

Therapeutic effects of revascularisation on the healing of free bone grafts in dogs

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Abstract

Introduction: The therapeutic effect of subcutaneous embedding and revascularisation on the repair of canine bone defects caused by open fracture was examined. **Material and Methods:** A total of 12 adult beagle dogs were randomly split into a control group (group C) and a test group (group T). A section of the radius was removed from each dog under general anaesthesia and the deficit supported by an orthopaedic implant. Group T had the section surgically implanted next to the blood vessel-rich saphenous vein and Group C had it cryopreserved at -80°C . After eight weeks, the bone was surgically implanted back into the matching radial deficit. Bone healing was evaluated by gross morphological and X-ray examinations, post-mortem histology, and successive blood measurements of key bone biochemical markers. **Results:** At 12 weeks, the bone healing boundary was disappearing more quickly in group T dogs than in their group C counterparts. X-ray and histological examinations showed that the cortical repair of group T subjects was complete and the bony plate arrangement was more regular than that in group C. The levels of bone biochemical markers also proved that the healing state of group T was better. **Conclusion:** The results showed that the degree of healing, osteoclast activity, and bone formation status of group T were better than those of group C, proving that the vascularised bone graft had a significantly shorter healing time than the cryopreserved bone graft.

Keywords: dog, vascularisation, bone defect, bone-callus, open fracture.

Introduction

Open fractures in injured pets are increasingly common in modern society and such injuries can be a challenging orthopaedic repair task. Such injuries are prone to infection, which may progress to osteomyelitis if not treated properly (23). Debridement surgery can reduce the incidence of postoperative infection, but open fracture surgery often results in bone deficit during debridement, and treatment becomes more difficult (16). There are many methods for repairing bone deficits, such as autologous or allogenic bone transplantation and bone extension or tissue engineering technology, but many of these treatment methods have not been used or proven in the veterinary field (1, 11, 14). Autologous bone grafting as the gold standard for the treatment of bone defects relies on donors and results in donor site deficits (10, 19). Human clinical treatment of bone defects has used a variety of biological materials, but the reuse of autologous long-segment free bone has been neglected. Most doctors have chosen to discard large sections of contaminated or

necrotic bone that has been severely damaged, resulting in wastage of potential graft material.

This study examined the matter of long-segment free bone re-use in open fractures, and used ectopic vascularisation and reimplantation in order to improve the success of clinical open fracture treatment in pets. This proved to be a safe and effective treatment for bone defects caused by debridement during the treatment of such injuries.

Material and Methods

Animals. A total of 12 healthy beagles of similar body condition and weight ($5\text{ kg} \pm 0.3\text{ kg}$), aged one to two years were selected for this trial. They underwent physical examination before the start of the experiment to confirm their health and the absence of limb deformities. The experimental dogs were provided by the Experimental Animal Base of Heilongjiang August First Land Reclamation University.

Experimental method. The dogs were randomly divided into two groups – the experimental group (group T) and control group (group C). Each dog received a general anaesthetic, the skin was cut along the lateral muscle groove of the forearm vein, the connective tissue and the radial extensor muscle were bluntly separated and the tibia shaft was fully exposed. A titanium-alloy plate was shaped to fit the radius, and holes were drilled at the distal and near ends of the radius, without initial fixation. The bone deficit model was then established by cutting the middle part of the radius ($15 \text{ mm} \pm 1 \text{ mm}$) with a wire saw. After rinsing, the plate was used to fix the two ends of the radius, keeping the bone deficit at a fixed distance, and allowing weight bearing. After haemostasis, the muscles and skin were sutured. Anti-inflammatory and analgesic treatments were performed postoperatively. All dogs were subcutaneously injected with ceftiofur sodium at a 50 mg/kg dose subcutaneously once a day for one week. Oral chewable firocoxib tablets at a 5 mg/kg dose were administered once a day for five consecutive days. All surgically harvested bone material was exposed to the external environment, and then placed in 10% povidone iodine for immersion sterilisation, rinsed with physiological saline, and drained for use. In group T, the extracted bone was embedded next to the saphenous vein of the thigh. In group C, the radial bone samples were frozen at -80°C for low-temperature aseptic storage.

After eight weeks, the two groups of bone grafts were surgically implanted back into the radial deficits of the respective dogs. The postoperative course was the same as in the first surgery. The bone healing of the two groups was evaluated by physical examination and observation and assessed by X-ray and histological examination and the measurement of bone biochemical markers over the 12 weeks following bone grafting.

Overall observation. Each dog had body weight, body temperature, appetite, mental state, activity levels, and wound healing recorded daily.

General morphological observation. At weeks 6 and 12 post implantation, two dogs from each group were randomly selected and given intravenous injections of lethal doses of sodium pentobarbital and potassium chloride for euthanasia, and then their radius complete with reimplanted section was removed. The healing of the autogenous bone graft, the fracture line and the bone shape in each sample were visually assessed.

X-ray examination. Four dogs were randomly selected from groups T and C at 4, 8, and 12 weeks after bone grafting and X-rayed (47 KV, 4.5 mA, 0.08 s). The bone healing was scored according to the Lane–Sandhu X-ray scoring standard (12), and the score results were analysed.

Histological examination. Bone specimens from the euthanised dogs were washed with physiological saline and fixed in 10% neutral formalin. After decalcification with hydrochloric acid, 3 mm sections of each sample were cut, dehydrated, embedded in paraffin, sliced, stained with haematoxylin-eosin (HE), and sealed with neutral gum. Sections were placed on

a NanoZoomer-SQ slide scanner (Hamamatsu, Hamamatsu City, Japan) for scanning, and the bone defect healing was scored according to the Lane–Sandhu histological system (9).

Measurement of bone biochemical markers. Blood samples from each dog were collected one day before surgery, post surgery at weekly intervals from weeks 1 to 6, and then after 8, 10, and 12 weeks. The plasma samples were stored frozen at -80°C . The plasma concentrations of osteocalcin (OC), bone-specific alkaline phosphatase (BSAP), canine type I procollagen carboxy terminal peptide (PICP), canine type I procollagen amino terminal peptide (PINP), and type I collagen C-terminal peptide (CTX) were determined by double-antibody sandwich ELISA to define the healing state of each bone sample.

Statistical analysis. Statistical analysis was performed using SPSS 18.0 (SPSS Inc, Chicago, IL, USA) statistical software. All data are expressed as mean \pm standard deviation. The two-samples *t* test was used for pairwise comparison and $P < 0.05$ was considered statistically significant.

Results

General observation. In both groups, swelling of the affected limb, lameness, and decreased activity were noticeable at one week after surgery. Body temperature did not increase significantly but appetite decreased slightly. At four weeks post surgery, all dogs' surgical wounds had healed, and they had increased physical activity and feed intake. At eight weeks post surgery, there were no wound site problems; dogs were active, and when the affected limb was touched, there was no pain reaction. At 12 weeks and the final observation, all dogs were thriving and active.

Gross morphology observation. At 12 weeks, the healing limit of the fracture end in dogs of group T had disappeared, the connection was tight, and the callus was well moulded. In comparison, group C still had an obvious fracture line at the end of the graft and the callus was poorly moulded.

X-ray examination. It can be seen from Fig. 1 that for group T at week four post surgery, low-density callus can be observed at the graft and the fracture ends were still present. By week eight, the fracture line had blurred, the density of the callus had increased, and the medullary cavity portion at the junction was connected. At week 12, recanalisation had occurred, the callus defect was filled, the cortical bone connection was completed, the overall shape was good, and the bone resorption was mild.

For group C at week four, the fracture line was observed to be clear and the amount of new callus was small. At week eight, the formation of callus had increased and there was slight bone resorption. At week 12, the continuity of the cortical bone was poor. The medullary cavity was basically recanalised, the fracture line was not obvious, and bone resorption could be clearly seen.

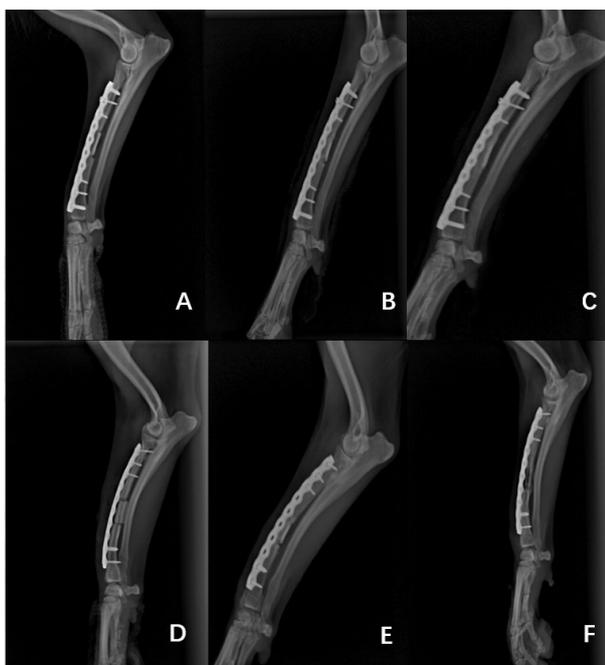


Fig. 1. Images of canine radius 4, 8, and 12 weeks after repair of bone defect. A, B, and C are the X-ray results for group T at 4, 8, and 12 weeks after reimplantation; D, E, and F are the X-ray results for group C at the same intervals after reimplantation

As can be seen from Table 1, at the same time point, the Lane–Sandhu X line score of group T was significantly higher than that of group C ($P < 0.05$).

Histological examination. As can be seen from Fig. 2A, osteoblasts (as indicated by the black arrow), bone pits of osteoclasts, TRAP cells, loose fibrous tissue, and trabecular bone were observed at 6 weeks in group T, but in comparison, no obvious osteoclastic activity at the same time can be seen in samples from group C in Fig. 2B. Chondrocytes, a small amount of trabecular bone, and loose fibrous oedema at the

junction (as indicated by the black arrow) can be seen, as well as new blood vessels and acute and chronic inflammatory cell infiltration. In Fig. 2C, a large amount of mature bone tissue can be seen in group T at 12 weeks, and a large number of osteoblasts are visible around the bone tissue (as indicated by the black arrow). The bone plates are arranged regularly (as indicated by the red arrow), and most of the bone connections are formed. In Fig. 2D, a large amount of new bone formation was observed at 12 weeks in group C, and the bone plates were arranged regularly (as indicated by the red arrow).

As can be seen from Table 2, the histological scores of Lane–Sandhu in the T group were better than those in the C group at the same point in time ($P < 0.05$).

Table 1. Comparison of the average scores of Lane–Sandhu X-rays at 4, 8 and 12 weeks after surgery ($n = 4$)

Group	4 weeks	8 weeks	12 weeks
T	4.4 ± 0.81*	7.9 ± 0.57*	11.95 ± 0.5*
C	2.95 ± 0.5	4.45 ± 0.95	8.55 ± 0.95

* – $P < 0.05$

Table 2. Comparison of mean scores of Lane–Sandhu histology bone formation scores at 6 and 12 weeks after surgery ($n = 2$)

Group	6 weeks	12 weeks
T	8.63 ± 0.64*	11.75 ± 0.71*
C	5.4 ± 0.93	8.25 ± 0.71

* – $P < 0.05$

Bone biochemical markers. As shown in Table 3, the values of BSAP and OC in group T peaked at the fourth week, and there was a significant difference compared with group C ($P < 0.05$). Both PICP and CTX peaked in the second week in group T after bone transplantation and again there was a significant difference ($P < 0.05$) compared with group C. The mean value of PINP increased in the second and eighth weeks, and once more there was a significant difference compared with group C ($P < 0.05$).

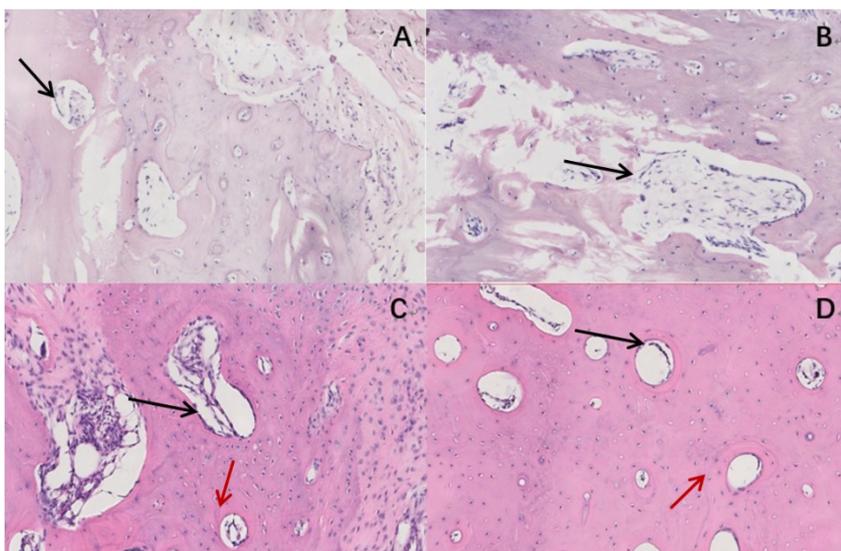


Fig. 2. Histopathological observation of free bone in groups T and C at 6 and 12 weeks after reimplantation. A and C are the histological observation at weeks 6 and 12 after reimplantation surgery for group T; B and D are the equivalent histological observation for group C. A – visible bone resorption, trabecular bone, and a small number of osteoblasts (black arrow) (HE, 200×); B – visible small trabecular bone, fibrous tissue oedema at the junction (black arrow) (HE, 200×); C – a large number of osteoblasts are present (black arrow) The bone plate is arranged regularly (red arrow) (HE, 200×); D – a large amount of new bone formation (black arrow) is visible, and the bone plate is arranged regularly (red arrow) (HE, 200×)

Table 3. Comparison of five markers in two groups of different time periods (n = 4)

Time	Group	BSAP (ng/L)	OC (ng/L)	PICP (ng/L)	PINP (ng/L)	CTX (ng/L)
1 day before surgery	T	13.71 ± 2.86	9.26 ± 4.35	40.36 ± 6.93	44.53 ± 7.88	44.83 ± 6.03
	C	14.57 ± 4.23	9.83 ± 4.28	42.77 ± 5.88	42.56 ± 5.63	44.52 ± 7.87
1 week after surgery	T	12.94 ± 2.01	8.93 ± 2.74	50.78 ± 7.53	47.39 ± 7.93	48.23 ± 5.19
	C	13.82 ± 3.52	9.48 ± 3.38	46.63 ± 4.65	43.79 ± 4.55	45.39 ± 7.63
2 weeks after surgery	T	14.29 ± 3.51	9.62 ± 2.85	58.79 ± 7.94*	55.37 ± 6.17*	54.42 ± 6.32*
	C	14.51 ± 4.89	9.66 ± 4.29	49.32 ± 4.58	44.93 ± 5.69	46.47 ± 4.34
3 weeks after surgery	T	15.94 ± 2.42	11.35 ± 4.47	52.54 ± 5.66	48.33 ± 7.88	52.14 ± 7.63
	C	13.64 ± 5.96	8.84 ± 3.23	55.52 ± 7.18	49.73 ± 6.64	49.65 ± 5.32
4 weeks after surgery	T	17.36 ± 4.57*	15.37 ± 3.63*	48.73 ± 6.37	45.62 ± 7.39	50.66 ± 7.54
	C	13.97 ± 4.25	9.93 ± 3.78	52.95 ± 7.88	47.65 ± 4.38	51.22 ± 7.24
5 weeks after surgery	T	16.44 ± 3.51	14.87 ± 4.16	44.84 ± 5.33	46.83 ± 7.91	48.35 ± 6.93
	C	15.27 ± 3.11	11.57 ± 4.31	48.44 ± 4.69	49.53 ± 5.33	47.12 ± 7.33
6 weeks after surgery	T	16.42 ± 4.12	14.47 ± 2.18	46.96 ± 5.85	48.49 ± 7.16	47.72 ± 4.66
	C	16.94 ± 5.88	13.22 ± 5.12	47.78 ± 6.15	44.57 ± 7.81	48.52 ± 4.39
8 weeks after surgery	T	15.51 ± 2.65	12.77 ± 4.54	50.74 ± 4.53	53.26 ± 4.47*	49.28 ± 5.62
	C	14.39 ± 3.83	12.26 ± 5.42	48.63 ± 5.77	45.44 ± 6.55	48.57 ± 7.94
10 weeks after surgery	T	14.62 ± 3.71	10.34 ± 3.82	51.33 ± 6.12	50.27 ± 6.97	48.28 ± 6.63
	C	15.24 ± 2.79	11.73 ± 5.15	52.73 ± 5.97	52.65 ± 6.27	49.21 ± 6.88
12 weeks after surgery	T	15.82 ± 4.97	9.66 ± 4.45	48.58 ± 4.78	47.88 ± 5.96	52.75 ± 6.19
	C	14.78 ± 4.37	11.13 ± 3.77	51.14 ± 4.31	51.18 ± 7.91	51.36 ± 6.35

* – P < 0.05

Discussion

In this experiment, gross morphological observations showed that at 12 weeks post surgery, the healing of the fractured end of the bone was superior in group T, and this was confirmed by X-ray examination. The reason may be that the bone grafts in group T had a shorter period of both haematoma and formation of the original bone callus, while the grafts in group C only received nutrients through the permeation of surrounding tissue fluid and the slow growth of blood vessels, resulting in the healing ability after grafting of cryopreserved bone being far less than that of vascularised bone (13, 17, 20). Histological observations showed that the development of bone callus, osteoblasts, and trabecular bone in group T was significantly more dynamic than that of group C, appearing at 6 weeks, when none was observed in group C. At 12 weeks, group T had more regular bone formation and bone connections, while group C had only reduced bone formation. The mean Lane–Sandhu histology bone formation scores of group T were higher than those of group C at the same time point (9). The histological observations were consistent with the results of gross morphological and imaging observations.

BSAP produced by osteoblasts is one of the classic and most commonly used biochemical markers for evaluating bone formation and bone turnover, and is positively correlated with osteoblast activity (5). In this experiment, BSAP levels showed a trend of first decreasing and then increasing, which was consistent with the trend of BSAP measured in the experiment of Stoffel *et al.* (18). The reason may be that the activity of osteoblasts after surgery was briefly inhibited. The plasma BSAP concentration in the experimental group peaked two weeks earlier than in the control group, suggesting that the osteoblasts were more active after vascularisation, allowing greater graft healing in a given period. OC is a non-collagen matrix protein synthesised

by osteoblasts and a major component of non-collagenous bone matrix (5). Delmas *et al.* demonstrated that OC as a specific index can directly reflect the rate and specific conditions of bone formation, and they found that elevating serum OC concentration indicates increased bone formation (6). In this experiment, the trend of increasing OC and BSAP was similar in both groups, and the OC concentration of group T peaked in the fourth week, two weeks earlier than that of group C, just as was the case for BSAP. As indicators that can directly reflect the rate of bone formation, these results confirmed that this rate is faster for the vascularised grafts in group T than for the frozen grafts in group C, and that the bone formation time is earlier, suggesting that vascularisation accelerates the process of bone healing (15).

In mature bone, type I collagen accounts for more than 90% of the bone organic matrix (2). Borys *et al.* (4) demonstrated the role of type I collagen metabolism in their experimental assessment of fracture healing. The results show that almost all PICP and PINP in healthy blood is derived from bone metabolism because this is faster than the metabolisms of other types of connective tissue. Therefore, PICP and PINP are other good indicators of bone formation. Borys *et al.* (3) analysed serum PICP concentrations in 25 cases of mandibular fractures and obtained results depicting an important role for PICP in the healing of fractures. Garcia-Perez *et al.* (8) found that PINP is highly specific to bone tissue and is closely related to bone resorption. In this experiment, the changes in PICP and PINP were almost the same. The PINP and PICP of group T reached their peaks and decreased first, indicating that bone tissue in these dogs ended the stage of organisation of haematoma earlier than that in group C and entered the original callus formation stage more quickly. In the experiment, the second increase of PINP at the eighth week may be due to the large amount of callus removed from the stress axis according to Wolff's law during the remodelling

phase (7). PICP also had a second increase but was not significantly different from the control group ($P > 0.05$).

CTX can reflect the early process of fracture healing, and its early concentration is closely related to the role of osteoclasts, being released by these cells during bone degradation (22). It is generally believed that CTX is positively correlated with bone turnover rate and is therefore considered to be a highly specific bone marker. Veitch *et al.* (21) found that CTX levels in patients with tibial fractures increased over about 3 days, peaked in 2 weeks, and remained at an elevated level even at 24 weeks (21). The trend of CTX in this experiment is consistent with the results of the research of those authors. The concentration of CTX in group T peaked in the second week ($p < 0.05$), indicating that the osteoclasts of this group were more active at the two-week time point than those of Group C, and the decrease during the subsequent week indicated the end of the haematoma mechanisation period. However, the concentration during the test was always higher than the baseline level, which may be due to reconstruction of the callus.

In this experiment, for bone defect repair the greater therapeutic effect of vascularisation of the bone until its reimplantation in group T over cryopreservation of bone in Group C was obvious in general morphological, imaging, and histological appraisals. From the molecular level of osteoblast activity, bone formation state, osteoclast activity, and bone resorption, bone biochemical markers verified that the bone reimplanted after vascularisation healed better than the cryopreserved bone graft. The reason is that the bone has been vascularised and has its blood supply guaranteed after *in-situ* reimplantation, so this technique spared the bone needing to go through a long creeping substitution, and healing time was significantly reduced. Recovery time after such surgery would be greatly shortened.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Heilongjiang Bayi Agricultural University, Daqing, China. All beagle experiments were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the School Council of Heilongjiang Bayi Agricultural University.

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