



Published in final edited form as:

J Orthop Res. 2021 August ; 39(8): 1825–1837. doi:10.1002/jor.24859.

A novel engineered purified exosome product patch for tendon healing: An explant in an ex vivo model

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Abstract

Reducing tendon failure after repair remains a challenge due to its poor intrinsic healing ability. The purpose of this study is to investigate the effect of a novel tissue-engineered purified exosome product (PEP) patch on tendon healing in a canine ex vivo model. Lacerated flexor digitorum profundus (FDP) tendons from three canines' paws underwent simulated repair with Tisseel patch alone or biopotentiated with PEP. For ex vivo model, FDP tendons were randomly divided into 3 groups: FDP tendon repair alone group (Control), Tisseel patch alone group, and Tisseel plus PEP (TEPEP) patch group. Following four weeks of tissue culture, the failure load, stiffness, histology and gene expression of the healing tendon were evaluated. The transmission electron microscopy (TEM) revealed that exosomes of PEP the diameters ranged from 93.70 to 124.65 nm, and the patch release test showed this TEPEP patch could stably release the extracellular vesicle over two weeks. The failure strength of tendon in the TEPEP patch group was significantly higher than that of the Control group and Tisseel alone group. The results of histology showed that the TEPEP patch group had the smallest healing gap and the largest number of fibroblasts on the surface of the injured tendon. Quantitative RT-PCR showed that TEPEP patch increased the

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Author Contributions Statement: G.S. designed the study, performed the experiments, acquired the data, and contributed to the writing and preparation of the manuscript. Y.W., Z.W., A.T., and D.J., helped perform the experiments and analyzed the data. G.S., P.A., and C.Z. designed experiments and interpreted the data. Z.W., D.S., A.B., S.M., and C.Z. contributed to revision and preparation of the manuscript. All authors have read and approved the final submitted manuscript.

Conflicts of interest

AB is a co-founder of Rion LLC.

expression of COL3, MMP2, MMP3, MMP14, and reduced the expression of TGF-beta1, IL-6. This study shows that the TEPEP patch could promote tendon repair by reducing gap formation and inflammatory response, increasing the activity of endogenous cells and the formation of type III collagen.

Keywords

Flexor digitorum profundus tendon; Tendon healing; Fibrin Sealant; Purified exosome products

1. Introduction

Flexor digitorum profundus (FDP) tendon injury accounts for a high proportion of hand trauma.¹ Although recently tendon repair techniques have been improved, the frequent occurrence of repair site catastrophic rupture and adhesion formation, especially in Zone II and III, remains a clinically challenging problem.^{2; 3} Previous reports have shown that a lot of patients have a repaired tendon re-rupture after repair of the flexor tendon. Although there are multiple factors that may lead to repaired tendon rupture, the hypocellular and hypovascular nature of the flexor tendon has been considered as the major underlying reason for the low intrinsic healing ability of this tendon.⁴ Therefore, the novel regenerative strategies to enhance the flexor tendon intrinsic healing capacity are demanding to prevent repair tendon gap formation or rupture, thus improving clinical outcomes following flexor tendon repair.

Exosomes are membrane vesicles with a diameter of 30–150 nm secreted by multiple types of cells.^{5–7} Exosomes are distributed in various body fluids such as blood⁸, saliva⁹, urine¹⁰, cerebrospinal fluid¹¹ and breast milk¹². Due to the different cellular sources, exosomes have the capacity to carry different kinds of proteins¹³, lipids¹⁴, messenger RNAs¹⁵ and play a critical role in intercellular communication. Recently, the purified exosome products (PEP) derived from human plasma was identified as a positive role in regulating tenocyte biological behaviors in vitro.¹⁶ Exosomes extracted from blood have broad potential applications in medical diagnosis, health monitoring and personalized medicine due to their biocompatibility and ease of crossing various physical barriers.¹⁷

Fibrin Sealant (FS) has been widely used as an adhesive for tissue repair during surgical treatment of various diseases.^{18–20} It consists of two human plasma-derived components: (1) highly concentrated fibrinogen complex (FC) and (2) high potency thrombin (TH). FS has been shown to be a suitable delivery vehicle for many different kinds of cells and exogenous growth factors that could be used to accelerate bone growth and vascularization.²¹ In addition, fibrin sealant is an effective and well-tolerated therapeutic option for patients with Crohn's disease and perianal fistula tracts.²² Previous studies have shown the fibrin sealant as a new type of scaffold for tendon and bone healing.²³ Due to its biocompatibility and biodegradability, it can be used as an excellent carrier for tissue engineering carrying a variety of cells and growth factors.^{24; 25} However, studies on the tendon healing with exosomes attached to the fibrin sealant scaffold have not been reported.

The aim of this study was to develop a novel biopotented tissue-engineered purified exosome product (TEPEP) patch and to evaluate its effects on flexor tendon healing in an ex vivo canine tendon tissue culture model. We hypothesized that augmentation of flexor tendon repair with the TEPEP patch would improve the tendon's intrinsic healing ability.

2. Method and materials

2.1 Study Rationale and Design

The flexor tendon ex vivo model has been widely used to investigate tendon intrinsic healing ability, as the lacerated viable tendon can heal in a culture media.²⁶ Although the healing strength was relative low compared to in vivo healing model, ex vivo model does provide important information regarding tendon intrinsic healing at the cellular level. In this study, we used a total of 50 flexor digitorum profundus tendons from 3 dogs (8 months, weighing 23.7 ± 2.3 kg) sacrificed from other IACUC approved projects that did not involve tendon related research. PEP (Good Manufacturing Practice grade, Rion LLC Rochester, MN) was manufactured and supplied by the Advanced Product Incubator (API) of Mayo Clinic. In brief, the production process includes separating multiple expired human donor plasma, separating exosomes from the separated plasma (filtration and centrifugation), and encapsulating the exosomes according to the patent description (US Patent 20160324A1). PEP exosomes are provided in room temperature stable lyophilized form with specific exosomal properties as previously described.¹⁶ The carrier we used for PEP was commercially available Tisseel (Baxter Healthcare Corp., USA). Among these flexor digitorum profundus tendons, 2 were utilized for tenocytes isolation and cellular studies, while other 48 were randomly divided into 3 groups: FDP tendon repair alone group (Control, n=16), Tisseel patch alone group (n=16), and Tisseel plus PEP (TEPEP) (n=16). Following four weeks of tissue culture, thirty repaired tendons (ten per group) were evaluated with mechanical properties and quantitative RT-PCR, others (six per group) were evaluated with histology. (Fig. 1-A)

2.2 PEP-Tisseel Patch Preparation

TEPEP patch was prepared by rehydrating lyophilized PEP within the Tisseel solution following the protocol illustrated in Fig. 1-B. In brief, the PEP was dissolved into 1 ml PBS in order to make a PEP solution representing 5×10^{12} vesicles per ml (100%). 400 μ l PEP mixed solution (2×10^{12} vesicles) and 600 μ l Fibrinolysis inhibitor solution were added to Sealer Protein Concentrate to make the Fibrinogen. Then, 1000 μ l Calcium Chloride Solution was added to Thrombin to make the Thrombin. Finally, both two reagents were mixed to get a TEPEP patch with 20% PEP concentration (1×10^{12} vesicles per ml).

2.3 Tendon Repair and Culture

All FDP tendons were harvested immediately following dog sacrifice and placed in tissue culture medium to maintain the tendon viability. Each tendon was transected in the middle of a 30 mm length centered at the proximal interphalangeal joint level. This section of the FDP tendon consisted of two collagen bundles. A simple suture of 6-0 Prolene sutures (Ethicon, USA) was placed in each bundle. Before tightening the suture loop, TEPEP patch, Tisseel patch or no patch were implanted between the tendon ends and then the

sutures were knotted to close the repaired tendon (Fig. 1-C). The PEP patch was a gel like material, and after closing the tendon end, the patch was scraped and no visible gap was observed following tendon repair. The repaired tendons were placed between 2 wire meshes with longitudinal grooves designed to maintain the tendons in a straight position without applying any tensional force on the tendon (Fig. 1-D). After 4 weeks cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco®, USA) containing 10% fetal bovine serum (FBS; Gibco®, USA) and 1% antibiotic-antimycotic (AA; Gibco®, USA), the tendons were assessed biomechanically and histologically. In the culturing process of the tendon model, a large number of adherent tenocytes were observed at the bottom of the square dish of different groups, all tenocytes were quantified by Image J (v1.48).

2.4 Transmission electron microscopy of PEP

According to the our previous proposal for exosome observation,¹⁶ 100% (v/v) concentration PEP solution was fixed with 1mL 2.5% glutaraldehyde in 0.1M sodium cacodylate solution for 1 hour at 4°C. After rinsed with 0.1M sodium cacodylate buffer and 2% osmium tetroxide for 1 hour, all samples dehydrated using a graded series of ethanol and stained with 2% uranyl acetate. Then, a total of 50 µL samples (5×10^{10} vesicles) per grid were viewed using a transmission electron microscope (TEM, JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV.

2.5 Release Test and Cellular Uptake of PEP

The release ratio of TEPEP patch was tested using a NanoSight System (NS300, Malvern, UK). Briefly, 20% TEPEP patch was mixed with 3 ml PBS, following incubation in a 15 ml tube at 37 °C. After incubating in a 37 °C incubator for 1 day, the supernatant PBS was collected and moved to another 6 ml collecting tube for testing. Add the new PBS and repeat until day 14. The particle concentration and size distribution was measured by nanoparticle tracking analysis (v3.20 software).

For the cellular uptake of PEP, tenocytes were harvested from fresh FDP tendons from Mixed-breed dogs mentioned above based on well-established protocol.¹⁶ Tenocytes (P4) were seeded with 300 cells per well on a Cell Culture Slides (Corning®, USA). After 2 days cultured in DMEM containing 10% FBS and 1% AA. The PEP was labeled with 1 mM Vybrant® CM-Dil solution (Invitrogen, USA). Then, the CM-Dil labeled PEP was incubated with tenocytes in serum-free DMEM at 37 °C for 2 h. After washed with PBS and fixed in 4% paraformaldehyde solution (Santa Cruz, USA), the tenocytes were stained with F-actin Alexa Fluor™ 488 Phalloidin (Invitrogen, A12379, USA) and Hoechst 33342 (Thermo, USA) respectively. Finally, the fluorescent images were observed and captured using confocal laser scanning microscopy (LSM 780, Zeiss, Germany).

2.6 Biomechanical Testing

After the 4 weeks culture, repaired tendons (n=10 each group) were mounted on to a custom-designed microtester to characterize mechanical properties of the repaired tendon healing. The testing apparatus included a load transducer, a stepper-motor driven stage, and a potentiometer that connected to a hook. The repaired tendon ends were placed with single loop suture that connected to the testing hooks (Fig. 1-E). Before testing, tendon repair

sutures were cut without disrupting the repair site, which enabled assessment of the strength of the healing tissue rather than the composite strength of suture and healing. The tendons were placed on a flat, plastic platform moistened with saline. Specimens were tested to failure at a rate of 0.1 mm/second. Failure strength and displacement were measured by the transducer and recorded at a sample rate of 20 Hz. Stiffness was defined as the slope of the linear region of the force/displacement curve.

2.7 Histology

After four weeks of culture, repaired tendons (n=6 each group) were used for histology described below. Tendon samples were embedded in optimal cutting temperature compound and cut at 7 μ m slices with a cryostat. Sections were stained with Hematoxylin and Eosin (Thermo, USA), Sirius Red (Polysciences, USA), and Masson staining (American Mastertech Scientific, USA) to evaluate the morphology and cellularity with light microscopy. Image J (v1.48) was used to quantify the cell number or healing gap of tendons.

2.8 Quantitative real-time RT-PCR

After biomechanical testing, the tendons tissue (n=10) were used for quantitative real-time PCR gene expression analysis. In addition, Dog's tenocytes of three above treatments also be harvested in order to investigate whether Gene expression difference compare with tendon tissue (Supplement Figure 1). All samples were performed on C1000 Touch™ Thermal Cycler using SYBR Green PCR Master Mix (Quantabio, USA) to measure the gene expressions of Collagen type I alpha 2 chain (COL1A2), Collagen type III alpha 1 chain (COL3A1), Transforming Growth Factor beta-1 (TGF- β), Interleukin 6 (IL-6), Matrix Metalloproteinase 2 (MMP2), Matrix Metalloproteinase 3 (MMP3), and Matrix Metalloproteinase 14 (MMP14). GAPDH is as an internal control. The primers are shown in Table 1. The relative quantification of the genes of interest was calculated using the 2^{-Ct} method.

2.9 Statistical Analysis

The outcomes of primary interest were the biomechanical parameters of ultimate failure strength and stiffness, relative gene expression, and histology outcomes of morphology and cellularity. The histology outcomes were reported descriptively only. Otherwise, outcomes comprised of continuous variables were compared between the 3 tendon repair groups using mixed-models analysis of variance, incorporating the dog ID as a random term to account for the within-animal correlation among the digits contributed by each dog. If the overall tests of the tendon repair group were statistically significant, pairwise contrasts were generated to identify which groups were different from the others. In order to guard against the increased type-I error rate associated with multiple comparisons, the p-values from the pairwise contrasts were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate. All statistical tests were two-sided and *p*-values less than 0.05 were considered significant.

3. Results

The morphological investigation of exosomes was performed by transmission electron microscope. The TEM micrographs showed that exosomes of PEP present hollow vesicle spherical structures with hypodense in the center and hyperdense in the around. (Fig. 2-A) The diameters of exosomes were ranged from 93.70 nm to 124.65 nm as measured with Image J software. We incorporated PEP into the Tisseel as a gel patch, which could release exosomes to the surroundings in a sustained manner. The particles concentration of PEP was calculated every day and the size distribution was 50~200 nm. (Fig. 2-B, 2-C and 2-D) The representative picture of NanoSight detecting process showed that flash light spots were particles in different focus planes. (Fig. 2-E) Fig. 2-F showed the TEPEP patch could release particles stably over a two-week observation period.

All F-actin and Hoechst 33342 showed a positive fluorescence in cytoplasmic and nuclear of tenocytes respectively. The nanoparticles showed a higher fluorescence in cytoplasm of tenocytes with 20% PEP compared to tenocytes without PEP. (Fig. 3-A and 3-B) In addition, in the culturing process of the tendon model, a large number of adherent tenocytes were observed in the TEPTP patch group at day 14, versus the smaller number of tenocytes observed in both Control and Tisseel patch group (Fig. 3-C and 3-D). During mechanical testing, all specimens failed at the repair site. The failure strength in the Tisseel patch (40.0243 mN, $p = 0.0025$) and TEPTP patch (57.9858 mN, $p < 0.0001$) group were significantly higher than that of the Control group (18.5197 mN), while the TEPTP patch was found superior to the Tisseel patch alone group ($p = 0.0223$) (Fig. 3-E). Tensile stiffness in the Tisseel patch (16.4584 mN/mm, $p = 0.0011$) group and the TEPTP patch (13.6093 mN/mm, $p < 0.0001$) group was significantly higher than that in the Control group (5.7209 mN/mm). There was no significant difference between the TEPTP patch group and the Tisseel patch group ($p = 0.06305$) (Fig. 3-F).

Partial healing was histologically documented in all the three groups (Fig. 4-A), with healing gap quantified among the three groups with Image J software (Fig. 4-B). The healing gap in the TEPTP patch group was significantly smaller than both the Control group ($p < 0.0001$) and the Tisseel patch group ($p = 0.0014$). There were no differences between the Control group and the Tisseel patch group ($p = 0.5561$) (Fig. 4-D). In addition, we also found a lot of fibroblasts migrated into the injury site of the flexor tendon in PEP-Tisseel group (Fig. 4-C). The cell number in TEPTP patch group was significantly higher than both Control group ($p = 0.0241$) and Tisseel ($p = 0.0139$) patch group (Fig. 4-E). While there were no differences between the Control group and the Tisseel patch group ($p = 0.4625$). Moreover, the Sirius red staining showed most of the collagen fibers belong to type I collagen and difference in collagen alignment was observed (Fig. 5-A). Masson trichrome staining showed most of the fibers are collagen fibers (Fig. 5-B).

For the gene expression in the dog tenocytes, the expression of COL3A1, MMP2, MMP3, and MMP14 was significantly increased in the TEPEP patch Group compared to other groups. For the expression of COL1A2, TGF- β and IL-6, there were no significant changes between TEPEP patch and Control groups (Fig. 6-A). Besides, the gene expression of COL1A2, COL3A1, MMP2, and MMP3 in the FDP tendon tissue were consistent with

above tenocytes study. The expression of TGF- β and IL-6 was significantly decreased in the TEPEP patch Group. And there were no significant changes in COL1A2 and MMP14 between TEPEP patch and Control groups (Fig. 6-B). Therefore, the role of PEP in this ex vivo tendon repair model may be to reduce inflammation and increase expression of type III collagen. (Fig. 6-C)

4. Discussion

A number of studies have been published recently on the effect of exosomes on tendon healing. For tendon repair, previous studies mainly focused on surgical techniques, growth factors, cell therapy, and tissue engineering scaffolds.^{27–29} Despite new biological scaffolds and other treatments designed to improve tendon healing and minimize scar formation, the mechanical properties and proliferation of tenocytes remain problematic.^{30; 31} In this study, we proposed a novel engineered purified exosome product patch for tendon healing, our results suggested that the Tisseel could improve biomechanics properties and the PEP could accelerate fibroblast migrates to the injury site of tendon gap and reduce the inflammatory microenvironment in the injured site. Moreover, PEP is a GMP grade product, also a traceable, safe, pure and effective drug. This makes the novel ex vivo model have potential clinical transformation prospects. Therefore, this study represented an innovative method in tendon repair strategies that could be translated for flexor digitorum profundus tendon injuries in human patients.

With the development of current biological scaffolds, composite materials such as biological materials combined with cells or various factors have been deeply explored.³² However, finding a bioactive material that can be rapidly degraded and stably release certain targeting factors has become a common problem faced by scientists worldwide. In this study, the TEPEP patch, which could release exosomes to the surroundings in a sustained manner within two weeks. The size distribution of particles was 50~200 nm and most of the particles released into the surrounding area could be well uptake by tenocytes. Although we didn't explore molecular or drug delivery capacity of PEP, there is no doubt that we could add certain mRNAs or related drugs via this way according to previous research.³³ In addition, this novel tissue-engineered tendon ex vivo model, it can mimic the intrinsic repair ability of tendon tissue and avoid the ethics or time constraints of in vivo experiments. In tendon explants, cells could still transfer mechanical stimuli and biological signals in a way that mimics the in vivo complexity of cell-cell and cell-matrix communication. This also makes it possible to study the histological morphology and mechanical mechanics of tendons. Different from our previous tendon model studies (bone marrow stromal cells,³⁴ growth and differentiation factor 5,³⁵ or different ratios of fibrin gels³⁶), this study first adopted PEP exosomes, a GMP grade product, as a safe and off-the-shelf biologics-based strategy to promote endogenous repair of tendon tissue.

Mechanical properties are one of the most important concerns for tendon healing. Our previous study attempted to use different concentrations of Fibrin gel (5–80 mg/mL Fibrinogen, 25–500 NIH units/mL Thrombin) to repair tendon, we found using high concentration of Fibrinogen as the patch have better mechanical properties (include the Failure Load, Failure Stress, and Tensile Stiffness) than the low one.³⁷ The methods of

mechanical testing and delivery scaffold were consistent with our research. In this study, compared with other groups, the mechanical properties of TEPEP group are better. This is most likely caused by the PEP's stimulation of tendon cell proliferation and the migration of fibroblasts on the tendon surface to the site of injury. Use of PEP (with no dose-to-dose variability) instead of bone marrow stromal cells, greatly reduced the inter-group error and enhanced the mechanical stability of the patch itself. Moreover, failure stress is also important indicator to evaluate the mechanical properties of tendon.³⁸ Therefore, a systematic biomechanical assessment of tendon recovery at the right time becomes particularly important.

The extracellular matrix of tendons is composed of several types of collagen molecules (e.g. Type I/III collagen), which play an important role in the repair and regeneration of injured tendon tissue. In this study, we found that the type III collagen increased in the TEPEP group compare with other groups. Due to collagen's unique triple helical structure, it can provide stable mechanical properties, good support, and elasticity for cells. Besides, it participates in various cell signaling pathways and cell differentiation. The synthesis of collagen III involves the early stages of wound repair because of a large number of fibroblasts migrate into the surface of the tendon rupture to form the fibronectin matrix.³⁹ This further confirms our hypothesis that PEP can promote accumulation in collagen III and accelerate the process tendon healing. On the other hand, after the tendon ruptures, its microenvironment is broken. In the healing process of tendons, a variety of cytokines and matrix metalloproteinases (e.g. MMP2, MMP3, MMP14), inflammatory factors (e.g. TGF- β , IL-6) and angiogenic factors constitute an inseparable interrelationship.⁴⁰ This project found that the application of PEP can effectively reduce the expression of IL-6 and TGF- β , further decrease the inflammatory response during tendon healing and increase the stability of the extracellular matrix.^{41; 42} Although we have not detected much gene expression changes in inflammatory factors and matrix metalloproteinases in cellular experiments, this may be due to the short exposure time of the cells to exosomes or due to an absence of immune cells in the ex vivo context. It should be noted that some gene expressions between tenocytes and tendon tissue were different. There may be several reasons. 1) Large amount of cells in the tenocyte model compared to tissue culture; 2) The PEP in cell culture medial may have a direct impact on the culture cells; 3) PEP intake by cells may be more predominant in cell culture model than the tissue culture; 4) extracellular matrix environment is different between cell culture and tissue culture. However, several gene expressions, such as type I and III collagen and IL-6, have shown the same trend between tenocyte model and repair tendon model.

The matrix metalloproteinases, synthesized and secreted by fibroblasts, are a class of enzymes that digests collagen and other structural molecules.⁴³ It has been reported that MMP2 plays an important role in the degradation of collagen type I.⁴⁴ This may further explain why we detected large amounts MMP2 of in tendon tissue but did not find much expression of type I collagen. In previous tumor invasion studies, MMP2 activation was furthermore shown to be closely related to MMP14 expression, consistent with the results detected in our project.⁴⁵ Above all, the specific role of PEP in different collagens by regulating various types of matrix metalloproteinases still requires further exploration.

The conclusions from this project is limited by the fact that the tendons utilized this study were of canine origin being treated with exosomes of human origin. Although the impact of PEP exosomes in humans would be anticipated to be stronger, the results reported herein will need further verification. Additionally, the ex vivo designs of this study provided a targeted focus on tenocyte mediated tendon healing. However, with the significant impact of microvasculature as well as the immune system on tissue regeneration, in vivo verification of the findings described is required.

In summary, application of the PEP loaded TISSEEL patch after flexor tendon repair increases the tendon intrinsic healing strength, cellularity, and tenocyte migration, while decreasing the repair gap formation and tenocyte inflammatory response in an ex vivo tendon healing model. Since our PEP is a fully off-the-shelf GMP grade biologics and TISSEEL is pharmaceutical agent, this approach has high potential for clinical translation. However, combination of PEP with a hydrogel delivery system in a pre-clinical in vivo study is required to validate the findings from the current study before clinical trial application can be pursued.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by grants from Orthopedic Research Review Committee of Mayo Clinic (94064024), NIH NIAMS AR57745, and Mayo Clinic Kelly Fellow Award. Mechanical testing was supported by the Mayo Clinic Biomechanics Core Facility. This study also thanks for the technical support from Dr. Zeling Long and Ramona. The authors Guidong Shi, Zhanwen Wang are funded by the China Scholarship Council.

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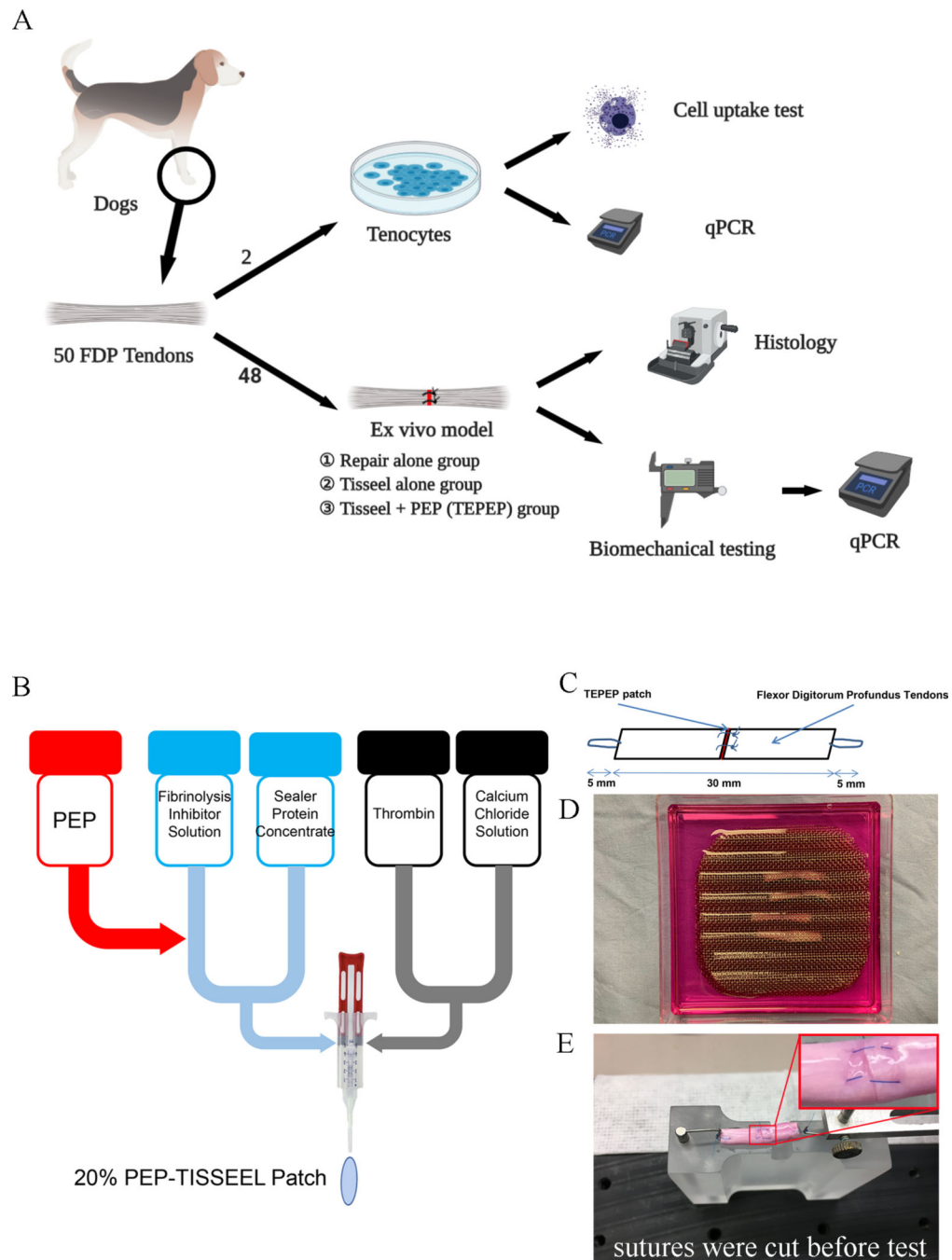


Figure 1:

An explant in an ex vivo model and mechanical evaluation. A. Study design of this study. B. PEP-Tisseel patches fabrication procedures. C. Each test specimen was 40 mm long, which included 5 mm of suture loop at each end and 30 mm of tendon. D. Repaired tendons were kept straight without tension between the grooves of custom-made wire meshes. E. Repaired tendon mounted on the experimental platform. (Sutures were cut before mechanical evaluation)

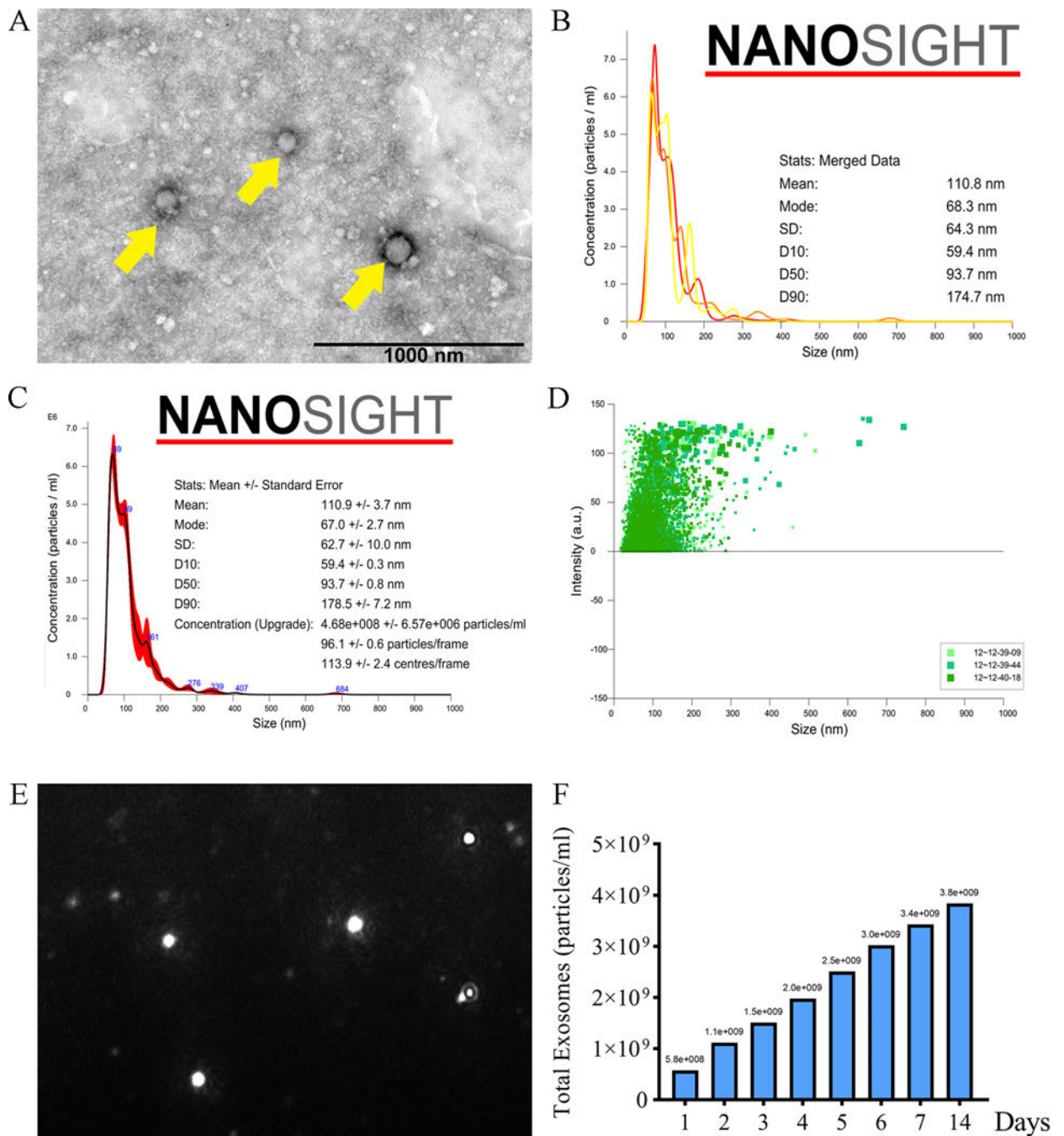


Figure 2: Characteristic and releasing test of the TEPEP patch. A. The structure of exosomes was observed by TEM. (Yellow arrows) B. Concentration of particles released by TEPEP patch in day 4. C. Mean size of particles released by TEPEP patch in day 4. D. Size distribution of TEPEP patch in day 4. E. Flash light spots were particles in different focus planes. F. Release test of TEPEP patch within two weeks.

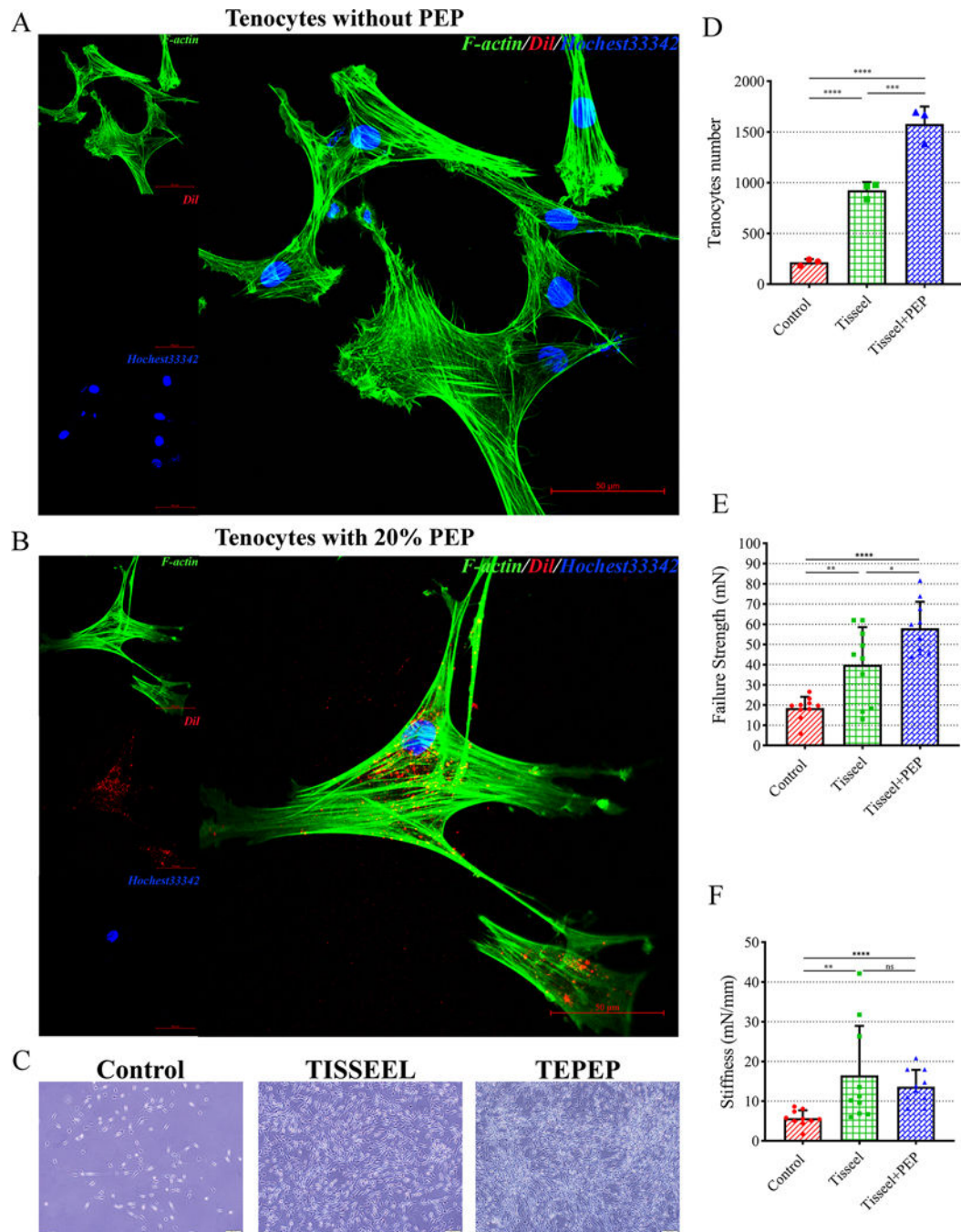


Figure 3:

Cellular uptake and mechanical testing results. A. Cellular uptake of PEP into tenocytes without PEP. (Scale bar: 50 μ m) B. Cellular uptake of PEP into tenocytes with 20% PEP. (Scale bar: 50 μ m) C. Tenocytes attached on the square dish in different groups at day 14. (Scale bar: 100 μ m) D. Quantification of the tenocytes number among three groups. E. Mean failure strength of the repaired tendon. F. Mean stiffness of the repaired tendon. Error bars represent standard deviation. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

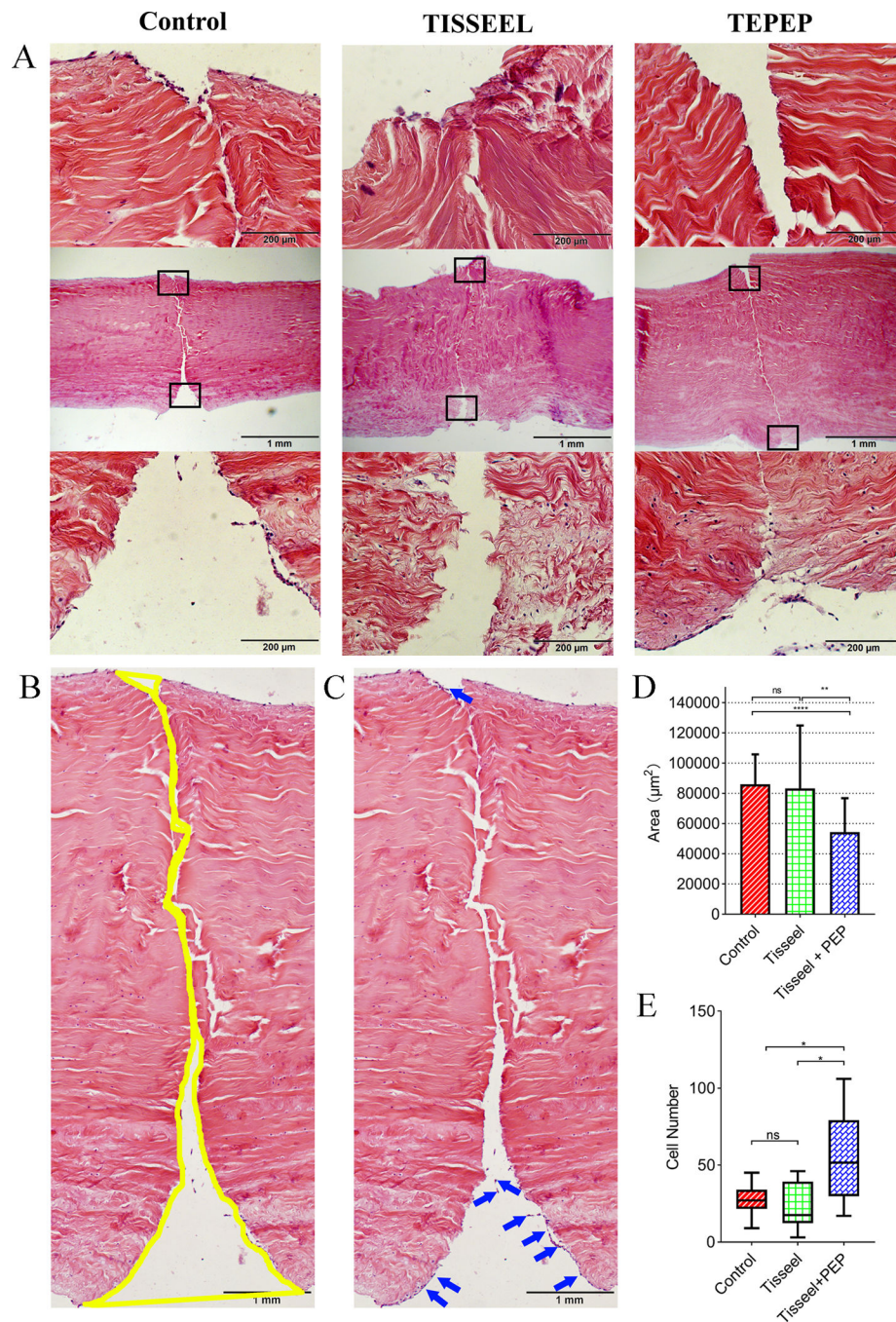


Figure 4: Histology results of healing tendons in treatment groups at 4 weeks. A: Representative H&E-stained images. B-C: Representative H&E-stained images from control group. Yellow area showed the healing gap of tendons. Blue arrows showed the fibroblasts migrate to the surface of injury tendon. (Scale bar: 1 mm) D. Quantification of healing among three groups. E. Quantification of the number of fibroblasts among three groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

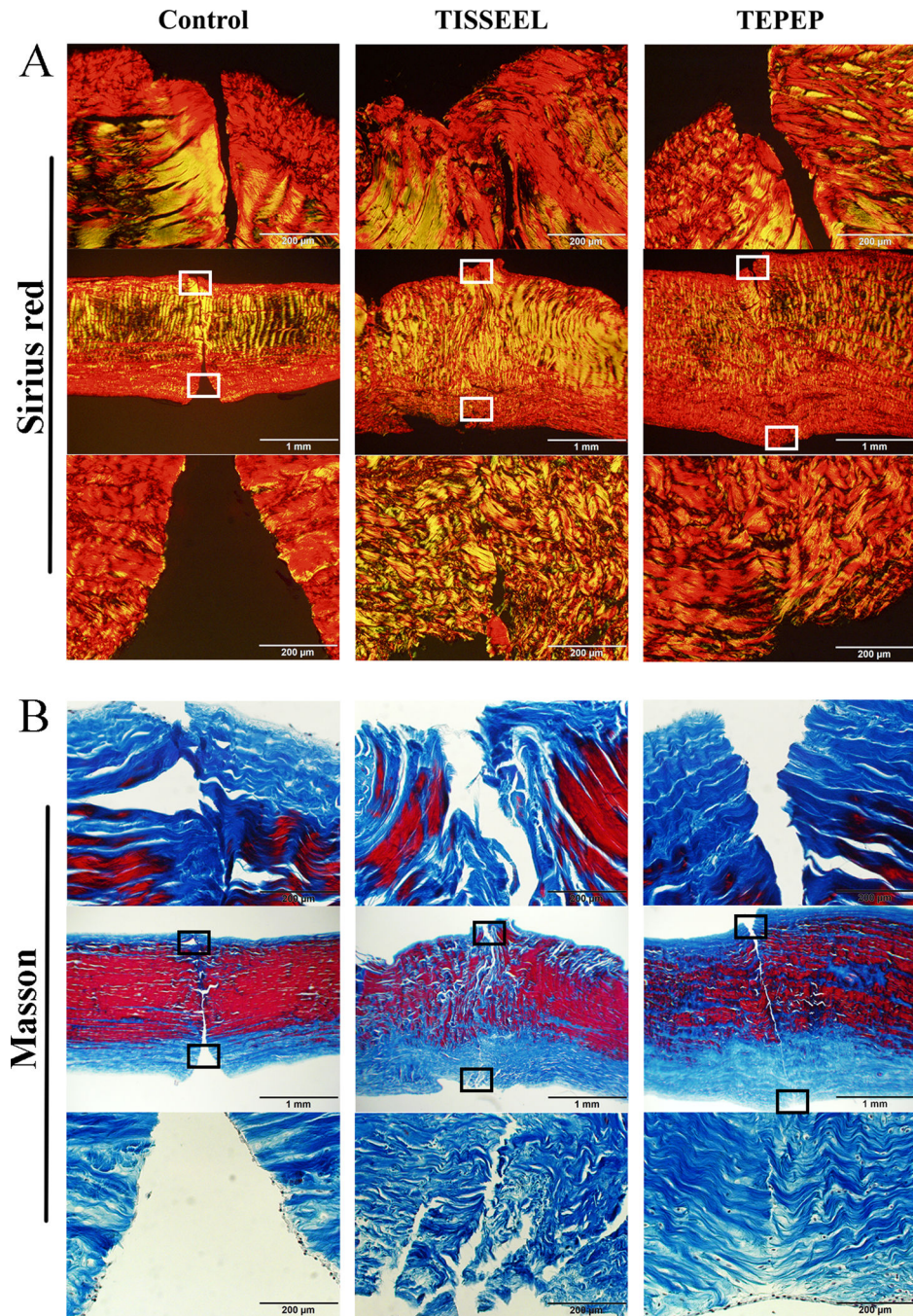


Figure 5: Histology results of healing tendons in treatment groups at 4 weeks. A. Representative Sirius red staining images at 4 weeks after surgery. B. Representative Masson staining images at 4 weeks after surgery.

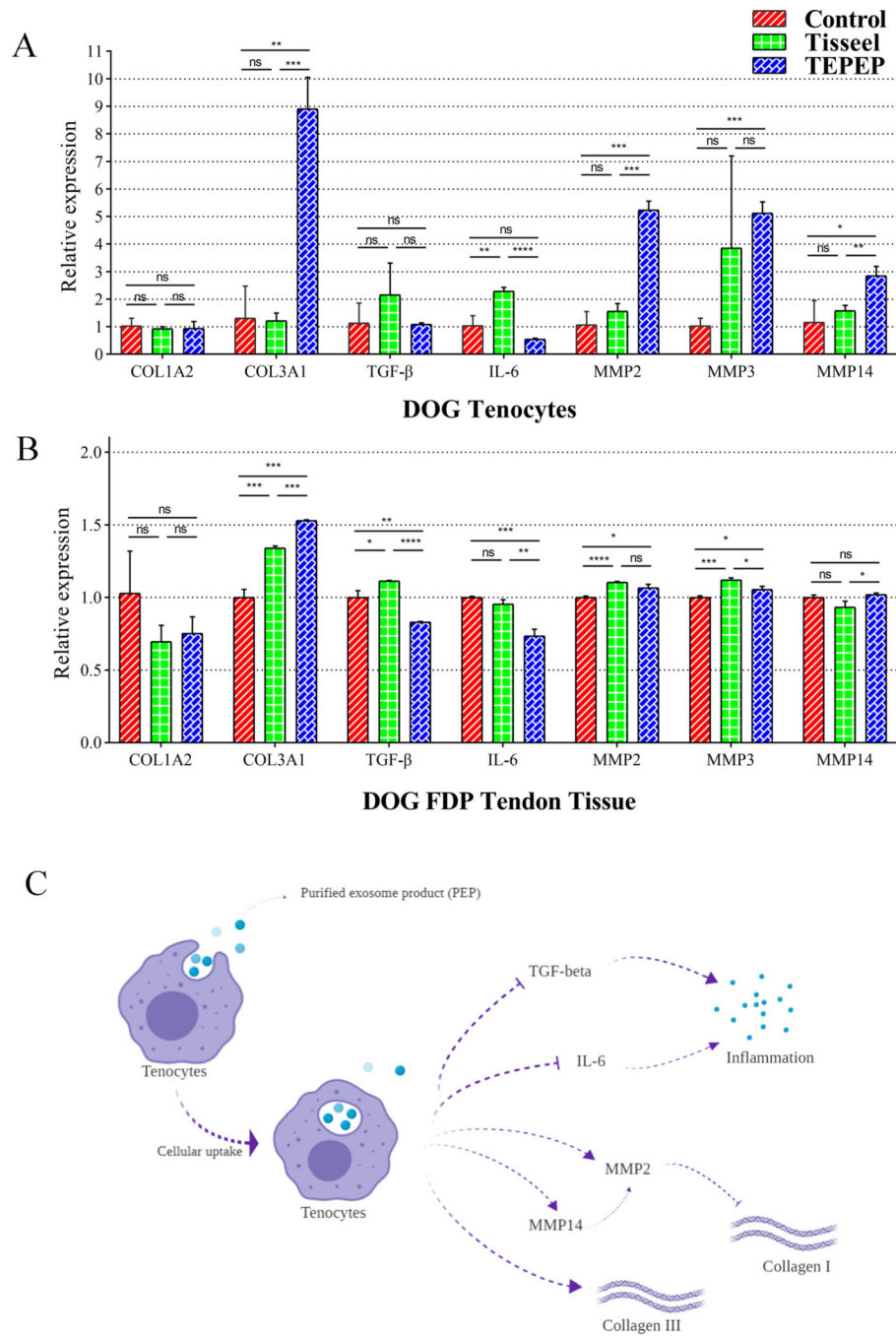


Figure 6A and B: Gene expressions of tenocytes and healing tendon tissue among three groups at 4 weeks. **C.** The role of PEP for tendon healing in the ex vivo model.

Table 1

Primer sequences used for quantitative RT-PCR.

Gene	Accession No.	Forward	Reverse	Product Length (bp)
COL1A2	NM_001003187	tggaagtcgtggtgatggtg	tctttaccagcagcaccagg	126
COL3A1	XM_845916.4	ccgtgccaaatatgcgtctg	aggaggagatgttgaggct	144
TGF- β	NM_001003309	tccggcagctctacattgac	ctccaaatgtagggcaggg	109
IL-6	NM_001003301.1	cggcaaaatctctgactga	actccacaagaccgtagtg	182
MMP2	XM_014109407	cacggccaactatgatgatg	agaatgctcagtcctcattg	112
MMP3	NM_001002967.1	cattccctgggtctcttca	ggaggaatcagaggaggtc	150
MMP14	AY534615.1	tgctgctctctctggatgc	ttttgggtactcgtgtcc	107
cGAPDH	NM_001003142	aacatcatcctgcttccac	ggcaggtcagatccacaact	130

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