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Radiological Evaluation of Regenerative Growth Plate Defect Treated with Platelet-Rich Fibrin Membrane in Rabbits

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ORIGINAL ARTICLE
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ABSTRACT

Bony bar formation after growth plate injuries leads to shortening and angulation of the long bone, which is considered one of the most critical sequelae affecting animals' and humans' lives in adulthood. The objective of the present study was to evaluate radiographically the role of using an autologous platelet-rich fibrin membrane in regenerating growth plate defects to prevent the formation of bony bars. A total of 20 kit rabbits, aged between 6-12 weeks and weighing 500-1100 g, were included in the current study. They were experimentally exposed to approximately 5×5×1 mm growth plate defects, which were filled with an autologous platelet-rich fibrin membrane previously prepared at the time of the surgery. A radiological follow-up was conducted weekly at the first, second, third, fourth, sixth, and eighth weeks post-surgery to examine the growth plate defect area. The tibial length and angulation were measured during this period of the study and compared to the contralateral limb of the same animal. The radiological results showed no bony bar formation in most cases and the presence of the growth plate up to the end of the study (week 8 post-surgery) in the injured area. In addition, no significant differences were identified in the tibial length and angulation of the affected limb in comparison to the contralateral limb of the same animal throughout the study. In conclusion, treating serious growth plate injuries by PRF membrane may prevent angular deformity and length discrepancy in limbs.

Keywords: Angulation, Bony bar, Growth plate, Platelet-rich fibrin membrane, Shortening

INTRODUCTION

Bone elongation is the main function of the growth plate located at the proximal and distal ends of long bones (Yu et al., 2019), and it is considered a weak area due to its composition compared to rigid bone, which renders it more prone to injuries, accounting for about 15-30% of the total skeletal system injuries in humans (Sabharwal and Sabharwal, 2018; Shaw et al., 2018; Shen et al., 2020). These injuries may lead to the formation of a bony bridge. A bony bar (bony bridge) is considered one of the most concerning issues in orthopedics since it can tighten both epiphyseal and metaphyseal bones, leading to various deformities such as the angulation and/or shortening of the affected limbs (Shaw et al., 2018; Ibrahim and Indra, 2022). These bony bars act as tethers that prevent certain areas of the growth plate from expanding (Khoshhal and Kiefer, 2005). Partial damage to the growth plate can result in shortening and progressive angular deformation of the bone, and more severe damage can lead to the complete arrest of longitudinal bone growth (Zhou et al., 2004). The early signs of bony bar formation are the structural disorganization and development of vertical septa, which eventually form the bony bridge, particularly in Salter's types III and IV (Wattenbarger et al., 2002; Xian et al., 2004). Radiological evaluation is used to identify bony and tendentious defects (Majeed and Hussein, 2017; Nazht, 2019; Hashim and Nazht, 2021) as well as cartilaginous defects (Yu et al., 2019). The growth plate is visible in radiographs of immature animals as a radiolucent area between the metaphysis and epiphysis due to its mostly hyaline cartilage composition (Kealy et al., 2010; Kazemi and Williams, 2021), and its defects may be seen on radiographs as a bony bar, which appears as a radiopaque area at the defect site (Gigante and Martinez, 2020). One of the long bones commonly affected by growth plate injuries during adolescence is the tibia, which is clinically evaluated via radiography (Tobita et al., 2002; Gültekin et al., 2020). An anteroposterior view of the hind limb best shows the formation of the bony bar and helps assess the tibial length and any resulting angulation (Sh et al., 2001; Wang et al., 2023). Various materials have been used to prevent bony bar formation in growth plate defects, such as scaffold materials (chitin and agarose) (Chen et al., 2003; Li et al., 2004; Azarpira et al., 2015), poly (lactic-co-glycolic acid) (Sundararaj et al., 2015), 3D-printed materials with or without autologous fat (Cheon et al., 2003; Yu et al., 2022), and autologous grafts (Al-husseni, 2008), with varying degrees of success. Other materials have also been used to treat cartilage defects, including ovine bone marrow-derived mesenchymal stem cells (Al-Mutheffer et al., 2023). However, the above methods have

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many limitations. These *in vitro* tissue engineering methods require the harvesting of cells from the patient and thus necessitate multiple procedures. Recent studies have attempted to treat growth plate defects by developing biomaterial scaffolds incorporating growth factors and stem cells, which may aid in regenerating growth plate defects.

Platelet-rich fibrin **(**PRF) is a simple and easily prepared material (Dohan et al., 2006a). PRF is a second-generation platelet concentration composed of cytokines, leukocytes, stem cells, and platelets, which acts as a scaffold, supports micro-vascularization, and serves as a transport medium for carrying cells, all important for tissue regeneration (Temmerman et al., 2018; Dirja et al., 2023). PRF functions as an autologous fibrin matrix used to enhance bone regeneration (Salih and Al-Falahi, 2018; Thanoon et al., 2019), improve tendon repair (Al-Falahi, 2016), and enhance the viability, differentiation, and migration of chondrocytes, demonstrating significant potential in cartilage repair (Wong et al., 2020; Dirja et al., 2023). A PRF clot is also defined as a fibrin network that traps platelets and leukocytes, and its matrix allows the slower and more elongated release of growth factors (Ehrenfest et al., 2009). The growth factors present in PRF include the platelet-derived growth factor (PDGF), the insulin growth factor (IGF), the basic fibroblastic GF (bFGF), the epidermal GF (EGF), and the transforming growth factor beta (TGF-β). It also contains the stem cells. The growth factors may enhance the differentiation and proliferation of chondroblasts (Barbon et al., 2019; Pavlovic et al., 2021).

The regeneration of growth plate defects is still one of the most challenging issues for researchers, as it may affect the quality of life for both animals and humans if not properly treated. Therefore, the objective of the current study was to radiographically evaluate the effect of employing an autologous PRF membrane in regenerating growth plate defects in rabbit models.

MATERIALS AND METHODS

Ethical approval

Ethical approval was granted before starting the study by the local committee for animal care and use in research at the College of Veterinary Medicine, University of Baghdad, Iraq (P-G/2558 dated Nov 11, 2023).

Study design

The present study included twenty healthy male and female rabbit kits (White New Zealand; aged 6-12 weeks, body weight: 500-1100 g). According to Yoshida et al. (2012), their mothers were allocated to private animal houses to calculate their ages properly. The reason for using immature animals was to ensure the presence of active growth plates, which become ossified upon reaching maturity. All the kits were housed under controlled temperature (22 ± 2C°) conditions, relative humidity (60-65%), 12-hour light-dark cycle, and were fed fresh vegetables (carrots, and lettuce), hay, and Alfalfa grass during the entire experiment. Each animal of the total number was considered for the treated and control groups: the left limb of each rabbit was exposed to a growth plate defect and then treated with a PRF membrane, while the contralateral (right limb) of the same animal served as the control and remained untreated.

Anesthetic protocol

Rabbit kits were initially anesthetized by intramuscular injection of 2% xylazine hydrochloride at the dosage of 5 mg/kg. After 10 minutes, ketamine hydrochloride (10%) was administered intramuscularly at a dosage of 35 mg/kg (Eesa, 2010). The heart rate was examined by stethoscope, and oxygen saturation was monitored with an oximeter.

Surgical procedure

The PRF was prepared according to Dohan et al. (2006b) by collecting 3 ml of blood sample via cardiac collection during the operation. The results were then transformed into plain glass tubes (glass tubes without anticoagulants). Immediate centrifugation at 3000 rotation per minute (rpm) for 10 minutes yielded three separated layers: red blood corpuscles (lower), PRF (middle), and acellular platelet-poor plasma (upper). The PRF was withdrawn with sterile forceps from the tube, cutting off the red blood corpuscles and squeezed between two sterile compresses to remove excess fluid (Huang et al., 2010). A 10×5 mm of the produced PRF membrane was used to fill the defect in the left limb of each kit (Figure 1A). The left limb was aseptically prepared for the surgery, and then an anteromedial longitudinal incision of about 3 cm was performed. The medial collateral ligament was identified and served as a landmark, and the growth plate of the proximal tibia was clearly distinguished as a white line, later confirmed by radiography. A drill bit with a diameter of 1 mm was perpendicularly directed to the growth plate and parallel to the joint to induce a defect of about (5×5×1) mm (width, depth, and length, respectively) according to Yu et al. (2019) (Figures 1B and C). Saline was irrigated through the drill track to cool and rinse out debris during drilling. The contralateral limb (right limb) of the same animal served as the control and was left untreated. Meanwhile, the PRF membrane (10 × 5 mm) (length and width, respectively) was inserted into the growth plate defect of the left limb (Figure 1D). Routine closure was performed, and antibiotics (Penicillin, 800,000 units administered intramuscularly twice a day for three days post-surgery) were administered until complete recovery was achieved. Stitches were removed 7-10 days post-surgery.

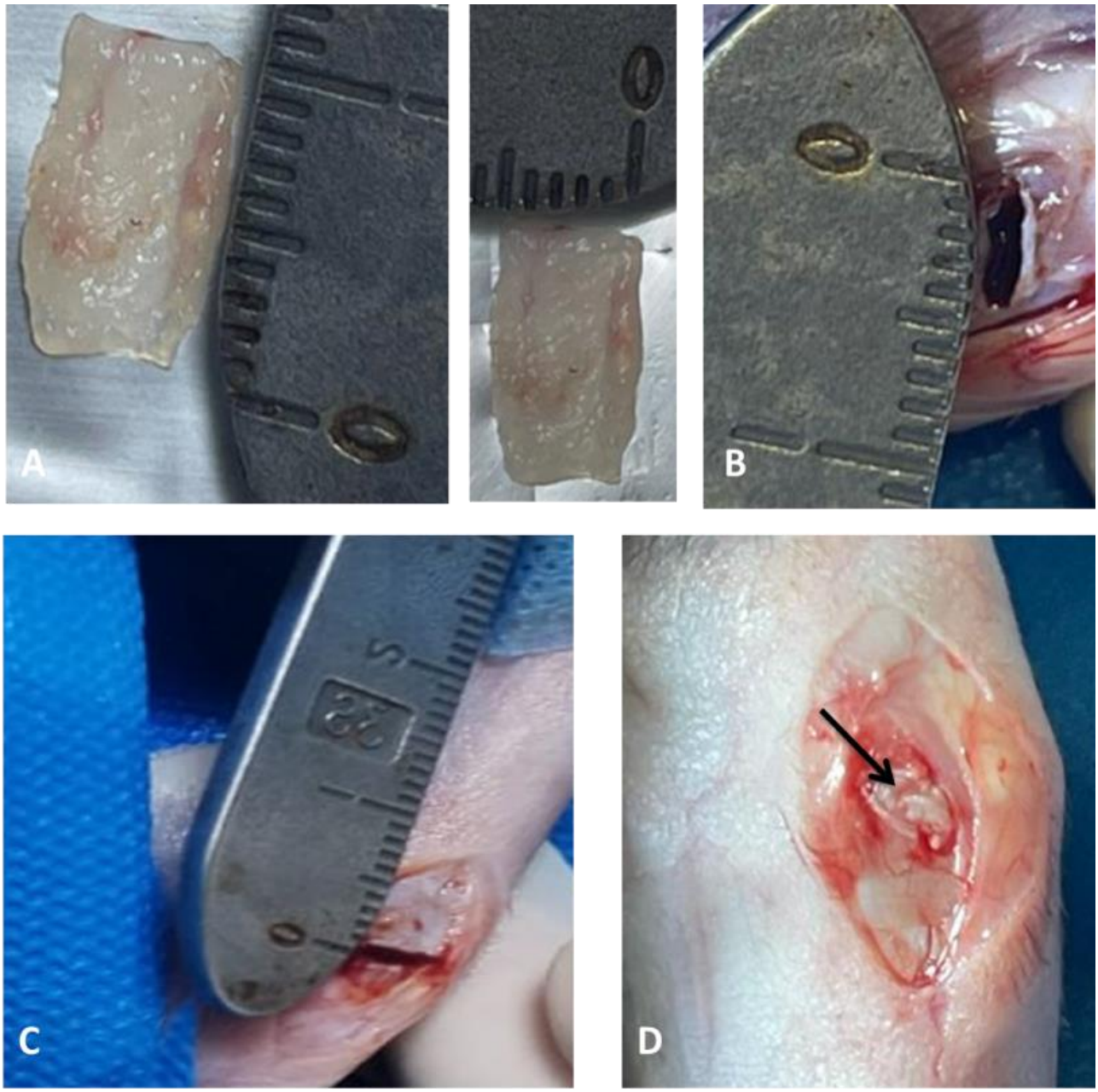

Figure 1. Preparation of the platelet-rich fibrin (PRF) membrane, measurement of the growth plate defect, and insertion of the PRF membrane into the growth plate defect in an immature White New Zealand rabbit. **A:** Platelet-rich fibrin membrane used in the defects. **B:** The length of the growth plate defect is 5mm. **C:** The width of the growth plate defect is 1 mm. **D:** The 10×5 mm area of the PRF membrane is inserted (black arrow) into the growth plate defect.

Radiological examination

An anteroposterior view of both hind limbs was examined after anesthetizing the animal with a DR machine (50 kV and 40 mA, Beam Limiting Device [Eco Ray] X-ray machine, Korea). The radiological examinations were conducted weekly up to eight weeks post-surgery. The opacity within the growth plate defect (ranging from a clear defect with radiolucent opacity to mild, moderate, and complete ossification), the growth plate area (normal, narrower, or wider than the adjacent unaffected growth plate), as well as a bony bar, shortening, and angulation of the affected limb (Figure 2) were evaluated weekly during the first, second, third, fourth, sixth, and eighth weeks and compared to the contralateral limb of the same kits within the population.

To diminish the effect of rabbit kit sizes on the tibial length measurement, images were calibrated before analyzing the tibial length and angle for both the affected and control limbs of each animal individually using ImageJ software (1.47v with Java 1.8.0-201). The tibial length was measured as the distance between the tibial distal growth plate and the proximal tibial plateau at 50% of the full width, while the tibial angle was assessed as the angle between the leg length measure and the average angle across the entire plateau. Tibial length and angle changes were assessed against the contralateral limb of the same animal within the whole population in line with Yu et al. (2022).

Statistical analysis

GraphPad Prism 5 (for Windows-Version 5.03) was used for data analysis with descriptive statistics including mean, standard deviation, and range as well as paired t-test at the level of $p \leq 0.05$. The data passed the normality test using the Gaussian distribution (Kolmogorov-Smirmov test with Dallal-Wikinson-Liliefor P value), and all data were confirmed to follow a normal distribution ($\alpha = 0.05$).

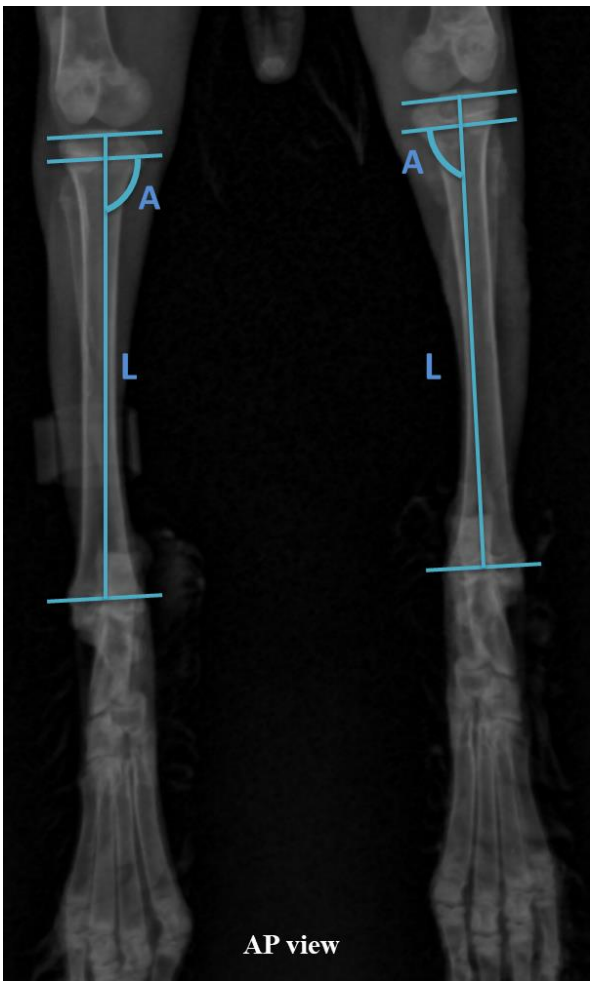

Figure 2. An anteroposterior (AP) radiograph view of an immature male White New Zealand Rabbit. Demonstrating the calculation of tibial length (L), identified as the distance between the tibial distal growth plate and the proximal tibial plateau at 50% of the full width. The tibial angle is measured as the angle formed between the length of the limb and the entire plateau (A).

RESULTS AND DISCUSSION

Rabbit kits were gradually returned to their feeding and drinking water a few hours after full recovery from anesthesia with normal movement and posture. The degree of the growth plate defect induced in this study has been previously classified as Salter's types III and/or IV. For this reason, leaving the defect without treatment would lead to bony bar formation as both epiphysis and metaphysis areas are affected (Xian et al., 2004; Jaimes et al., 2014; Yu et al., 2019).

In the current study, when the left affected limbs were radiographically examined via anteroposterior view pre-surgery, images showed normal growth plate shape and opacity within the proximal tibial growth plate. However, immediately post-surgery, the images showed an obvious defect in all subjects, with a mild increase in its opacity and a clear defect area within the proximal tibial growth plate, located medially after applying the PRF membrane (Figure 3). The anteroposterior view was also considered for identifying the growth plate defect area in the rabbit model by others for determining the changes within the defect (Yu et al., 2022) or the posteroanterior view in humans (Nguyen et al., 2017). In addition, Salter's second-degree classification requires an extra view, the lateral view, as the anteroposterior or posteroanterior views may not capture miner defects (Chen et al., 2015).

At the end of the first week, the defect area was visible and widened at the medial aspect of the affected limb in 73% of the subjects. Opacity within the growth plate defect remained unchanged at 45.5%, increased mildly at another 45.5%, and was significantly higher in 9% of cases treated with the PRF membrane (Figure 4A). Widening of the growth plate after trauma has been identified as indicative of a growth plate defect (Nguyen et al., 2017). Moreover, others have noted that irregularities in growth plate edges might also be associated with the widening of the growth plate after injury, indicating a growth plate defect, which is often diagnosed through more accurate techniques such as magnetic resonance imaging (MRI) (Jawetz et al., 2015) since radiography is less precise in detecting minor or early defects, such as fibrous

bar (Ecklund and Jaramillo, 2001; Jawetz et al., 2015). Furthermore, the anti-compression pad effect of the PRF membrane observed in this study might initially result in high opacity within the defected area.

By the second week post-surgery, 29% of cases of defect treated with PRF membrane retained visible defect borders, while 71% exhibited slight narrowing of the defect compared to the contralateral limb of the same animal. Radiographically, moderate opacity was observed in 57% of cases, mild opacity in 14%, and no detectable opacity in 29% (Figure 4B). The opacity and narrowing area of the growth plate defect, observed in about 71% of cases, possibly demonstrates the existence of the PRF membrane as a pad. However, the effect of releasing platelet-derived growth factor-AB (PDGF-AB) continues until the beginning of the second week to stimulate chondrocyte proliferation (He et al., 2009; Xiao et al., 2014; Kobayashi et al., 2016). Others referred to the PRF as a biological tool that delivers growth factors and cytokines to the site of injury, representing its role in the proliferation and differentiation of chondrocytes *in vitro* and its important role in cartilage repair (Brandl et al., 2010). This likely explains the high percentage of opacity in more than two-thirds of the population observed in the present study.

Both the third and fourth weeks post-surgery in the treated groups showed the existence of the growth plate defect radiographically, with no obvious higher opacity at about 57%. However, the remaining 43% exhibited higher opacity. On the other hand, the results of the area of the growth plate defect paralleled the opacity results, showing a slight narrowing in 57%, while 43% displayed a normal growth plate area (Figure 4C). It is hypothesized that the PRF membrane persisted up to the fourth week, explaining the continued opacity up to the fourth week. Previous studies have also indicated that the PRF membrane can remain functional for up to four weeks in experimental models (He et al., 2009).

By the sixth week post-surgery, the growth plate defect treated with the PRF membrane showed a normal growth appearance with no opacity in about 57% of the total population. The remaining 4343%, however, lacked clear radiographic identification of the growth plate but showed no evidence of bony bar formation (Figure 4E). Moreover, by the end of the work (the 8th-week post-surgery), approximately 57% of the cases displayed normal growth plate appearance without increased opacity, while 43% had some opacity and a narrow growth plate defect area (Figure 4F).

It is believed that the PRF membrane did not function as a decompressed pad by the 8th-week post-surgery but acted as a regenerative material as more than half of the total number of the treated group showed the existence of the growth plate by the eighth week. To explain the above, the decompressing effect of the PRF membrane should be ended by Week Four, as it has been suggested by others that the PRF membrane exists for a maximum of four weeks within the body after implantation (He et al., 2009). Therefore, the growth plate was not replaced by the bony bar up to the end of the study, which was similarly confirmed by Wong et al. (2020). Additionally, Dirja et al. (2023), emphasized the role of growth factors in PRF membranes in stimulating chondrocyte proliferation for improving the repair of articular cartilage defects, which was explained by the role of PRF in enhancing the viability, differentiation, and migration of chondrocytes.

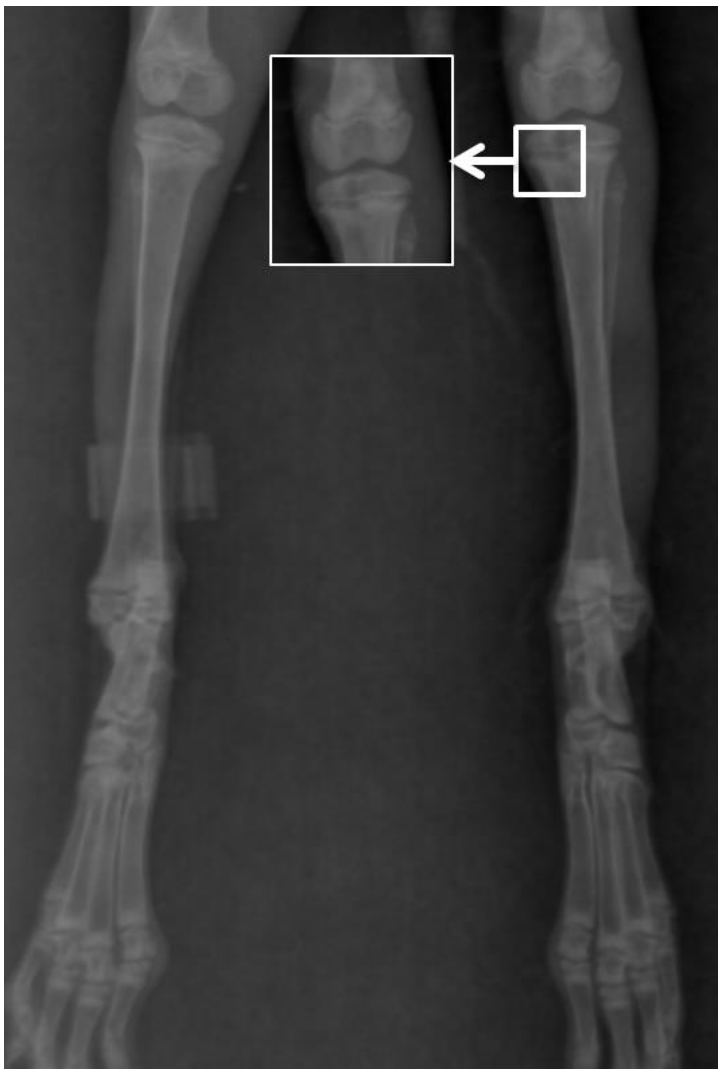

Figure 3. The shape of the growth plate in the medial part of the proximal tibia of an immature White New Zealand rabbit in the anteroposterior view. Demonstrating mildly increased opacity and a clear defect area after inserting the PRF membrane immediately post-surgery.

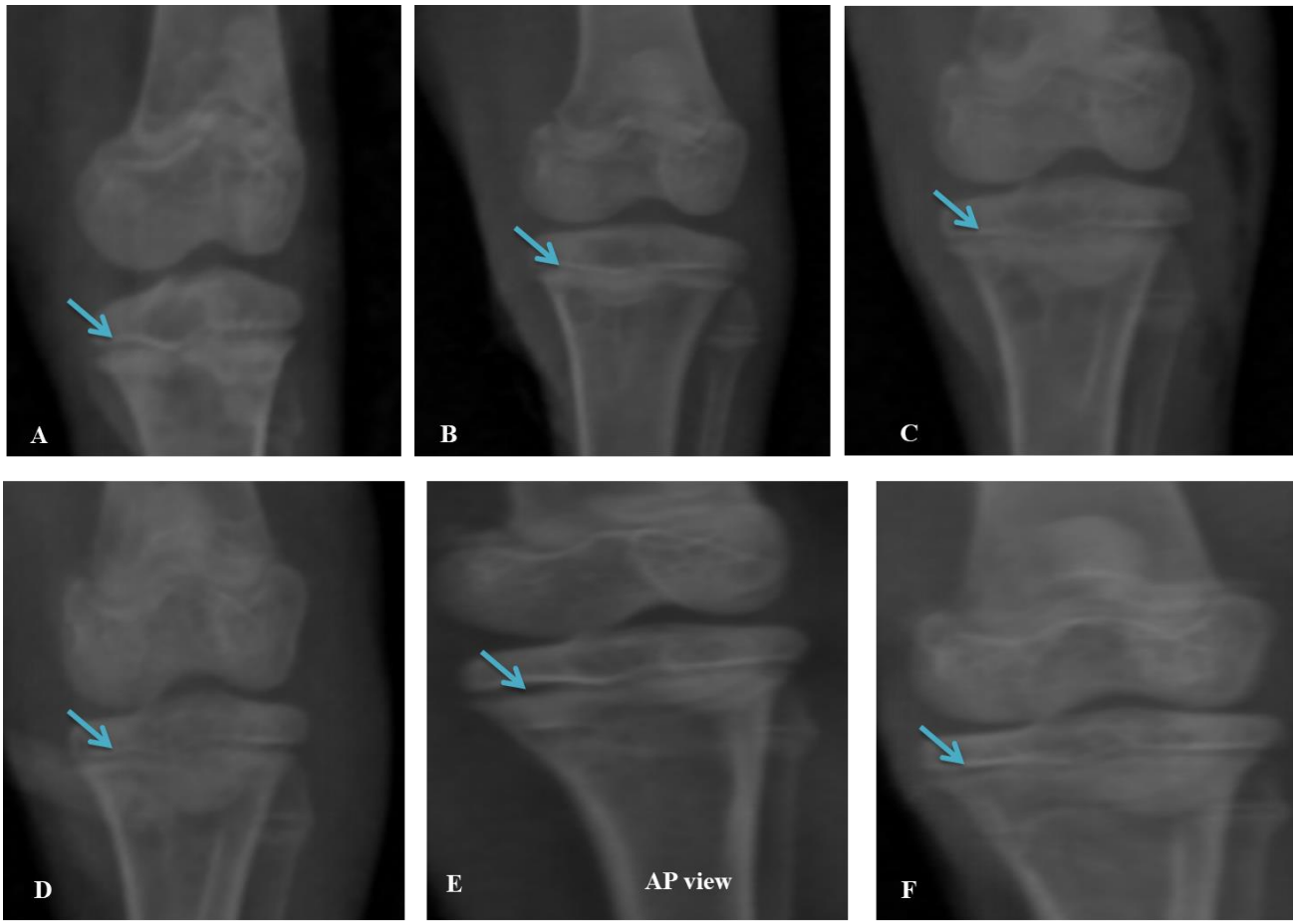

Figure 4. The growth plate defect area of an immature White New Zealand rabbit. The defect area is seen clearly (wide) in the first week post-surgery with mildly increased opacity at the medial aspect of the proximal tibial growth plate (**A**); In the second-week post-surgery, the defect area is seen slightly narrow with moderately increased opacity (**B**); the third week represents normal growth plate at the medial aspect in comparison to the lateral aspect of the proximal tibial growth plate with no obvious higher opacity (**C**); at the 4th (**D**), 6th (**E**), and 8th (**F**) weeks, the growth plate area appears normal with no obvious signs of bony bar formation from the epiphysis and metaphysis and no sign of bony bar formation at the growth plate defect.

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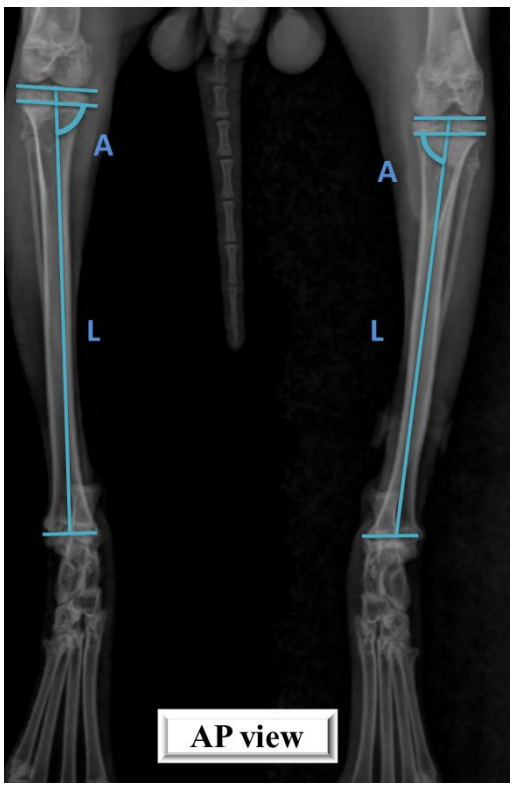

Figure 5. Anteroposterior view radiograph of an immature male White New Zealand rabbit representing the hind limbs eight weeks post-surgery. **L** shows the limb length, with no significant difference between the left treated limb and the contralateral limb of the same animal, and **A** shows angulation, with no significant difference between the left treated limb and the contralateral limb of the same animal, showing no sign of shortening and angulation deformities.

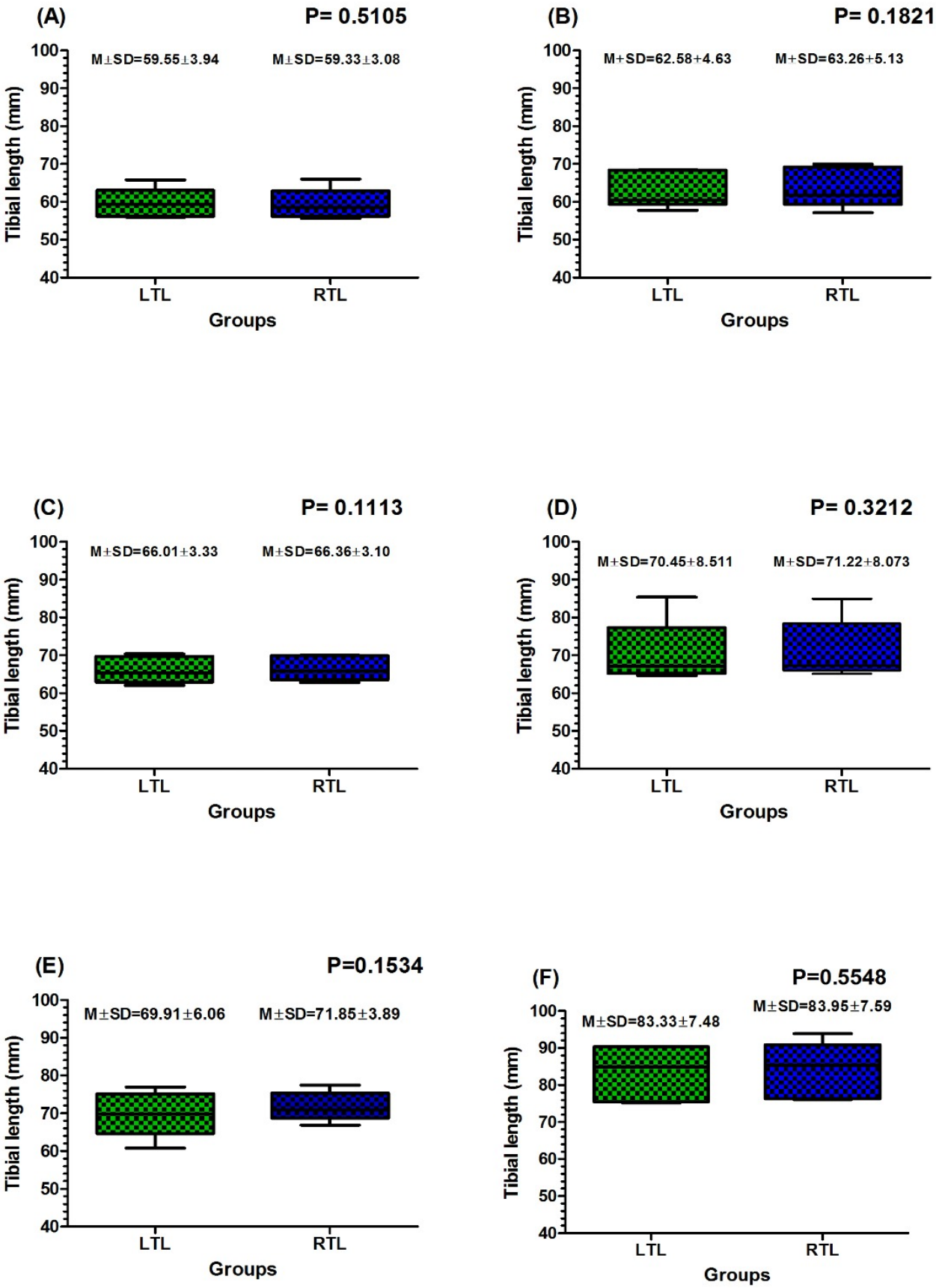

Figure 6. Box and whiskered vertical graph (with min-max values) of immature male and female White New Zealand rabbits representing the tibial length throughout the study. **A**: One-week post-surgery, **B**: Two weeks post-surgery, **C**: Three weeks post-surgery, **D**: Four weeks post-surgery, **E**: Six weeks post-surgery, and **F**: Eight weeks post-surgery. No significant differences ($p > 0.05$) were observed between the treated and contralateral limbs of the same individual White New Zealand rabbit kids. LTL: Left Tibial Length; RTL: Right Tibial Length.

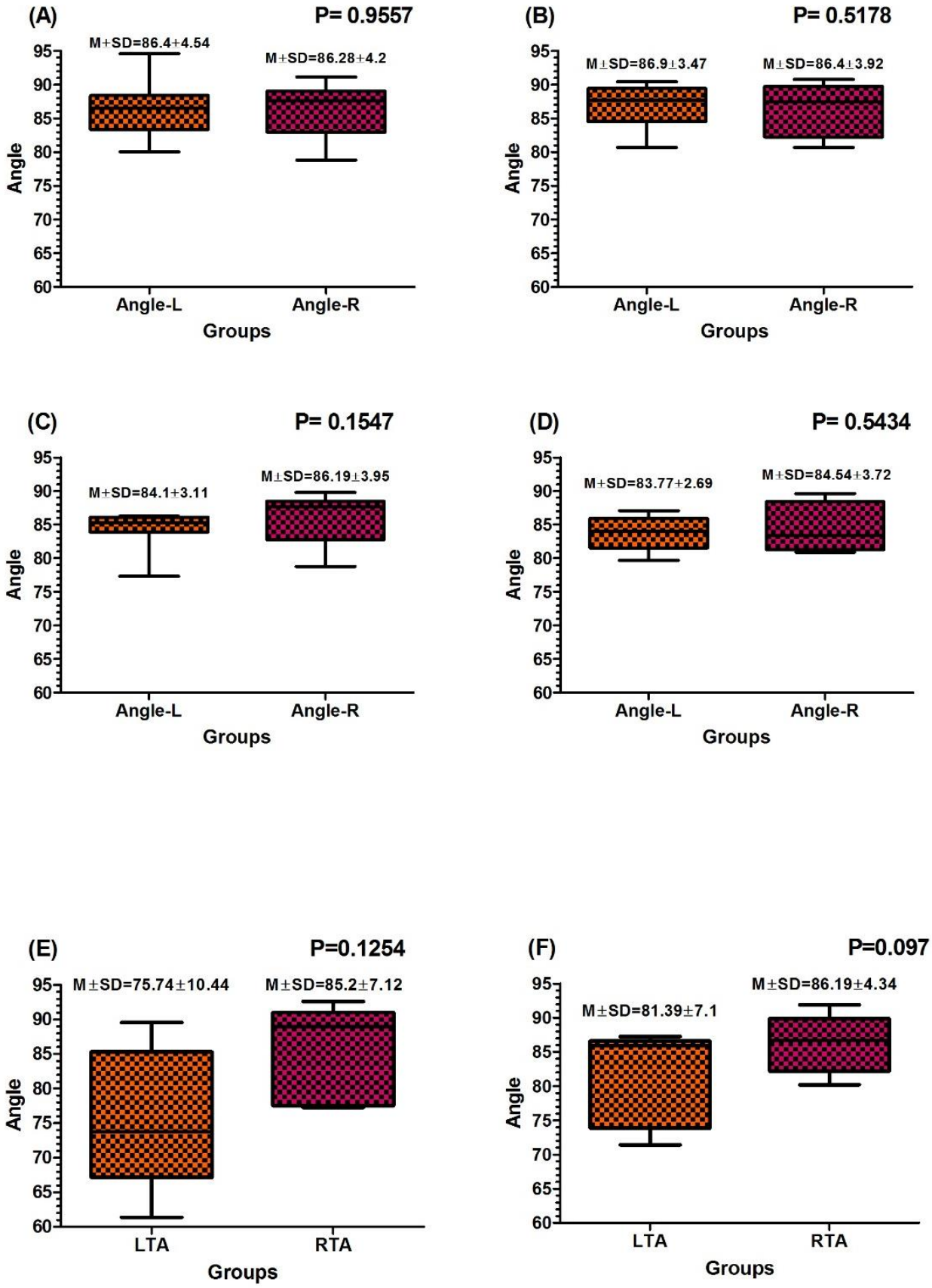

Figure 7. Box and whiskered vertical graph (with min-max values) showing the angle of the treated limb in comparison to the contralateral limb of the individual rabbit kid. There are no significant differences ($p > 0.05$) between the treated and contralateral limbs of the individual White New Zealand rabbit kid. **A**: One-week post-surgery, **B**: Two weeks post-surgery, **C**: Three weeks post-surgery, **D**: Four weeks post-surgery, E: Six weeks post-surgery, and F: Eight weeks post-surgery. LTA: Left Tibial Angle, RTA: Right Tibial Angle.

Radiographic evaluations of limb length and angulation throughout the study showed no significant differences between treated and contralateral limbs at eight weeks (P = 0.554 for length, P = 0.097 for angulation; Figures 5, 6, and 7). When chondrocytes of the iliac crest seeded on demineralized bone matrix as a scaffold were considered by a group of researchers, shortening and angulation were also significantly prevented when examined 16 weeks post-treatment (Jin et al., 2006). It is believed that the composition of the PRF membrane reduces angulation in the sixth- and eighth weeks post-surgery. Nonetheless, the decompressive effect of the PRF membrane during the first weeks may have a great effect in preventing the shortening of the limb and its further angulation. In contrast, other studies have used autologous bone marrow to treat growth plate defects, resulting in the osseous formation and limb shortening by the third-week post-surgery (Al-husseni, 2008). McCarty et al. (2010) used autologous bone marrow-derived mesenchymal stem cells (MSCs) for growth plate defect regeneration and showed positive results as they referred to the self-renewing and multi-lineage differentiation features of the mesenchymal stem cells (MSCs) for growth plate regeneration. Tobita et al. (2002) cultured autologous chondrocytes in atelocollagen gel and transplanted them into a growth plate defect, demonstrating that shortening and angulation were also significantly avoided after examination from 2 weeks up to 52 weeks. Chondrocytes implanted in atelocollagen gel may have mechanical properties to prevent a collapse with time and can proliferate and synthesize the extracellular matrix (ECM). In the present study, the shortening and angulation of the affected left limb is significantly avoided when compared to the contralateral limb in the same animals (P = 0.554). It is believed that the PRF membrane has released growth factors that might play a significant role in preventing the bony bar and reducing limb deformities. The above finding has also been supported by other studies which pointed to the effect of releasing transforming growth factor-β1 (TGF-β1) at the beginning of the third week in line with previous study (He et al., 2009) to stimulate the synthesis of ECM by chondrocytes and enhanced cartilage defect repair (van der Kraan and van den Berg, 2007). Other researchers used chitin as a scaffold for mesenchymal stem cell (MSC) transplant, which may prevent significant angulation as early as 2 weeks up to 16 weeks for shortening after excision growth plate (Li et al., 2004). It is probable that polyheterosaccharide found in chitin, similar to glycosaminoglycan (GAG), is structurally a major component of the ECM of cartilage. The angulation was also prevented. However, using other materials, such as bone wax and tissue-engineered construct (TEC), contributed to the significant prevention of angulation for up to eight weeks in rabbits (Yoshida et al., 2012), which is also explained by the significant role of bone wax as a decompressive pad from a side with extra positive results for TEC in regenerating growth plate defects. Nevertheless, using transplanted allogeneic chondrocytes for repairing growth plate defects may reduce bone angulation and shortening when examined for up to 16 weeks (Li et al., 2013). It is believed that these transplanted chondrocytes may release TGF-β1, playing a role in the chondrogenesis of bone mesenchymal stem cells (BMSCs) into chondrocytes to repair the injured growth plate and preventing collapsed growth plate defects and bony bar formation.

CONCLUSION

The present study suggests that the PRF membrane not only functions as a pad to reduce the possibility of epiphyseal and metaphyseal gap compression but also plays a role in regenerating the chondrocytes of the growth plate. This is evidenced radiographically by the prevention of bony bar formation, likely facilitated by the growth factors contained in the PRF membrane. In addition, stem cells and the above-said mechanism may play a positive role in cartilage regeneration by stimulating chondrocyte differentiation and proliferation, preventing the angulation and shortening of the limb. To further enhance the treatment outcomes, it is recommended to combine the PRF membrane with a scaffold, which could provide additional rigidity and support in the treatment of growth plate defects.

DECLARATIONS

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Author's contributions
Sura H. Abd-Alkhaleq and Aseel Kamil Hussein contributed to the planning and performed the study procedures as study design and writing. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials

The authors confirm that all data supporting the findings of this study are available upon reasonable request.

Ethical considerations

This article is not submitted anywhere else, and the findings are analyzed and written under the supervision of all authors. All authors wrote the article and checked the last draft of the manuscript for the similarity index.

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