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Platelet-rich plasma, bone marrow and chitosan in minimally invasive plate osteosynthesis of canine tibia fractures – a randomized study

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Abstract: The goal of this study was to analyse the effects of percutaneous application of platelet rich plasma (PRP), autologous bone marrow concentrate (BM) and chitosan gel (CHI) on bone consolidation following minimally invasive plate osteosynthesis (MIPO) of the fractures of the tibia in dogs. Client-owned dogs ($n = 30$) with tibial fracture were divided into four treatment groups – Group 1 (control), Group 2 (BM), Group 3 (PRP) and Group 4 (CHI). The biomaterial specific to each group was injected at the fracture site immediately after the MIPO procedure. Serial radiographs were used to determine the fracture line and the development of periosteal callus immediately after surgery and at 15, 30, 60, 90 and 120 days post-surgery. There was no significant difference ($P > 0.05$) in the degree of oedema or grade of lameness between the groups. Grade 4 (minimum) or 5 lameness (absent) was observed in 70% of animals from all groups at 15 days post-surgery. The biomaterials PRP, BM and CHI combined with MIPO contribute to bone consolidation of tibial fractures in dogs and do not cause adverse reactions or fracture complications. Bone marrow concentrate results in shorter bone consolidation time.

Keywords: bone consolidation; surgery; biomaterials; dogs; percutaneous application

Minimally invasive plate osteosynthesis (MIPO) enables greater preservation of the biological environment and, thus, maximizes the healing potential of the bone and the damaged soft tissue (Boero Baroncelli et al. 2012). It results in fast recovery of limb func-

tion and reduces post-operative pain in long bone fractures in cats and dogs (Pozzi et al. 2013).

The biomaterials used in veterinary orthopaedics can help greatly in the consolidation as adjunct applied in the fracture gap during the surgical proce-

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ture (Azevedo et al. 2014). In recent years, there has been an increase in research studies on bone marrow concentrate (Vaz et al. 2010), platelet-rich plasma and chitosan (Guzman et al. 2014) as osteostimulating biomaterials. These materials are easy to obtain, simple to use and do not require specialized equipment (Tan and Marra 2010). Furthermore, they can be applied percutaneously, as their consistency is compatible with the use of a syringe and needle. Their application can be minimally invasive and of low morbidity to the patient (Connolly and Shindell 1986).

Complications of orthopaedic surgeries are often difficult to resolve and there has been a continuous search for ways to enable and promote the process of bone healing. The objective of this study was to analyse the percutaneous application of platelet enriched plasma, bone marrow concentrate and chitosan in minimally invasive plaque osteosynthesis in dogs. We hypothesized that the use of biomaterials would be to provide bone healing faster than osteosynthesis without the percutaneous application of the biomaterial.

MATERIAL AND METHODS

Specimens and groups. This study was approved by the Ethics Committee in the Use of Animals (CEUA) of the institution (Protocol No. 017930/12) and all owners signed a consent form to include the animal in the study.

The study was randomised in the period of four years (2012–2015) and used thirty dogs with tibial fractures that had been admitted to the Small Animal Surgical Service of the School Hospital. The inclusion criteria were: young or adult dogs with fractures of the tibia, in conjunction or without fractures of the fibula, preferably comminuted or with more than one fractured segment. Animals that showed signs of systemic disorders that could compromise bone consolidation were excluded from the study.

All animals were subjected to minimally invasive plate osteosynthesis (MIPO) and divided into four experimental groups of eight animals ($n = 8$), with the exception of Group 4 ($n = 6$). The groups were classified according to the biomaterial to be injected: Group 1 – control (only MIPO), Group 2 – bone marrow concentrate, Group 3 – platelet-rich plasma, and Group 4 – chitosan gel. The group 4 had

eight animals, but two died for various reasons before the end of the postoperative follow-up.

In this study we used straight stainless steel plates (Caomedica, Campinas, Sao Paulo, Brazil) and locking screws 2, 2.7, 3.5 and 4.5 mm long. The size was chosen based on the weight and bone structure of the patient.

Biomaterial preparation. Bone marrow concentrate (BM): Bone marrow (10 ml) was extracted from the greater tubercle of the humerus using heparinized syringes and centrifuged (Centrifuge Baby – Model 206, Fanem, Sao Paulo, Brazil) at 1500 rpm for 10 min in order to concentrate the mononuclear cells (Del Carlo et al. 2004). The plasma supernatant was discarded and the bone marrow concentrate taken to the surgical theatre to be used in the osteosynthesis. Nucleated cells were counted before and after centrifugation, using a Neubauer chamber and bone marrow (40 μ l) diluted in Turk's solution (800 μ l). This procedure was performed at the time of MIPO.

Platelet-rich plasma: Blood from each patient (4.5 ml) was collected into tubes containing sodium citrate (Vacuum blood collection tubes with 3.2% sodium citrate, Labor Import, Osasco, Sao Paulo, Brazil) and centrifuged twice. The samples were centrifuged at 1200 rpm for 10 min, the plasma and buffy coat layer aspirated and transferred to a sterile tube with no additive and further centrifuged at 1600 rpm for 10 minutes. Approximately 80% of the supernatant was discarded, leaving only the concentrated platelet portion and the platelet button at the bottom of the tube. Platelets were activated by gentle agitation and calcium chloride (Calcium chloride 10%, Injectcenter, Ribeirão Preto, São Paulo, Brazil).

Chitosan gel: The gel was obtained according to the protocol by Chenite et al. (2000), in which 200 mg chitosan (high molecular weight and degree of deacetylation higher than 75%; Sigma-Aldrich, CAS No. 9012-76-4) was dissolved in 9 ml of 0.1 M chlorhydric acid solution. Subsequently, 560 mg of disodium glycerol phosphate, dissolved in 1 ml of distilled water, was slowly added under gentle agitation (Glycerol phosphate disodium salt hydrate – Sigma-Aldrich, CAS No. 55073-41-1), resulting in a white gel. The gel was sterilised in an autoclave and kept at room temperature (25 °C) until needed.

Anaesthetic procedure. Patients were premedicated intramuscularly with chlorpromazine chlorhydrate (Longactil 25 mg/ml, Cristalia, Produtos

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Quimicos Farmaceutica Ltda, Itapira, Sao Paulo, Brazil) (0.3 mg/kg) and morphine (Dimorf 10 mg/ml, Cristalia, Produtos Químicos Farmaceutica Ltda, Itapira, Sao Paulo, Brazil) (0.25 mg/kg). After 20 min, anaesthesia was induced with Propofol (Profol 1%, Cristalia, Produtos Quimicos Farmaceutica Ltda, Itapira, São Paulo, Brazil) (4 mg/kg, *i.v.*) and the animals were intubated with an orotracheal tube. Anaesthesia was maintained by a mixture of iso-fluorane (Forane, Abbott, Sao Paulo, Brazil) and 100% oxygen. Subsequently, the animals were positioned for local epidural anaesthesia with 2% lidocaine (Xylocaina 2%, Hipolabor Farmaceutica Ltda, Sabara, Minas Gerais, Brazil) without vasoconstrictor (4 mg/kg) combined with 0.75% bupivacaine (Neocaina 0.75%, Cristalia, Produtos Quimicos Farmaceutica Ltda, Itapira, Sao Paulo, Brazil) without vasoconstrictor (2 mg/kg) and tramadol chlorhydrate (Tramal 5%, Hipolabor Farmaceutica Ltda, Sabara, Minas Gerais, Brazil) (0.5 mg/kg) in the space between the 7th lumbar and the 1st sacral vertebrae (L7–S1).

Surgical procedure. Fracture reduction was performed using a closed and indirect method, with minimum manipulation. Bone alignment and length were achieved based on anatomical references of the limb and by comparison with the contralateral limb. No intra-operative image analysis was used. Surgical access was restricted to two small incisions on the medial face of the tibia, one proximal and the other distal to the fracture site. The plate was inserted at the proximal incision and slid through the tunnel of soft tissue adjacent to the bone surface, over the fracture site. Once the plate was adequately positioned on the bone surface, holes were drilled for the insertion of locking screws with the aid of a perforation guide and a long screw bit. The number of screws used was determined based on each fracture. After insertion of the screws, the surgical wound was sutured in three layers. Poliglecaprone 2-0 suture was used for stitching the muscle layers and reduction of the dead space (subcutaneous) using simple continuous suture pattern. Nylon 3-0 was used for the simple interrupted suture of the skin.

Following skin suture, a 40 × 12 mm diameter needle (Needle 40 × 12 mm, Becton Dickinson Industria Cirurgica Ltda, Curitiba, Parana, Brazil) was inserted percutaneously through palpation within the bone defect at the fracture site and, based on each treatment, a single 2 ml dose of biomaterial

was injected. The control group did not receive any injection. The 2 ml volume was standardised as the amount that was obtained after preparation BM and PRP processes regardless of weight of the animal.

Post-operative care. The animals were prescribed Cephalexin (Cefalexina, Eurofarma Laboratorios Ltda, Sao Paulo, Brazil) (25 mg/kg every 12 h, for ten days), meloxicam (Maxicam injetavel 0.2%, Ouro Fino Saude Animal Ltda., Cravinhos, Sao Paulo, Brazil) (0.2 mg/kg on the first day and 0.1 mg/kg on subsequent days, every 24 h, for three days) and dipyrone (Dipirona sodica, Laboratorio Geyer, Porto Alegre, Rio Grande do Sul, Brazil) (25 mg/kg every eight hours, for seven days) and tramadol chlorhydrate (Tramal 5%, Hipolabor Farmaceutica Ltda, Sabara, Minas Gerais, Brazil) (3 mg/kg every eight hours, for five days) for pain management. The wound was cleaned with saline solution and rifampicin (Rifocina spray, Sanofi Aventis Farmaceutica Ltda, Suzano, São Paulo, Brazil) was applied topically once a day, for 15 days. Skin sutures were removed on the 15th day post-surgery.

Post-operative evaluations. The surgeon and the specialist who evaluated the post-operative condition did not participate in the surgical procedure and therefore did not know which treatment was received by which animal (a blinded study).

Patients were radiographically evaluated by the same veterinary specialist in imaging diagnosis before the surgery (Time 0 – T₀) and at 15 (T₁₅), 30 (T₃₀), 60 (T₆₀), 90 (T₉₀) and 120 days post-surgery (T₁₂₀).

The surgeon evaluated the lameness at each time point (T₀, T₁₅, T₃₀, T₆₀, T₉₀ and T₁₂₀) graded according to the adapted scores (Scott and Witte 2011) as follows: grade 1 represents severe lameness with no weight bearing on the affected limb at stance; grade 2, lameness present at walk and trot; grade 3, mild lameness at walk but worsens at trot; grade 4, lameness is present but only evident at trot and, grade 5, absence of lameness at walk or trot.

Serial craniocaudal and mediolateral radiographies obtained during the immediate post-operative period and at 15, 30, 60, 90 and 120 days post-surgery. In order to analyse the process of bone consolidation, the following variables were evaluated: radiopacity of the fracture line (RFL), localization of the periosteal callus (LC), presence of bone bridge (PBB), reestablishment of the cortices (RC), reestablishment of the medullary canal (RMC), callus remodelling (CR), callus volume (CV) and charac-

teristics of the fracture line (Scale of Radiographic Evaluation – SRE – scores 1 to 6 (Souza et al. 2011).

Statistical analysis. Analysis was performed using the General Linear Model (GLM) of the Statistical Analysis System software (SAS 9.1, SAS Institute, Cary NC, USA) and profile multivariate analysis because it simultaneously analyses relationships between the multiple temporal measures of the time variable for each animal with some other variable, in order to compare the treatments over time.

The data (groups, time, radiographic variables and degree of claudication) underwent the normality test of Shapiro-Wilk. After, this study used with analysis of time-repeated measures with the split-plot design, in which the factor treatment (4 levels – groups) was tested on the plots (degree of lameness × radiographics variables) and the factor time (6 levels – T_0 , T_{15} , T_{30} , T_{60} , T_{90} and T_{120}) on the subplots, with 5 repetitions per treatment. If there were significant differences ($P < 0.05$) between the means, they were compared by Tukey test at 5% significance.

RESULTS

Out of the 30 animals studied 40% were younger than 12 months, 57% were crossbreeds; 43% were of specific breeds (Blue Heeler, Border Collie, Dachshund, Fila Brasileiro, Labrador Retriever, Pinscher and Poodle); and 46% were males and 54% females. The diaphysis was the most affected section of the tibia, corresponding to 85% of the fractures. There were 63% transverse or oblique, 10% spiral and 27% comminuted fractures. Approximately 47% of the animals were small (1 kg to 10 kg), 26.5% medium (11 kg to 20 kg) and 26.5% large (21 kg to 50 kg).

Age was homogeneous in Group 1 (control) and Group 2 (BM), in which 50% of animals were younger than 12 months old. Group 3 (PRP) and Group 4 (CHI) had 75% and 67% of animals older than 12 months, respectively.

The types of fractures within each group were heterogeneous, with the exception of Group 4, and classified according to Piermattei et al. (2006). Group 1 had 37.5% of type A, 25% B and 37.5% C fractures; Group 2 had 75% A, 12.5% B and 12.5% type C fractures; Group 3 showed 50% A and 50% type C; and Group 4 had 100% type A fractures.

There was no significant difference ($P > 0.05$) in the degree of oedema or grade of lameness be-

tween the groups. In the present study, 70% of the animals showed lameness grade 4 or grade 5 at 15 days post-surgery. At T_{30} , there were no signs of lameness (grade 5).

No significant difference ($P > 0.05$) was observed in use of the limb between the treatment groups (Figure 1); however, Group 2 showed the highest percentage of weight bearing (60% of grade 5 – unrestricted use of the limb) at 15 days post-surgery.

There was no significant difference ($P > 0.05$) in the radiopacity of the fracture line (RFL), localization of the periosteal callus (LC), presence of bone bridge (PBB), reestablishment of cortices (RC), remodelling of the callus (RC) or volume of callus (VC) between the different treatment groups. However, the variables reestablishment of the medullary canal (RMC) and scale of radiographic evaluation (SRE) were significantly different ($P < 0.05$) in Group 2 and occurred earlier (T_{30}) than in other groups.

The radiopacity of the fracture line was initially absent; in other words, the fracture line became apparent as time progressed (T_{90} and T_{120}) and the callus developed, increasing the radiodensity of the fracture line (Figure 1). Similarly, the reestablishment of the medullary canal was absent at first (T_0 and T_{15}) in all groups; at T_{30} it was present in Group 2 (BM) and in half of the animals from Group 3 (PRP) but absent in Groups 1 and 4, with significant differences ($P < 0.05$) between them. At T_{60} , T_{90} and T_{120} it was present in all groups, with no significant difference ($P > 0.05$) being observed.

There was no significant difference ($P > 0.05$) in the scale of radiographic evaluation between the groups. However, Group 2 (BM) showed significantly shorter ($P < 0.05$) bone consolidation time (46.87 days) than Groups 1, 3 and 4 (69.37, 67.50 and 57.50 days, respectively).

One animal from Group 1 developed an inflammatory reaction to the suture (poliglecaprone 25), which was later replaced by nylon. Another animal from the same group showed a defect on the implant at 60 days post-surgery, with break of the proximal screw. Bone consolidation was delayed and occurred at 120 days post-surgery. However, at this time, there was exposure of the bone plate, which was removed and replaced by rigid external immobilization for 15 days. Another complication was the insertion of a screw in the tibia-tarsal joint of an animal from Group 3.

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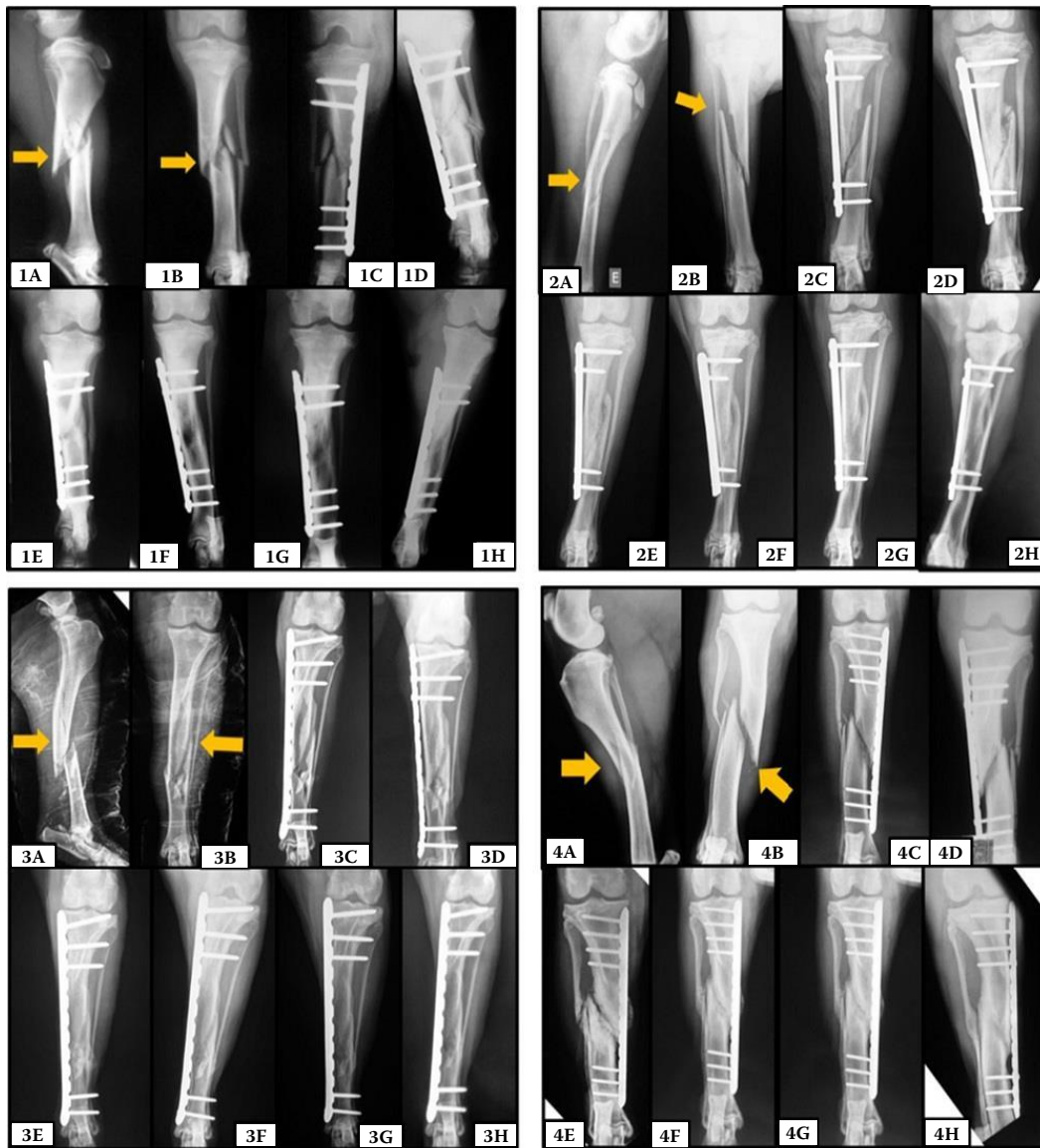


Figure 1. Photographic images of the radiographs of a patient group 1, 2, 3 and 4 in the experimental period

Patient group 1: (1A) – comminuted fracture in middle third of the tibia (arrow) in mediolateral projection; (1B) – cranio-caudal projection indicating a comminuted fracture (arrow); (1C) – immediate postoperative period; (1D) – 15 days postoperatively with early bone callus formation; (1E) – 30 days postoperatively with clinical fracture union; (1F) – 60 days after surgery; (1G) – 90 days after surgery; (1H) – 120 days postoperatively

Patient group 2: (2A) – long oblique fractures in the middle third of the tibia (arrow) in mediolateral projection; (2B) – cranio-caudal projection indicating the long oblique fracture (arrow); (2C) – postoperative immediate with good alignment of the bone shaft; (2D) – 15 days postoperatively with early bone callus formation; (2E) – 30 days postoperatively with clinical fracture union; (2F) – 60 days after surgery; (2G) – 90 days after surgery; (2H) – 120 days postoperatively

Patient group 3: (3A) – comminuted fracture in middle third of the tibia (arrow) in mediolateral projection; (3B) – cranio-caudal projection indicating the comminuted fracture (arrow); (3C) – Immediate postoperative period with good alignment of the bone shaft; (3D) – 15 days postoperatively with early bone callus formation; (3E) – 30 days postoperatively with clinical fracture union; (3F) – 60 days after surgery; (3G) – 90 days after surgery; (3H) – 120 days postoperatively with bone remodeling

Patient group 4: (4A) – Long oblique fracture in middle third of the tibia (arrow) in mediolateral projection; (4B) – cranio-caudal projection indicating the long oblique fracture (arrow); (4C) – Immediate postoperative period; (4D) – 15 days after surgery; (4E) – 30 days after surgery; (4F) – 60 days after surgery; (4G) – 90 days postoperatively with clinical fracture union; (4H) – 120 days postoperatively

DISCUSSION

The principles of MIO (minimally invasive osteosynthesis) are better applied to non-reducible fractures, especially those that are complex and comminuted, many of which are accompanied by extensive soft tissue damage. However, its use is not contraindicated in other types of fractures as long as adequate spatial alignment is obtained (Beale and McCally 2012). In the present study, MIPO was used to successfully correct complex comminuted fractures as well as simpler oblique ones, corroborating with Schmokel et al. (2007), Beale and McCally (2012), Boero Baroncelli et al. (2012), Hudson et al. (2012) and Adelina et al. (2013) who reported the use of MIPO in simple line fractures and stressed that care must be taken when inserting screws close to the fracture line.

The present study did not use trans-operative radiography or fluoroscopy for the visualization of the fragments during the surgical procedure; nevertheless, bone consolidation was obtained in all types of fractures, even in the simple ones. Many studies consider intra-operative image analysis indispensable (Hudson et al. 2012; Adelina et al. 2013) as it enables quick visualization of several exact projections of the bone surface and is thus considered the most useful method in evaluating alignment (Peirone et al. 2012).

Young animals have shorter bone consolidation time (Piermattei et al. 2006), however, as the groups in the present study were composed of animals of homogeneous age, age was not considered a relevant factor. Boero Baroncelli et al. (2012) compared MIPO to open reduction and, even though there were discrepancies in age within the groups (i.e. MIPO group had only 25% of animals younger than 12 months), there was no influence of age on bone consolidation time.

In the present study, MIPO was combined with the percutaneous application of bone marrow concentrate, platelet-rich plasma and chitosan gel at the reduced fracture site to minimize bone consolidation time, and thus reduce patient morbidity. These biomaterials stimulate bone and cartilage regeneration through a minimally invasive procedure (Connolly and Shindell 1986) of low cost and that only requires a syringe and needle for application. Furthermore, studies on the combined use of MIPO and biomaterials are limited.

Hernigou et al. (2005) reported the percutaneous use of biomaterials in cases of non-fusion

of the bone, which was considered effective and safe, especially when bone marrow concentrate was used. Differently from the study mentioned above, the biomaterials in this study were used to evaluate their influence on bone consolidation of recent fractures and on the clinical results, with the aim of reducing the post-operative complications that may occur in tibial fractures. This study did not analyse signs of bone infection and only one animal developed complications in fracture reduction, in which delayed fusion was observed in a patient of the control group.

MIPO enables a quick recovery of the function of the limb, as it minimizes the damages to soft tissues and is thus more advantageous than open reduction internal fixation (ORIF; 1). There was no significant difference on the use of the limb between the groups; however, Group 2 showed greater percentage (60%) of grade 5 lameness (no lameness) at 15 days post-surgery. This may be due to the osteogenic and osteoinduction properties of the bone marrow, as it is composed of mesenchymal stem cells that can differentiate into osteoblasts and act on bone formation and repair (Kraus and Kirker-Head 2006). However, no significant difference was observed between the groups, attributing the lack of lameness to the stability obtained with the technique and implants used.

PRP did not contribute negatively to fracture consolidation as the consolidation time observed for that group was similar to the control (67.50 and 69.37 days, respectively). On the other hand, Batista et al. (2011) who used PRP and BM combined to β -tricalcium-phosphate in damaged rabbit tibia, concluded that PRP resulted in better consolidation by enabling greater formation of compact bone than BM. In the current study, BM resulted in lower consolidation time than the other treatments. In humans, percutaneous injection of concentrated bone marrow aspirate has been successfully used in promoting pseudoarthrosis in cases of exposed fractures of the tibia with non- or delayed fusion (Le Nail et al. 2014). Furthermore, these authors report that due to its efficacy, low rates of post-surgical complications, preservation of bone stock and low cost, the concentrated BM should be considered as an alternative therapy to the management of complicated fractures of long bones.

In this study, chitosan was used in gel form, which enabled its percutaneous application. This type of application offers lower morbidity to the patient,

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the ability to carry therapeutic agents and the absence of residual substances (Naderi et al. 2011). The number of randomized studies with its use in fractures is scarce and only a few studies in tendons (Santana et al. 2014) and cartilage can be found (Martins et al. 2013).

Chitosan resulted in bone consolidation time similar to the control and PRP, indicating its potential use in fractures. Chitosan activates macrophages, which in turn modulate bone consolidation and release cytokines and growth factors that aid in tissue healing (Gorzelanny et al. 2010). Furthermore, chitosan did not cause any inflammatory reaction nor did it negatively contribute to bone consolidation time.

There was no significant difference on the scale of radiographic evaluation between the groups; however, Group 2 (BM) showed significantly shorter bone consolidation time, at 46.87 days. This consolidation time is considered fast and is a result of the association of minimally invasive techniques (MIPO combined with percutaneous application of bone marrow concentrate), which preserved the biological environment of the fracture and provided undifferentiated mesenchymal stem cells that increased the chances of faster tissue healing with reduced complication risks. This bone consolidation time is similar to those reported by Guiot and Dajardin (2011), who observed clinical fusion within 45 days post-surgery when using MIPO without the use of adjuvants to stimulate healing.

The biomaterials platelet-enriched plasma, bone marrow concentrate and chitosan contributed to bone consolidation when combined with minimally invasive osteosynthesis of the tibia in dogs, with bone marrow concentrate resulting in lower consolidation time. The radiography scores are essential in determining the time of clinical fusion and consolidation of the fracture, especially the scale of radiographic evaluation. No significant difference was observed in clinical evaluation or bone callus between the groups. The MIPO technique can be adequately performed without trans-operative image analysis and chitosan does not cause inflammatory reaction or undesired complications.

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