously published rat femoral fracture studies.

Results Cadaveric validation confirmed that the optimal blade travel distance, set as half the femoral diameter, consistently produced standardized transverse fractures without comminution. A significant correlation between body weight and femoral diameter (Femoral diameter = 1.0276 ln [Body weight] − 1.349) allowed accurate preoperative determination of optimal blade travel distance. Live animal testing demonstrated consistent fracture patterns, stable intramedullary pin fixation, and no complications during surgical procedures. Statistical analysis revealed significantly lower coefficients of variation for healing parameters in this study compared to previously published models ($p<0.05$). Histological analysis indicated the fracture type was transverse. Callus was found around fracture site.

Conclusion Our validated three-point bending system significantly enhances reproducibility, consistency, and methodological rigor in animal fracture research. This standardized model provides an ideal foundation for future preclinical studies investigating diabetic fracture healing mechanisms and potential therapeutic interventions.

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Keywords Three-point bending system, Diabetic fracture model, Rat femur, In vivo fracture induction, STZ diabetes model

Background

Fracture healing impairment in patients with diabetes mellitus (DM) presents a significant clinical challenge due to an increased incidence of delayed union and nonunion [1, 2]. Diabetes currently affects approximately 537 million adults worldwide, a number projected to reach 643 million by 2030 and 783 million by 2045 [3]. Globally, fracture incidence remains high, with roughly 37 million fragility fractures occurring annually among older adults [4]. Such a considerable clinical burden necessitates reliable and reproducible preclinical models to facilitate research into the biological mechanisms and treatment of impaired fracture healing associated with diabetes.

Experimental animal models play a crucial role in investigating the underlying mechanisms of delayed fracture healing and evaluating potential therapeutic interventions. Despite extensive use, current fracture induction systems for small animals often exhibit significant methodological limitations. Most notably, these systems frequently lack detailed calibration data and thorough validation processes, resulting in inconsistent fracture types, locations, and severities [5, 6]. These methodological shortcomings introduce substantial variability, hindering accurate comparisons across different studies and diminishing the reliability of results.

This study addresses these critical gaps by designing and rigorously validating a custom three-point bending system for producing standardized mid-shaft transverse femoral fractures in rats. The primary aim is to establish a reproducible fracture induction method that ensures consistent fracture morphology and biomechanical conditions, significantly reducing experimental variability. Precise calibration based on the correlation between rat body weight and femoral diameter is utilized to further enhance consistency. By validating fracture induction first in cadaveric specimens and subsequently in diabetic and non-diabetic live animals [7], we demonstrate the effectiveness and reproducibility of this novel system, thus providing a robust methodological foundation for future research into diabetic fracture healing mechanisms and therapeutic interventions.

Materials and methods

Ethical approval

All experimental procedures involving animals were reviewed and approved by the Ethical Committee of the University of Dundee. The study was conducted in accordance with institutional and national guidelines for the care and use of laboratory animals.

Three-point bending system: final design and functionality

The three-point bending fracture induction system utilized in this study was custom-designed and developed specifically to address methodological limitations in previously reported fracture models. The original conceptual foundation of this system was based on the classic fracture induction concept introduced by Einhorn and colleagues in 1984 [5]. Building upon their initial idea, we precisely engineered our fracture induction device using AutoCAD® software, followed by production and assembly assistance provided by the Department of Orthopaedic & Trauma Surgery at the University of Dundee. The detailed design, calibration, and systematic validation of this custom system represent significant methodological advances, enhancing reproducibility and consistency in animal fracture research.

Briefly, this custom-designed system consists of several essential components, each contributing crucially to precise and reproducible fracture induction. These key components include an adjustable blade travel control system, adjustable femur supporters, an anti-rotational constraining mechanism, and a controlled weight-driven impact system (Fig. 1). Each component's structure and function will be described individually in the subsequent sections.

The blade travel control mechanism of the custom-designed three-point bending system consists of adjustable rods, enabling precise and reproducible control of blade penetration depth. Initial cadaveric testing indicated that setting the blade travel distance to approximately half the femoral midshaft diameter consistently produced complete transverse fractures, effectively preventing excessive penetration and avoiding comminuted fractures.

The system includes two adjustable femoral supporters that provide stable positioning of the femur during fracture induction. These supporters can be adjusted to accommodate femurs of varying lengths, ensuring consistent placement perpendicular to the impact blade. Specifically, the greater trochanter and lateral condyle rest on opposing supporters, stabilizing the femur securely and maintaining precise alignment necessary for reproducible fracture patterns.

To maintain the precise vertical orientation and prevent undesirable rotation of the impact blade, a constraining rod was incorporated into the design. Blade rotation upon impact can result in irregular fracture patterns, such as spiral or oblique fractures, thereby undermining reproducibility. Thus, this constraining rod

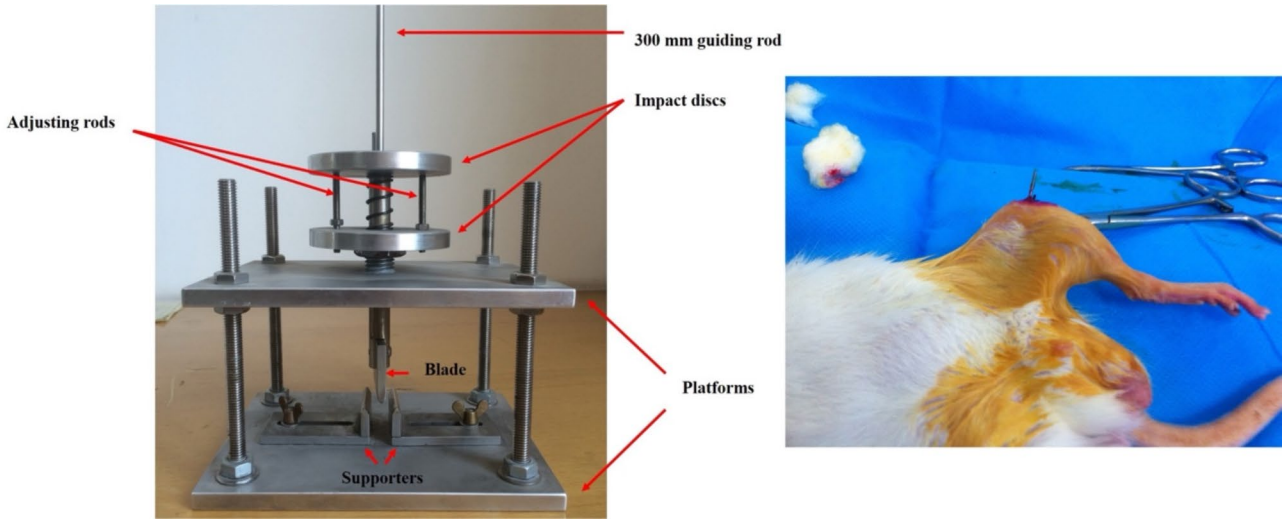

Fig. 1 Self-designed three-point bending system and intramedullary pinning in rats. Left: The custom-built three-point bending system, showing its key components for controlled fracture induction. Right: The surgical procedure for intramedullary pin fixation in rats before fracture induction

ensures uniform force application, enhancing the accuracy and consistency of fracture morphology.

A weight-driven impact mechanism was carefully implemented using a standardized 500-gram weight released from a fixed height of 300 mm. This controlled force application guarantees consistent fracture induction. Additionally, the integrated guiding track ensures linear motion of the weight, effectively eliminating lateral displacement or deviation during blade descent. This design guarantees that fracture induction occurs under precise and reproducible biomechanical conditions, significantly reducing variability and ensuring consistency across experiments.

Surgical procedure: retrograde intramedullary pin fixation and postoperative care

All femurs underwent retrograde intramedullary pin fixation to ensure stability during and after fracture induction (Fig. 1). The surgical procedure was performed on both cadaveric and live rats, with additional anesthesia and postoperative care protocols applied to live animals.

Preoperative preparation and anesthesia (For live animals only)

For live animal procedures, anesthesia was administered before surgery to ensure proper analgesia. Rats were anesthetized using inhalation anesthesia (isoflurane) in an induction chamber. Once stabilized, a sciatic nerve block was administered to provide regional pain control during and after surgery.

Intramedullary pin fixation (For both cadaveric and live animals)

The femur was surgically exposed via a longitudinal incision medial to the ligamentum patellae. The patella was retracted laterally, exposing the distal femoral joint capsule. Using a hand drill, a 1.0 mm K-wire was inserted retrograde through the distal femur, passing into the medullary cavity and stopping at the proximal femoral cortex. This ensured rigid internal fixation before fracture induction. The protruding wire end was trimmed to prevent joint mobility restriction. Then the incision was closed.

Postoperative treatment (For live animals only)

After surgery, rats were given analgesic treatment, consisting of ibuprofen supplemented in drinking water for five days postoperatively. Animals were closely monitored for signs of distress, infection, or weight loss, with daily assessments of wound healing and locomotor activity. No external fixation was required, as the intramedullary pin provided sufficient stabilization.

Cadaveric validation of the system

A total of 22 cadaveric normal rat femurs were used to validate the three-point bending system before in vivo application. Each femur was positioned on the adjustable supporters, with the greater trochanter placed on one supporter and the lateral condyle resting on the other supporter to ensure proper alignment perpendicular to the impact blade. The blade was driven by a 500 g weight falling from a 300 mm height. The travel distance of the blade was set to be half the diameter of rat's mid-femur. The blade performed a sudden impact on the middle and medial part of rat's femur to induce fracture. After fracture induction, the femurs were carefully dissected and extracted from the surrounding soft tissues for direct visual inspection of the fracture morphology (Fig. 2). Another 22 rat cadavers from different age groups were collected from our colleagues. All 44 cadavers'

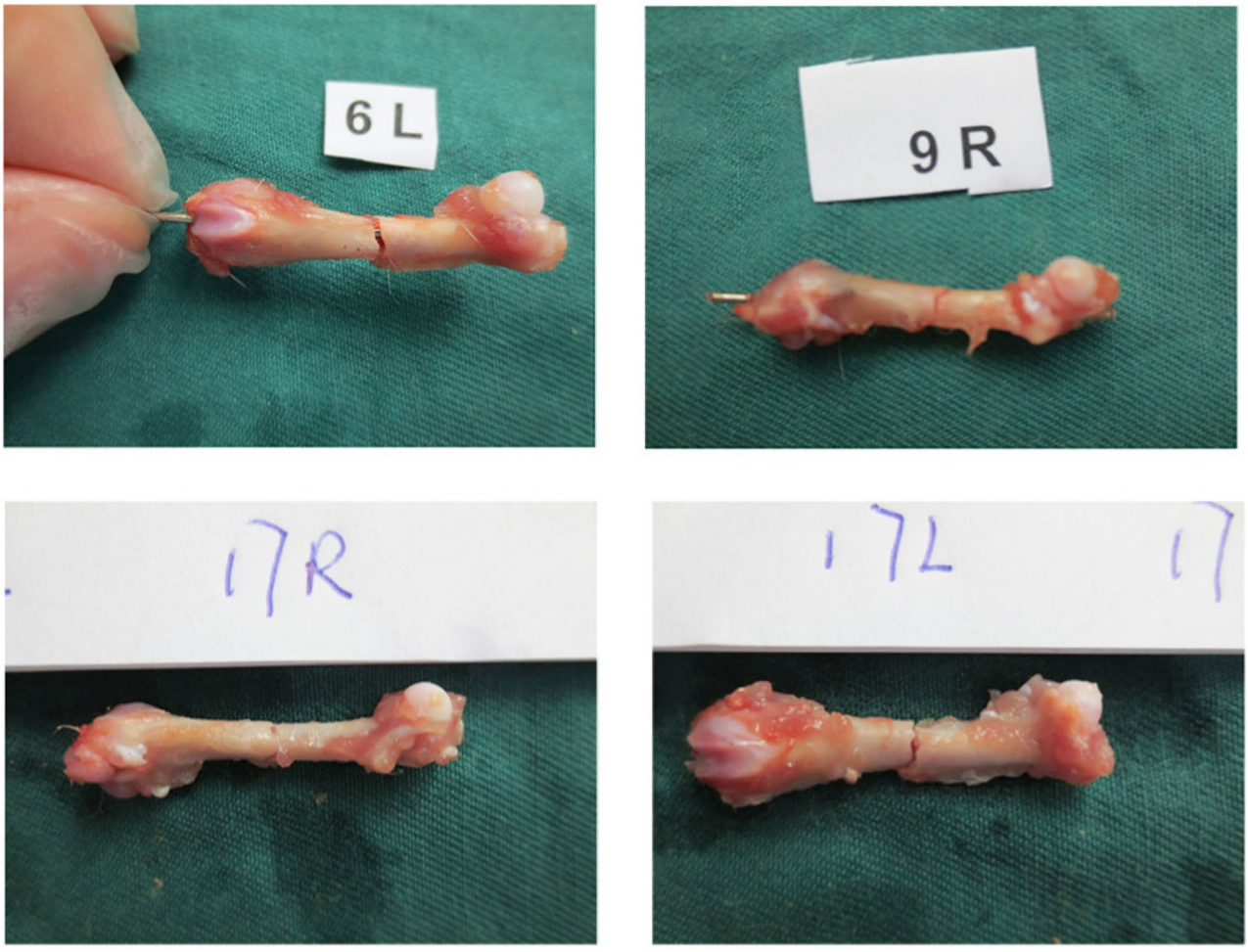

Fig. 2 Fractured femurs from cadaver test. Harvested cadaver femurs with soft tissues removed, displaying transverse fracture patterns induced by the three-point bending system

body weight and femoral diameter were recorded and analyzed.

Application in live diabetic and non-diabetic rats

Healthy male Sprague-Dawley rats weighing 255 ± 7 g were randomly assigned to diabetic group ($n = 22$) and a non-diabetic control group ($n = 22$), according to previous study [4] and statistical power analysis. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ). Two weeks after STZ injection, fractures were successfully induced with the same technique described above and confirmed by X-ray. Radiological, histological and mechanical tests were performed at the end of the 2nd, 4th and 8th post-fracture weeks to compare the healing procedure of diabetic and non-diabetic rats.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, CA, United States). Data passing normality tests were expressed as mean ± standard deviation (SD). Student's t-test was used for two-group comparisons, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted for multiple-group comparisons. Pearson's correlation coefficient was calculated to evaluate correlations between continuous variables. Statistical significance was defined as a p-value of less than 0.05.

To further assess reproducibility and methodological consistency of our custom-designed three-point bending fracture induction system, we calculated and analyzed the coefficient of variation (CV) for key fracture-healing parameters, specifically including callus diameter, callus area, and maximum bending force at re-fracture. These CV values obtained from our study were compared directly with similar parameters reported in previously published rat femoral fracture models, as summarized in Table 1. Comparative analysis included studies from Breitbart et al. (2010) [8], Dedania et al. (2011) [9], Campos et al. (2022) [10], and Azad et al. (2009) [11].

Table 1 Coefficient variation of fracture healing parameters (%)

	This research	Previous research
Fracture healing parameters	**Coefficient variation**	**Coefficient variation**
callus diameter/femoral diameter at 4th week:		
DM control	8.35% ($n=8$)	
Gene therapy	21.38% ($n=10$)	
Empty vector	16.07% ($n=4$)	
Calcification ratio of calluses at 4th week:		
DM control	12.16% ($n=8$)	
Gene therapy	8.45% ($n=10$)	
Empty vector	10.81% ($n=4$)	
Biomechanical endurance (KPa) at 4th week:		
Non-DM Control	18.22% ($n=5$)	
DM control	23.68% ($n=6$)	
DM Gene therapy	12.12% ($n=7$)	
Empty vector	18.7% ($n=4$)	
		Azad et al., 2009
Total new bone area at the 3rd week(mm^2):		
Non-DM + buffer		33.88% ($n=4$)
Non-DM + rhBMP-2		20.55% ($n=4$)
DM + buffer		46.43% ($n=4$)
DM + rhBMP-2		12.67% ($n=4$)
Biomechanical Testing at 9th Week in rhBMP-2:		
Torque to Failure (Nm)		56.28% ($n=4$)
Torsional Rigidity (Nm/rad)		20.94% ($n=4$)
Shear Modulus		74.17% ($n=4$)
Maximum Shear Stress (MPa)		105.91% ($n=4$)
		Breitbart et al., 2010
Bone area: Endosteal bone at 4th week (mm^2):		
Control		56.52% ($n=7$)
DM + mesenchymal stem cell (MSC)		39% ($n=5$)
Bone area: Periosteal bone (mm^2):		
Control		148% ($n=7$)
DM + mesenchymal stem cell (MSC)		91.52% ($n=5$)
		Dedania et al., 2011
Bone area: Endosteal bone at 4th week (mm^2):		
Control		27.86% ($n=6$)
DM + insulin		25.47 ($n=7$)
Bone area: Periosteal bone (mm^2):		
Control		72% ($n=6$)
DM + insulin		68.97% ($n=7$)
		Campos et al., 2022
TRAP positive area at callus% at 4th week:		
SHAM		17.24% ($n=7$)
SHAM + VT		32.32% ($n=7$)
DM		33.53% ($n=7$)
DM + VT		26.92% ($n=7$)

CV, coefficient variation; DM, diabetes mellitus; rhBMP-2, recombinant human bone morphogenetic protein-2; VT, vibration therapy

Results

A significant correlation was identified between the body weight of the rats and the diameter of the femoral midshaft, allowing for a predictive approach to setting the blade travel distance in the three-point bending system. Statistical analysis confirmed that larger body weights corresponded to increased femoral diameters, following a logarithmic growth pattern. This relationship was mathematically expressed as: Femoral diameter = 1.0276 ln (Body weight) – 1.349 (Fig. 3). Using this equation, the blade travel distance was adjusted preoperatively based

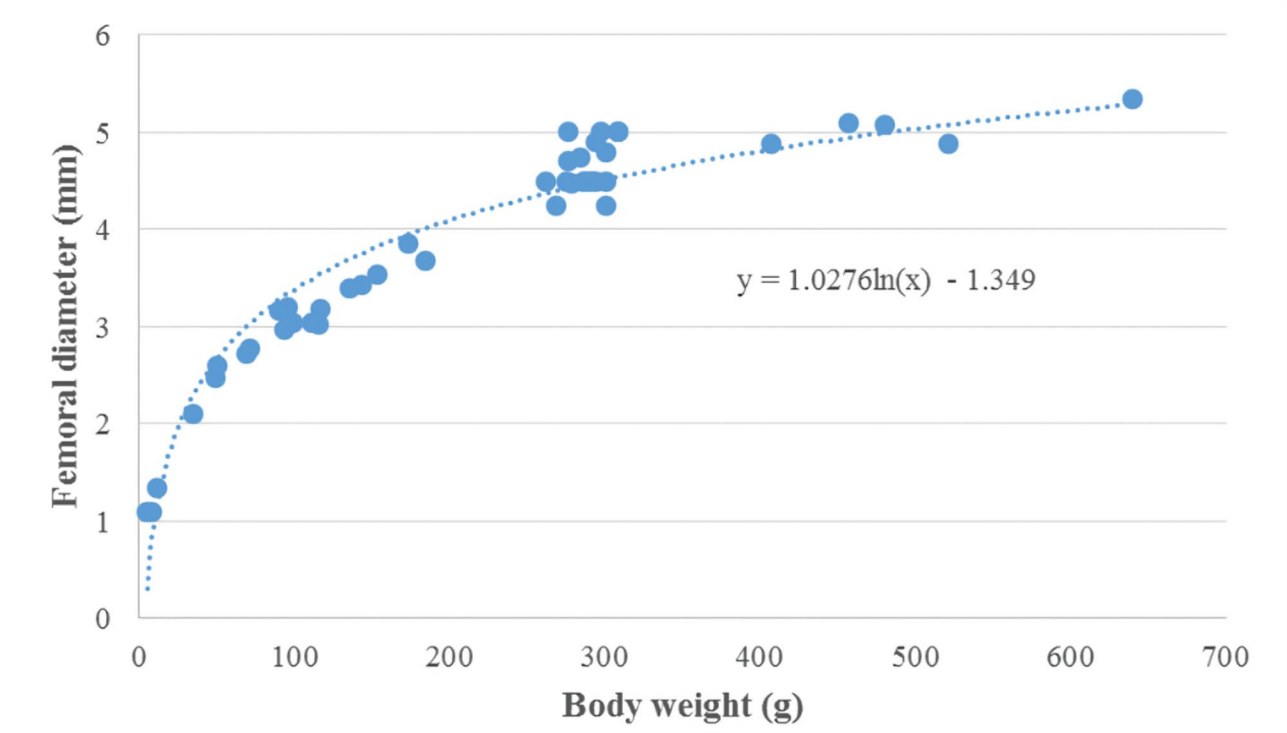

Fig. 3 Correlation between rats' body weight and femoral diameter. A logarithmic correlation between body weight and femoral midshaft diameter, allowing preoperative estimation of blade travel distance

on body weight, ensuring consistent and precise fracture formation across different animal sizes.

The cadaveric validation of the system further confirmed its effectiveness and reproducibility. The fracture type was consistently transverse, with no evidence of comminution or spiral deformation (Fig. 2). Additionally, all fractures were located at the midshaft region, aligning with the intended impact site. The intramedullary pin fixation remained stable in all samples, preventing displacement at the fracture site.

After successful cadaveric validation, the custom-designed three-point bending system was applied in vivo to induce femoral fractures in rats, including both diabetic and non-diabetic animals. All surgical procedures were successfully completed without complications, and fractures were confirmed via postoperative X-ray imaging, which demonstrated consistent mid-shaft transverse fractures in the left femur of each rat (Fig. 4a, b). The intramedullary pins were accurately placed, ensuring stable fixation at the fracture sites. The healing procedure of fracture was also evaluated via histological tests (Fig. 4c). In our previously published comparative study, diabetic rats exhibited significantly impaired healing outcomes when compared to their non-diabetic counterparts. Specifically, histological analyses indicated reduced callus formation in diabetic animals, and mechanical testing confirmed that the force required to re-fracture the callus was significantly diminished in the diabetic group, highlighting compromised biomechanical integrity in diabetic fracture healing [6]. These findings underscore the utility of our validated three-point bending system in reliably producing standardized fractures and facilitating robust preclinical investigations into diabetic fracture healing mechanisms.

The reproducibility and methodological consistency of fracture induction and healing achieved by our custom-designed three-point bending system were quantitatively assessed by calculating coefficients of variation (CV) for key fracture-healing parameters. Specifically, CV values were determined for callus diameter, calcification ratio and maximum bending force at re-fracture in our rat femoral fracture model. To objectively evaluate our model's performance, these CV values were directly compared with those reported in previously published studies employing similar fracture models. The comparative analysis included studies conducted by Breitbart et al. (2010) [8], Dedania et al. (2011) [9], Campos et al. (2022) [10], and Azad et al. (2009) [11] (Table 1).

Statistical comparisons demonstrated that the CV values obtained from our custom-designed three-point bending fracture induction system were significantly lower ($p < 0.05$) than those reported in these previous studies (Fig. 5). The lower CV values indicate superior reproducibility, reduced variability, and improved

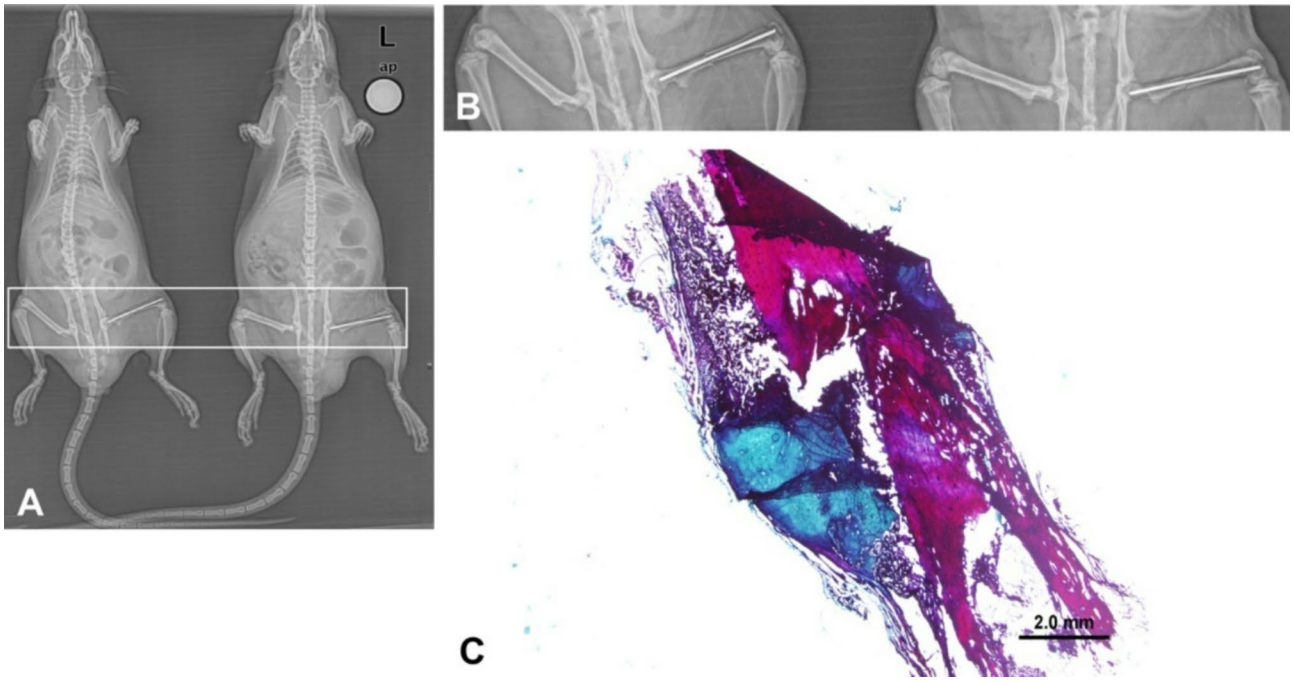

Fig. 4 Radiographic and histological assessment of the custom three-point bending fracture model in rats. (**A**) Representative postoperative X-ray image showing three rats with successfully induced mid-shaft transverse femoral fractures in the left femur using the custom-designed three-point bending system. Intramedullary pins are precisely placed, ensuring stable fixation without displacement or comminution. (**B**) Magnified X-ray view of the fracture site, highlighting the controlled transverse fracture pattern and accurate alignment of the intramedullary pin. This zoomed-in image provides a detailed view of the fracture morphology and confirms the consistency of the fracture model. (**C**) Histological analysis of the fracture callus stained with Masson's trichrome. Cortical bone is stained red, while cartilaginous tissue appears blue. The histological evaluation reveals callus formation at the fracture site, facilitating assessment of bone regeneration

consistency in fracture patterns and healing outcomes in our experimental model. These statistical findings confirm the methodological advantages of our system, particularly highlighting its reliability and potential as a robust platform for future research on impaired fracture healing, especially in diabetic animal models.

Discussion and conclusion

In this study, we successfully developed and comprehensively validated a custom-designed three-point bending system for inducing precise and reproducible mid-shaft transverse femoral fractures in rats. Unlike previously reported fracture induction methods, which often lacked rigorous technical documentation and reproducibility, our system directly addresses these critical methodological limitations through meticulous engineering, detailed calibration, and extensive validation.

A particularly novel aspect of this research is the establishment of a robust correlation between rat body weight and femoral midshaft diameter. This correlation, defined mathematically as Femoral diameter = 1.0276 ln (Body weight) – 1.349, allowed precise preoperative determination of the optimal blade travel distance. Statistical analysis confirmed the strength and reliability of this predictive relationship, significantly enhancing procedural accuracy, reducing unwanted fracture patterns, and ensuring biomechanical uniformity across specimens. Consequently, this correlation significantly enhances the consistency and reliability of experimental outcomes, crucial for comparative studies on fracture healing.

Cadaveric validation results demonstrated that our system consistently produced uniform, controlled mid-shaft transverse fractures, effectively eliminating the occurrence of unwanted spiral or comminuted fractures. Subsequent in vivo application further validated the system's practicality and reproducibility. Radiographic examinations consistently confirmed precise fracture morphology, accurate intramedullary pin placement, and stable fracture fixation in all animal specimens. These results reinforce the suitability of the system for rigorous preclinical studies, especially those focusing on conditions such as diabetes mellitus (DM), where fracture healing impairment is a recognized clinical issue.

In our previously published study utilizing this validated fracture model, diabetic rats exhibited significantly impaired healing outcomes compared to non-diabetic counterparts. Histological analyses demonstrated dramatically reduced callus formation in diabetic animals, consistent with slower and compromised healing processes. Correspondingly, mechanical testing indicated

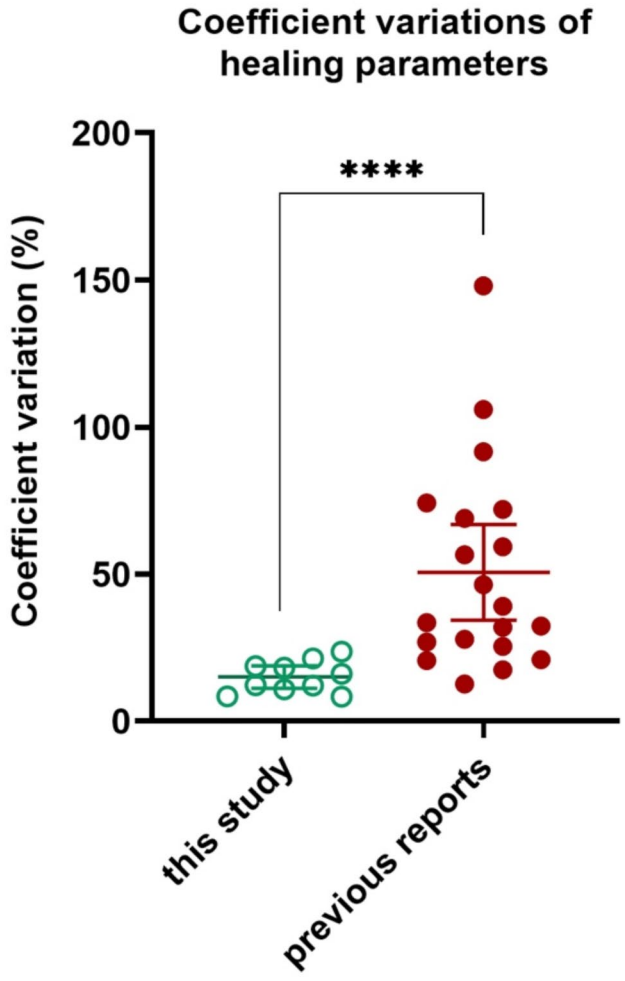

Fig. 5 Difference in coefficient variations of the healing parameters in this study and previous reports in the literature. Significantly smaller coefficient variations in this study reveal advantages in the healing parameters. ****, $p < 0.0001$

significantly reduced strength of the healed bone callus, as reflected by lower re-fracture force values in diabetic rats. Such results clearly demonstrate the utility of this fracture model in precisely replicating diabetes-induced delays in fracture repair, providing clinically relevant insights into diabetic fracture healing impairment.

Importantly, the reliability and reproducibility of animal models significantly influence research quality and interpretability, a point reinforced by similar studies. For example, Manti et al. utilized a rigorously defined animal model to evaluate the effects of amniotic membrane treatment on Achilles tendon healing, emphasizing that consistent methodological approaches markedly improve reliability and comparability of histological and clinical outcomes [12]. In line with their findings, our standardized fracture induction system similarly ensures methodological rigor and reproducibility, greatly enhancing the reliability and scientific robustness of fracture healing research, particularly in pathological states such as diabetes.

Additionally, statistical analyses of fracture consistency further underscore the advantages of our system. Specifically, coefficient of variation (CV) analysis of fracture-related parameters demonstrated significantly smaller variations compared to previously published fracture models. Statistical analyses, conducted using GraphPad Prism 9 software, included Student's t-test and one-way ANOVA for inter-group comparisons and Pearson's correlation for determining the relationship between continuous variables. A threshold of statistical significance was set at $p < 0.05$. The observed lower variability highlights the superior methodological consistency and reliability of our custom fracture induction model, an essential attribute for preclinical studies requiring precise comparative analyses.

The primary advantages of our custom-designed fracture induction system include detailed technical documentation, reproducibility of fracture induction, reduced experimental variability, and adaptability to animal size variations. By overcoming critical methodological gaps identified in previous animal fracture studies, our system provides an invaluable experimental foundation for accurately investigating fracture healing mechanisms and evaluating novel therapeutic strategies, especially in diabetic models.

Future research directions include leveraging this validated model to explore a wide array of therapeutic interventions, including pharmacological, biomaterial-based, and gene therapy approaches aimed at enhancing fracture healing in diabetic conditions. Furthermore, investigating healing responses under different diabetic severities and durations may offer deeper insights into diabetes-induced impairment mechanisms, thus broadening the clinical implications of these findings.

In conclusion, this study presents a significant methodological advancement through the comprehensive validation of a novel, custom-designed three-point bending system for standardized fracture induction in rats. By ensuring reproducibility, biomechanical consistency, and precise control, this validated model offers a robust platform for future orthopedic research, particularly focused on understanding and improving diabetic fracture healing outcomes.

Abbreviations

ANOVA	Analysis of variance
AutoCAD	Automatic Computer-Aided Design
CV	Coefficient of variation
DM	Diabetes mellitus
DM	Diabetes mellitus
MSC	Mesenchymal stem cells
rhBMP-2	Recombinant human Bone Morphogenetic Protein-2
SD	Standard deviation
SHAM	Surgical control group without intervention
STZ	Streptozotocin

STZ	Streptozotocin
TRAP	Tartrate-resistant acid phosphatase

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Author contributions
QDG reviewed the literatures, designed the research, performed statistical analysis, and wrote the manuscript. WJW and ZG designed the research and critically revised the manuscript. The author(s) read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Academy and Ethics Committee of University of Dundee and conformed to the Guide for the Care and Use of Laboratory animals.

Consent for publication
This paper is approved by all authors for publication.

Competing interests
The authors declare no competing interests.

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