■ TRAUMA

The effect of haemorrhagic shock and resuscitation on fracture healing in a rabbit model

AN ANIMAL STUDY

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Aims

Little is known about the effect of haemorrhagic shock and resuscitation on fracture healing. This study used a rabbit model with a femoral osteotomy and fixation to examine this relationship.

Materials and Methods

A total of 18 male New Zealand white rabbits underwent femoral osteotomy with intramedullary fixation with 'shock' (n = 9) and control (n = 9) groups. Shock was induced in the study group by removal of 35% of the total blood volume 45 minutes before resuscitation with blood and crystalloid. Fracture healing was monitored for eight weeks using serum markers of healing and radiographs.

Results

Four animals were excluded due to postoperative complications. The serum concentration of osteocalcin was significantly elevated in the shock group postoperatively (p < 0.0001). There were otherwise no differences with regard to serum markers of bone healing. The callus index was consistently increased in the shock group on anteroposterior (p = 0.0069) and lateral (p = 0.0165) radiographs from three weeks postoperatively. The control group showed an earlier decrease of callus index. Radiographic scores were significantly greater in the control group (p = 0.0025).

Conclusion

In a rabbit femoral osteotomy model with intramedullary fixation, haemorrhagic shock and resuscitation produced larger callus but with evidence of delayed remodelling.

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Trauma is the leading cause of mortality in the young and causes significant morbidity with an accompanying cost to health systems and society.[1] More than 60% of patients who suffer major trauma have an orthopaedic injury that requires surgery.[2] About 70% of transfusion protocol activations are for patients with orthopaedic injuries following blunt trauma.[3] Furthermore, despite modern methods of treatment, at least 25% of lower limb long-bone fractures develop delayed union or nonunion,[4] indicating that our understanding of fracture healing, particularly when associated with major trauma, remains incomplete.

The healing of a fracture is mediated by local processes that are influenced by the body's systemic inflammatory milieu.[5] There is likely to be some overlap between the first phase of healing and the systemic response to haemorrhagic shock and resuscitation, as both involve inflammatory processes.[6,7] A better understanding of the effect of haemorrhagic shock on the healing of fractures may allow improved treatment strategies that achieve expeditious union with fewer delayed unions and nonunions, with more rapid functional recovery for patients, and reduced economic burden.

The authors of clinical[8] and basic science[9] studies have attempted to define the optimal timing for the surgical management of major musculoskeletal injuries in patients experiencing haemorrhagic shock, in order to minimize complications. However, our knowledge of the effect of haemorrhagic shock and its resuscitation on fracture healing is limited. Animal studies have produced conflicting results,[10-13] have been limited by short follow-up and have used methods of fixation and resuscitation that poorly reflect clinical practice.

Wichmann et al[13] found a detrimental effect of haemorrhagic shock on fracture healing based

upon plasma osteocalcin concentration and osteocyte necrosis at three days post-tibial fracture in 18 mice. These findings were supported by Lichte et al[11] who used histology and micro-CT to show delayed healing in the femora of 28 mice at three weeks post-injury. However, Bumann et al[10] found that the fractured tibiae of rats that experienced haemorrhagic shock had better blood flow in the first 24 hours post-injury and superior mechanical properties at four weeks than those that were not shocked. Starr et al[12] found no effect of haemorrhagic shock on the healing of goat tibiae four weeks post-injury, based upon biomechanical, radiological and histomorphometric parameters.

The aim of this study was to investigate the effect of haemorrhagic shock and resuscitation on fracture healing using an animal model and clinically relevant methods of resuscitation and fixation. The hypothesis was that haemorrhagic shock and resuscitation enhance fracture healing, as assessed by biochemical and radiological parameters. This hypothesis was based upon the possibility that the systemic inflammatory state triggered by haemorrhagic shock[6] may stimulate the inflammatory processes that mediate fracture healing.

Materials and Methods

The study had ethical approval. A total of 18 male New Zealand white rabbits, weighing between 2.5 kg and 3.5 kg, were randomly allocated to shock (n = 9) and control (n = 9) groups.

Surgical procedure. Using previously described methodology, the rabbits were anaesthetized, intubated, ventilated and monitored.[9] A central venous line was inserted in the shock group through the external jugular vein as previously described.[14] Those in the control group underwent a sham procedure with a skin incision and superficial dissection only. Subcutaneous enrofloxacin 10 mg/kg (Baytril, Bayer Corporation, Leverkusen, Germany) was given as antibiotic prophylaxis. In order to create an osteotomy, animals were placed in the lateral position, the right hind-limb was shaved and a medial parapatellar incision made under sterile conditions. The patella was dislocated laterally and the distal femoral articular surface exposed. Using a 3 mm manual drill, the medullary canal was accessed and reamed. A retrograde intramedullary nail (RabbitNail, RISystem, Davos, Switzerland) was introduced using an aiming device.[15] A longitudinal incision was made over the lateral aspect of the mid-shaft of the femur and a 0.44 mm Gigli Wire Saw (RabbitNail) placed around the femur. Using a guide (RabbitNail) mounted on the aiming device, a transverse mid-shaft femoral osteotomy was undertaken. When the saw reached the nail it was backed out and the osteotomy completed. The nail was then reintroduced and locked with one proximal and two distal pins. The patella was reduced and the wounds were sutured and dressed.

Haemorrhagic shock and resuscitation. Following the osteotomy, haemorrhagic shock was induced in the study group. The total blood volume (TBV) of New Zealand white rabbits has previously been found to be 54 ml/kg ± 2 ml/kg.[16] Using a pump (Harvard Apparatus, Cambridge, Massachusetts), 35% of the TBV was withdrawn through the central venous line over 15 minutes. Hypovolaemic shock was maintained for an additional 45 minutes, during which withdrawn blood was stored in a syringe at body temperature. 250 IU/kg heparin was administered intravenously prior to the induction of hypovolaemia in order to prevent withdrawn blood from clotting. In order to measure the effect of haemorrhage, mean arterial pressure (MAP), arterial base excess and haematocrit were recorded at regular intervals using methods previously described.[9]

After one hour of induced hypovolaemia, the withdrawn blood was reinfused over a period of an hour. Crystalloid resuscitation was given using Hartmann's Solution to a volume of 5% of TBV per hour, for four hours. Animals in the control group also received crystalloid resuscitation to compensate for the loss of volume relating to the osteotomy, fixation, urine and respiration. The total anaesthetic time after the osteotomy was five hours in both groups.

Postoperative care. After resuscitation, anaesthesia was withdrawn and the rabbits returned to their pen. Subcutaneous analgesia involved carprofen 4 mg/kg regularly for the first 48 hours. They were monitored for signs of pain and additional analgesia was given subcutaneously, as needed, using buprenorphine 0.05 mg/kg. Enrofloxacin 10 mg/kg (Baytril) was administered subcutaneously every 12 hours for seven days. The rabbits were cared for by the investigators and university staff daily until death eight weeks postoperatively.

Serum immunoassays. Venous samples, equal in volume to 1% of TBV, were obtained pre- and postoperatively and weekly for eight weeks. Serum was separated and stored at -85°C and later thawed for use with commercially available immunoassays to test for markers of bone healing, namely osteocalcin (OC; Quidel Corporation, San Diego, California), bone-specific alkaline phosphatase (B-ALP; Quidel Corporation) and N-terminal polypeptide (PINP; Mybiosource, San Diego, California). B-ALP is an enzyme released by osteoblasts, mainly in early fracture healing. OC is a circulating protein that is synthesized by osteoblasts and is a measure of their activity. PINP is cleaved from pro-collagen during the formation of type I collagen and increases in concentration as type III collagen is replaced by type I late in fracture healing.[17]

Radiological monitoring. Plain radiographs of the femur were obtained within 12 hours of surgery to confirm the creation of a transverse mid-shaft osteotomy and the adequacy of fixation. Radiographs were then taken weekly until death at eight weeks. The rabbit was sedated using intravenous propofol 5 mg/kg and anteroposterior (AP) and lateral radiographs were undertaken using a Philips General Bucky Table x-ray facility (Philips, Eindhoven, Netherlands) and processed by a Fuji Profect CS reader (Fujifilm, Tokyo, Japan) on computed radiological digital imaging plates.

Using AMICAS (Merge Healthcare, Chicago, Illinois), a web-based picture archiving and communication system (PACS), weekly radiographs for each rabbit were independently reviewed in chronological order, by two blinded radiologists. A scoring system adapted from previous studies was used to assess the extent of healing, with points awarded for periosteal reaction, union and remodelling, to a possible maximum of ten (Table I).[18,19]

Table I. The scoring system used by two blinded radiologists to assess the degree of fracture healing each week until eight weeks postoperatively

Category	Description	Score
Periosteal reaction	None	0
	Minimal (localized to gap)	1
	Medium (extends over gap > 1/4)	2
	Moderate (> 1/2 but < 3/4)	3
	Full	4
Bone union	None	0
	Mild bridge (< 50%)	1
	Moderate bridge (> 50%)	2
	Union	3
Remodelling	No remodelling	0
	Early remodelling	1
	Moderate remodelling	2
	Advanced remodelling	3
Maximum score		10

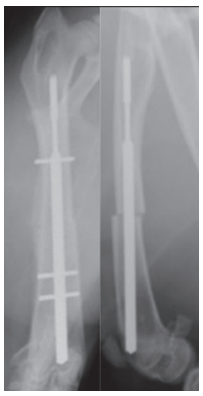

Fig. 1a

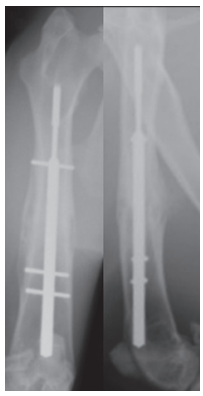

Fig. 1b

Anteroposterior and lateral radiographs taken a) postoperatively and b) at eight weeks showing the osteotomy, intramedullary nail, locking pins and development of callus.

The callus index is the ratio of the maximum diameter of the callus to the diaphyseal diameter.[20,21] The mean of the radiologists' scores for a radiograph was accepted if they differed by only one point. If the difference between scores was > one point, a score was reached by consent. Each radiograph was reviewed in chronological order and the callus index calculated independently each week by two blinded investigators using that week's maximum diameter of callus and the diaphyseal diameter 4 mm proximal to the osteotomy, measured from the immediate postoperative radiographs. The diaphyseal diameter was always calculated from postoperative films as callus impaired the subsequent accurate measurement of the diaphyseal cortex. For each radiograph, the mean of the two investigator's measurements was accepted if the interobserver variation was < 0.5 mm. All investigators remeasured callus index independently if the interobserver variation for a radiograph was ≥ 0.5 mm. If this variation remained ≥ 0.5 mm after remeasurement, a final value was reached by consent.

Statistical analysis. Statistical analysis was performed using SPSS version 19 (IBM Corp., Armonk, New York) and GraphPad Prism version 6 (GraphPad Software, La Jolla, California). Data were assessed for normality using the Shapiro–Wilk test and analyzed using the two-way analysis of variance technique. Data are presented as mean and range, unless otherwise indicated. A p-value of < 0.05 was considered significant.

Results

A total of 18 rabbits underwent a femoral osteotomy and stabilization. However, due to postoperative complications, four were excluded (one in the shock group and three in the control group). Of these, one in the shock group and two in the control group, died from respiratory failure on postoperative days 17, 12 and 14, respectively. Post-mortem examination indicated that the likely cause of death was obstructive bronchitis secondary to lower respiratory tract infection. One rabbit in the control group was killed by euthanasia on the first postoperative day due to an unsuitable osteotomy as identified by radiographs

Thus, a total of 14 rabbits completed the study with eight in the shock group and six in the control group. All had a standard transverse mid-shaft femoral osteotomy and were weight-bearing within 24 hours postoperatively. All surviving rabbits showed radiological evidence of callus formation (Fig. 1).

The state of shock in the shock group involved significant reductions in base excess, haematocrit and mean arterial pressure compared with the control group ($p < 0.0001$) (Fig. 2).

The shock group had a significant increase in OC compared with the control group at the postoperative time-point only (mean 112.7 ng/ml, 86.7 to 137.9 *vs* 67.9 ng/ml, 44.0 to 135.0; $p < 0.0001$). OC levels were decreased from baseline in both groups at all subsequent times and there was no difference

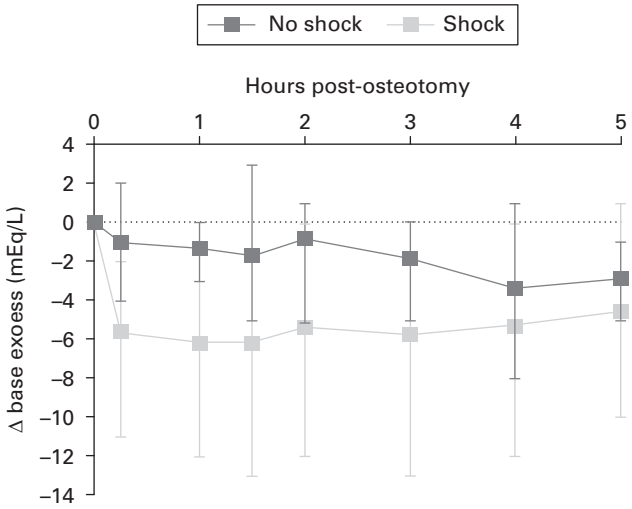

Fig. 2a

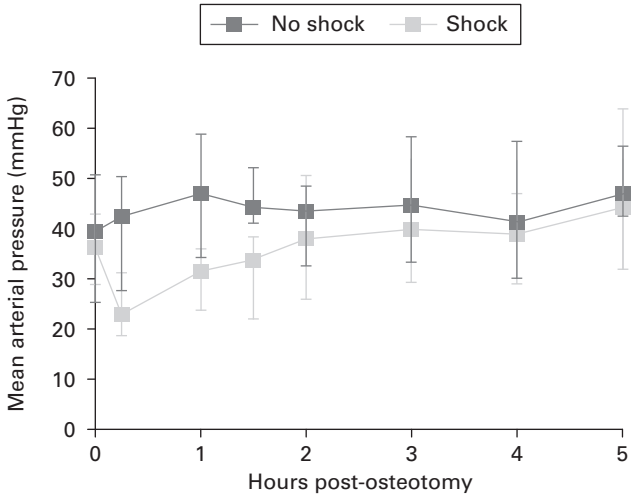

Fig. 2b

Intraoperative measures of a) base excess and b) mean arterial pressure.

between groups overall (p = 0.6912). B-ALP was also reduced in both groups from the first week postoperatively, and there was no difference between groups with regard to B-ALP (p = 0.4491) and P1NP (p = 0.1128) values overall (Fig. 3).

The mean callus index was higher in the shock group beyond week three postoperative (Fig. 4). Although this was not significant at any individual time point in *post hoc* testing, the mean callus index was overall significantly higher in the shock group on AP (p = 0.0069) and lateral (p = 0.0165) views. The mean callus index on both views fell earlier in the control group, which also had significantly higher radiological scores four weeks postoperative (mean 4.9, 3.0 to 6.5 *versus* 3.5, 2.5 to 5.0; p = 0.0401). The radiological scores in the control group were also higher than those of the shock group every week from postoperative week three and this was significant overall (p = 0.0025) (Fig. 5).

The mean difference in callus index measurements between the two blinded investigators (JB and AB) was 0.8 mm (0.0 to 6.9) and there was interobserver agreement of 75%, with 64 of 252 radiographs exceeding the 0.5 mm threshold and requiring remeasurement. The difference between the repeat measurements for 20 radiographs (8%) remained > 0.5 mm, requiring the investigators to reach consensus upon the callus index for these radiographs. The median difference in radiological scores between the radiologists was one point (0 to 5) and there was interobserver agreement of 68% with 41 of 126 pairs of AP and lateral radiographs requiring the radiologists to reach consensus.

Discussion

This study used biochemical and radiological parameters to examine the effect of haemorrhagic shock and resuscitation on fracture healing. Intraoperative measures of base excess, haematocrit and MAP confirmed that the shock which was induced in the shock group correlates with a class III haemorrhage in humans.[22] Our volume-based model of haemorrhage was also consistent with previous studies in which 30%, and between 45% and 50% of TBV, was bled from goats and mice, respectively.[12,13] The 60-minute duration of hypovolaemic shock was similar to that of four previous investigations, which ranged from 40 to 95 minutes.[10-13]

Serum immunoassays of OC, B-ALP, P1NP did not show any significant effect due to shock and resuscitation on the biochemical processes associated with fracture healing. The relevance of the immediate postoperative rise in OC in the shock group is uncertain, but the return of values comparable to the control group one week postoperatively indicates an acute phase response as the most likely mechanism, rather than any sustained effect on fracture healing, whether inflammatory or otherwise. This finding is contrary to that of Wichmann et al,[13] who recorded a significantly lower plasma level of OC at 72 hours in mice in whom shock and a tibial fracture had been induced, compared with controls. However, the serum level of OC was not measured at baseline, postoperatively, or beyond 72 hours.

The reduced levels of OC during the healing period is consistent with the findings of others who reported that OC is reduced for a sustained period following a major physiological injury.[23] Although, to our knowledge, this has not been shown for B-ALP, the reduction of both markers could be explained by the suppression of bone metabolism due to the stress response to trauma. However, the levels of P1NP, a proxy for type I collagen synthesis, were sustained throughout the study period indicating that this aspect of bone metabolism was not affected by the period of hypovolaemic shock.

Our finding of a greater callus index in the shock group after the third postoperative week may reflect hypertrophic callus formation due to the systemic inflammatory state that follows

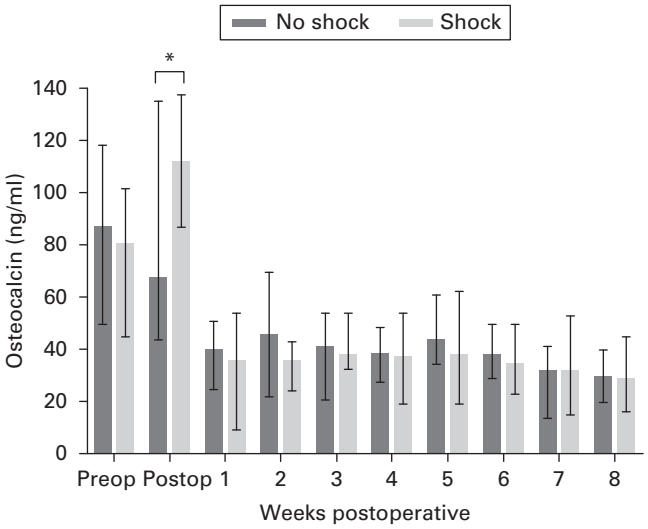

Fig. 3a

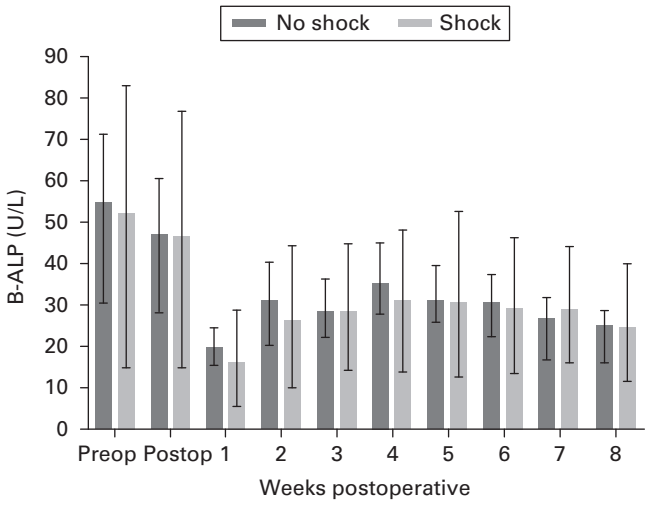

Fig. 3b

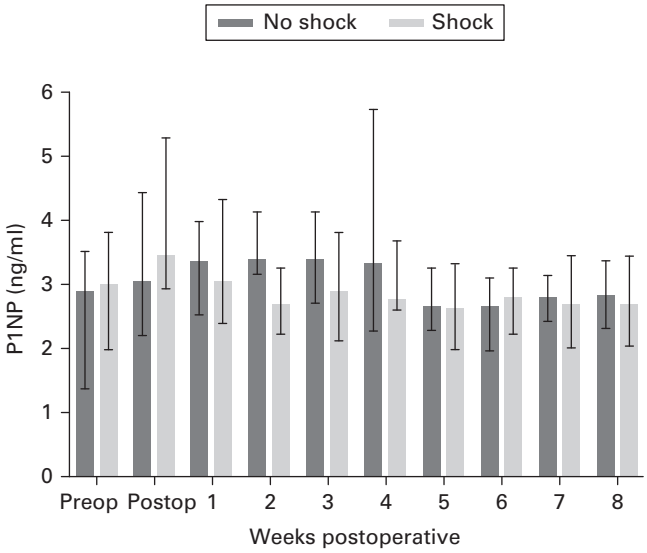

Fig. 3c

Serum levels of a) osteocalcin (OC), b) bone-specific alkaline phosphatase (B-ALP) and c) N-terminal polypeptide (PINP) up to eight weeks postoperatively. There was an increase of OC in the shock group compared with the control group postoperatively ($p < 0.0001$). OC and B-ALP were suppressed in both groups during all postoperative weeks. There was no difference in P1NP levels between the groups or over time.

haemorrhagic shock. This hypothesis is supported by the findings of Lichte et al,[11] who, in a rat model, showed that the levels of pro-inflammatory cytokines, IL-6, KC, MCP-1 and TNF-α are significantly elevated six hours after a fracture. However, the earlier decrease in callus index in the control group may indicate an earlier onset of remodelling and therefore, faster healing despite developing smaller callus over all. This is supported by radiological scores showing earlier union and remodelling in the control group. This situation is analogous to that of hypertrophic nonunion in which the size of the callus does not correlate with the degree of union.

Our findings of earlier and larger callus formation during the inflammatory phase of healing and delayed maturation and remodelling of the callus in the shock group are consistent with the well characterized immunological state which is seen in patients after major trauma.[6] These patients develop a severe early systemic inflammatory response with delayed apoptosis of neutrophils in association with a catabolic state. Although there is the appearance of inflammation, the adaptive immune system is suppressed with a decreased ability to fight infection during the recovery from injury with optimized repair processes and anabolic changes.[24] This similarity between localized and

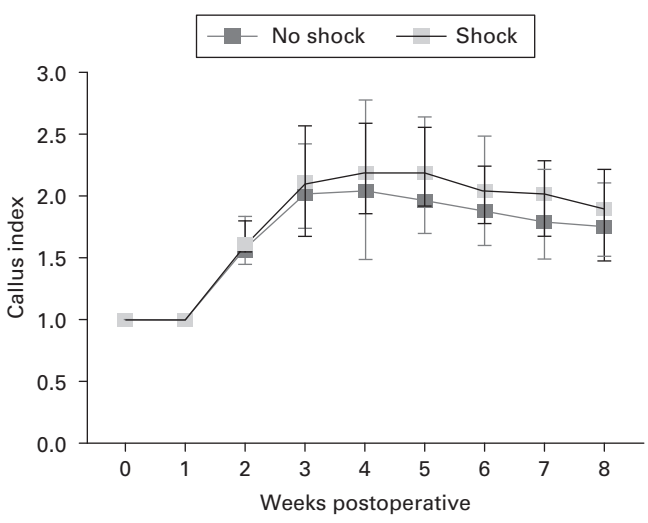

Fig. 4a

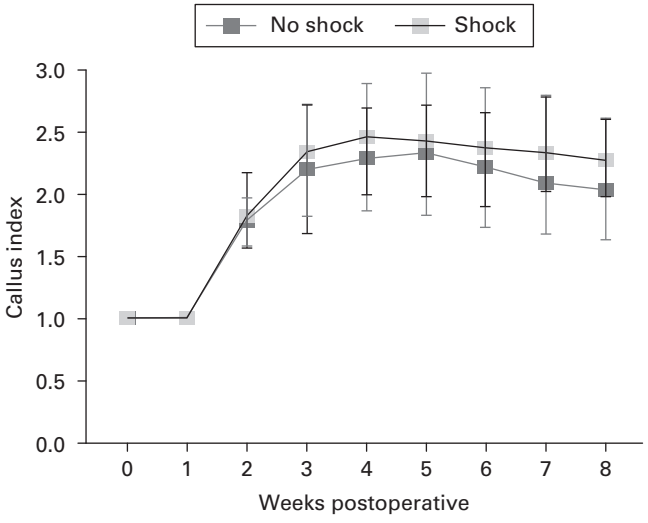

Fig. 4b

The mean callus index in the shock group was consistently elevated compared with the control group in a) anteroposterior (p = 0.0069) and b) lateral (p = 0.0165) radiographs. There was an earlier decrease of the callus index in the control group.

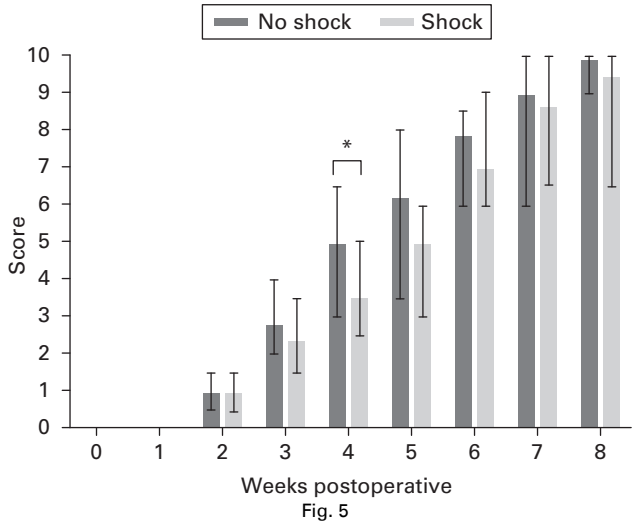

Fig. 5

The radiological scores were increased in the control group compared with the shock group, four weeks postoperatively (p = 0.0401) and were consistently elevated from the third week (p = 0.0025).

systemic responses suggests an opportunity whereby optimizing the immunological state after severe injury could potentially lead to improved rates of union of fractures.

Two previous studies have used radiological methods to investigate the effect of haemorrhagic shock and resuscitation on fracture healing. Starr et al[12] recorded differences in the size and density of callus on radiographs in eight male goats with bilateral mid-shaft tibial fractures and found no difference between the groups. Lichte et al[11] used micro-CT and found no effect of shock and resuscitation on the density of callus. However, these studies only included postoperative measurements at four and three weeks, respectively, which is the time at which the differences shown in the present study began to appear.

The key limitation of our study is the lack of histological and biomechanical data after the animals were killed. Previous studies have shown a detrimental effect following shock and resuscitation on the histology at the fracture site at both 72 hours and three weeks postoperatively.[11,13] Starr et al[12] showed no difference in either histological or biomechanical parameters at four weeks. Lichte et al[11] showed no effect on the biomechanics of callus at three weeks. Bumann et al[10] used a closed mid-shaft tibial fracture model in 42 rats and found superior biomechanical properties at four weeks in the callus of those with haemorrhagic shock and resuscitation compared with controls. They postulated that this was due to more rapid restoration of blood supply to the fracture site in shocked animals, which they demonstrated using blood flowmetry.

A further limitation of this study is the absence of micro-CT data. These are considered to be the benchmark for radiological analysis and were used by Lichte et al.[11] However, our

radiological analyses were rigorous, with two independent, blinded reviewers for each observation with low thresholds for remeasurement and rescoring. While it is possible that the differences in callus index and radiological scores in the groups, and with the passage of time, may be partly due to measurement error, the reliability of our findings is supported by analyses of interobserver agreement.

A final limitation of this study is the original sample size of 18 rabbits and the loss of four, which resulted in a final sample of 14. However, this sample was greater than or similar to two prior studies on this subject and compensated for by other key methodological strengths. A strength of the study was the longer-term follow-up to eight weeks, allowing healing to be monitored until remodelling was underway, as compared to previous studies, which included follow-up to a maximum of only four weeks.

While the absence from our model of soft-tissue damage that typically accompanies femoral fractures may be seen as a limitation, the use of a standardized osteotomy has its advantages as it eliminated the pattern of the fracture as a variable and maximized the internal validity of the findings. This study is also the first to use a purpose-designed reamed and locked intramedullary nail in an animal model, and to use whole blood for the resuscitation, in an attempt to replicate clinical practice.

In conclusion, haemorrhagic shock and resuscitation may lead to larger early callus formation but may delay remodelling at a femoral osteotomy site compared with controls in a rabbit model.

Take home message:

- Haemorrhagic shock and resuscitation may lead to larger early callus formation, but may delay remodelling

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Author contributions:

J. Brady: Designing the study, Conducting the experiments, Collecting and interpreting the data, Drafting the manuscript.

B. M. Hardy: Designing the study, Assisting with experiments, Statistical analysis, Interpreting the data, Critically revising the manuscript.

O. Yoshino: Designing the study, Assisting with experiments, Critically revising the manuscript.

A. Buxton: Assisting with experiments, Radiography, Critically revising the manuscript.

A. Quail: Study design, Assisting with experiments, Interpreting the data, Critically revising the manuscript.

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