
بسم الله الرحمن الرحيم

باسمه تعالی

گواهی دفاع از رساله دکتری تخصصی

دانشکده: دامپزشکی

گروه آموزشی: جراحی

هیات داوران رساله دکتری آقای : رافد الخلیفه در رشته: جراحی با عنوان : ارزیابی پیوند تاندون منجمد پوشش داده شده با سلول های بنیادی مزانشیمی مشتق شده از مغز استخوان خودی و پلاسمای غنی از پلاکت جهت حفظ طول تاندون در بره گوسفند" را در تاریخ: ۱۳۹۷/۰٤/۰٦ با درجه: عالی ارزیابی نمود.

مشخصات هیات داوران	نام و نام خانوادگی	مرتبه دانشگاهی	دانشگاه یا موسسه	امضاء
۱- استاد راهنمای اول	جناب آقای دکتر داود شریفی	استاد	دانشکده دامپزشکی دانشگاه تهران	
۲- استاد مشاور (حسب مورد)	جناب آقای دکتر عباس توسلی	استاد	دانشکده دامپزشکی دانشگاه تهران	
۳- استاد مشاوردوم (حسب مورد)	جناب آقای دکتر مجید مسعودی فرد	دانشیار	دانشکده دامپزشکی دانشگاه تهران	
٤- داور داخلی	جناب آقای دکتر فریدون صابری افشار	دانشیار	دانشکده دامپزشکی دانشگاه تهران	
٥- داور داخلی	جناب آقای دکتر فرهنگ ساسانی	استاد	دانشکده دامپزشکی دانشگاه تهران	
٦- داور خارجی	جناب آقای دکترسیف اله دهقانی	استاد	دانشکده دامپزشکی دانشگاه شیراز	
۹-نماینده تحصیلات تکمیلی دانشکده (عضو هیات علمی و رئیس جلسه)	جناب آقای دکتر فرهنگ ساسانی	استاد	دانشکده دامپزشکی دانشگاه تهران	

تذکر : این برگ پس از تکمیل توسط هیات داوران در نخستین صفحه پایان نامه درج می گردد.

دانشگاه تهران
دانشکده دامپزشکی

ارزیابی پیوند تاندون منجمد پوشش داده شده با سلول‌های بنیادی مزانشیمی مشتق شده از مغز استخوان خودی و پلاسمای غنی از پلاکت جهت حفظ طول تاندون در بره گوسفند

نگارش:
رافد مجید نعیم الخلیفه

اعضاء هیأت داوران:

جناب آقای دکتر داود شریفی استاد دانشکده دامپزشکی دانشگاه تهران، استاد راهنما
جناب آقای دکتر عباس توسلی استاد دانشکده دامپزشکی دانشگاه تهران، مشاور اول
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پایان نامه برای دریافت درجهٔ دکترای تخصصی در رشتهٔ جراحی دامپزشکی

سال تحصیلی: ۹۷-۱۳۹۶ شماره ثبت: ۷۸۹

تعهد نامه اصالت اثر

اینجانب **رافد مجید نعیم الخلیفه** متعهد می شوم که مطالب مندرج در این پایان نامه حاصل کار پژوهشی اینجانب است و دستاوردهای پژوهشی دیگران که در این پژوهش از آنها استفاده شده است، مطابق مقررات ارجاع در فهرست منابع و مآخذ ذکر گردیده است. این پایان نامه قبلاً برای احراز هیچ مدرک هم سطح یا بالاتر ارائه نشده است. در صورت اثبات تخلف (در هر زمان) مدرک تحصیلی صادر شد توسط دانشگاه از اعتبار ساقط خواهد شد.

رافد مجید نعیم الخلیفه

امضاء

چکیده:

ارزیابی پیوند تاندون منجمد پوشش داده شده با سلول‌های بنیادی مزانشیمی مشتق شده از مغز استخوان خودی و پلاسمای غنی از پلاکت جهت حفظ طول تاندون در بره گوسفند

دانشجو: رافد مجید نعیم الخلیفه

استاد راهنما: دکتر داود شریفی

زمینه و هدف: پیوند تاندون معمولاً برای التیام جراحت وارد بر تاندون خم کننده مورد نیاز است و به دلیل اهمیتی که این تاندون دارد، وجود روش‌هایی که به کارایی مجدد و قدرت آلوگرافت تاندون سرعت بخشد، ضروری می‌باشد. هدف از مطالعه حاضر تعیین تأثیر استفاده از پلاسمای غنی از پلاکت (PRP) و سلول‌های مزانشیمی مشتق از مغز استخوان در بهبود فرآیند بازسازی پس از پیوند تاندون خم کننده انگشتی سطحی (SDF) با تاندون آلوگرافت در بره می‌باشد. پانزده بره چهار ماهه برای جایگزینی ۵ سانتی متر SDFT با تاندون آلوگرافت منجمد همسن با بره‌ها، انتخاب شدند. **مواد و روش کار:** تحت شرایط کاملاً آسپتیک، ۵ سانتی‌متر از قسمت میانی تاندون SDF از هر دو اندام قدامی برداشت و بلافاصله با تاندون آلوگرافت منجمد جایگزین شد. تاندون آلوگرافت از کشتارگاه تهیه و در دمای ۲۰- درجه سانتی گراد به مدت ۴۵ روز نگه-داری شد. حیوانات مورد مطالعه به سه گروه تقسیم شدند و در هر گروه ۵ بره حضور داشت. گروه اول، گروه کنترل بود. در گروه دوم، ۱ میلی‌لیتر از PRP در ناحیه آناستوموز تزریق شد و در گروه سوم ۱ میلی‌لیتر از سلول‌های مزانشیمال در ناحیه آناستوموز وارد شد. دو ماه پس از جراحی، محتویات هیدروکسی پرولین و استحکام کششی مورد سنجش قرار گرفت و ارزیابی‌های هیستوپاتولوژی و سونوگرافی انجام شد. **نتایج:** به علت شباهت ضایعات، از لحاظ بالینی تمام بره‌ها درجات یکسانی از لنگش را نشان دادند. پس از برداشت گچ در روز پانزدهم پس از جراحی، لنگش بیشتر خود را نشان داد، اما به تدریج در هفته سوم در گروه درمان بهبودی مشاهده شد و وزن بره‌های این گروه در زمان تولد طبیعی بود. در بررسی سونوگرافی، افزایش قابل توجهی در سطح مقطع عرضی تاندون در گروهی که از MSCs استفاده شده بود، وجود داشت، اما، تفاوت قابل توجهی در ضخامت بین گروه PRP و MSCs مشاهده نشد. از لحاظ درجه فیبر، کاهش چشمگیری در گروه PRP وجود داشت. با این حال، از لحاظ میزان اکوژنیسیتی و عرض، تفاوت قابل توجهی بین هیچ یک از گروه‌ها مشاهده نشد. آزمایش هیدروکسی پرولین، تأثیر مثبت MSCs را در گروه سوم با 134.322±2.123 mg/g ماده خشک نشان داد. با مقایسه مقدار طبیعی 137.171±5.291 mg/g ماده خشک، این دو گروه مقادیر زیادی کلاژن برای جهت‌گیری فیبر در دسته‌جات اولیه تاندون را نشان دادند. محتویات هیدروکسی پرولین، در گروه کنترل بسیار کم بود (87.694±6.502 mg/g) و گروه PRP با 99.116±1.839 mg/g ماده خشک. در مطالعات بیوشیمیایی، RMN در گروه کنترل، 11.42±3.44 N ثبت شد، در حالی‌که، در گروه PRP، 19.11±6.20 و در گروه سوم 31.98±5.06 بود. ضریب سختی یا الاستیسیته (N/mm) در گروه کنترل 1.89±0.90 بود، در حالی‌که در گروه دوم (PRP) 3.36±0.55 و در گروه سوم 4.20±1.90 بود. تغییر شکل (εFmax mm) در گروه کنترل

7.00±2.41 mm، در گروه دوم 12.66±4.90 و در گروه سوم (MSc) 16.64±4.78mm بود. آخرین متغیر، انرژی جذب شده بود (w uptoFmaxNmm) که در گروه کنترل 84.54±38.32، در گروه دوم 90.62±54.31 و در گروه سوم که از MSCs استفاده شده بود، 221.87±48.44 بود. از لحاظ Stress RMN و انرژی جذب شده، افزایش قابل توجهی در گروه سوم (MSCs) ($P<0.05$)، در مقایسه با گروه PRP و گروه کنترل مشاهده گردید. مطالعه هیستوپاتولوژی نشان داد که MSCs می‌تواند باعث افزایش تکثیر سلولی، سازمان‌دهی کلاژن و غیره شود که پس از آن، نتایج گروه PRP از گروه کنترل از لحاظ هیستوپاتولوژی بهتر بود. **نتیجه گیری:** نتایج این مطالعه نشان داد که MSCs بازسازی تاندون آلوگرافت را افزایش داده و به آن سرعت می‌بخشد و هم‌چنین، در بهبود کیفیت آن مؤثر است (قدرت کششی، محتوای کلاژن و غیره). تجویز PRP به تنهایی تأثیر کمی در بهبود فرآیند التیام تاندون آلوگرافت دارد.

واژگان کلیدی: سلول‌های بنیادین مزانشیمی، پلاسمای غنی از پلاکت، تاندون آلوگرافت منجمد و بره.

مقدمه:

ساختار کلاژنی تاندون‌ها، نیروی کششی را از عضلات به استخوان‌ها منتقل می‌کنند. رنگ آن‌ها سفید درخشان بوده و قوام فیبروالاستیکی آن‌ها باعث شده که بتوانند فشار مکانیکی زیادی را تحمل کنند.

همانند منیسک و غضروف مفصلی، میزان خونرسانی، گردش سلولی و متابولیسم در بافت تاندون اندک است. به همین دلیل، التیام ضایعات تاندون طولانی بوده و معمولاً اسکار جراحت یا خود زخم باقی می‌ماند. در ارتوپدی، التیام پارگی حاد تاندون و تغییرات دژنراتیو، مشکلات زیادی را برای کلینیسین‌ها ایجاد می‌کند. روش‌های جدید برای التیام بافت تاندون شناسایی شده و روش‌های درمانی جدید و بیولوژیک برای همتراز کردن تاندون توسعه پیدا کرده است.

تاندون دارای ماتریکس فیبریلار بسیار سازمان یافته‌ای است که حاوی کلاژن تیپ ۱ و کلاژن‌های مینور متنوع، پروتئوگلیکان و گلیکوپرتئین می‌باشد. ماتریکس تاندون توسط تنوسیت‌های مقیم نگه‌داری می‌شود. جراحات تاندون عمدتاً ماهیت دژنراتیو داشته و اغلب به درمان پاسخ نمی‌دهند، به‌طوری‌که که به آهستگی التیام یافته و به ندرت قدرت و الاستیسیته اولیه خود را بدست می‌آورند.

فرآیند التیام در اثر تشکیل بافت اسکار رخ داده و نتیجه ضعیفی در بیمار مبتلا دارد. در حال حاضر، روش‌های درمانی که به واسطه جراحی و غیر آن وجود دارند، منجر به التیام ضعیف تاندون شده و هریک از آن‌ها عوارضی را برجای می‌گذارند. بنابراین، برای التیام و بازگرداندن ساختار تاندون، پیوند تاندون مورد نیاز می‌باشد که به همین جهت، اتوگرافت‌ها، آلوگرافت‌ها و گزنوگرافت‌ها مورد استفاده قرار می‌گیرند. هر یک از این پیوندها، محدودیت‌های مختلفی چون درد و عوارض جانبی مربوط به دهنده، زیست سازگاری ضعیف، انتقال بیماری و واکنش ایمنی که منجر به پس زدن و شکست پیوند می‌شود، دارد.

بسیاری از مطالعات تجربی، بر روی التیام تاندون خم کننده، روش بخیه زدن، مواد بخیه و توان‌بخشی بعد از جراحی متمرکز بودند، اما افزایش توجه به التیام ابتدایی تاندون خم کننده منجر به انجام تحقیقات بیشتر در مورد اینکه برای بهبود فرآیند التیام چه کارهایی می‌توان انجام داد، گردید.

پیوند تاندون معمولاً به التیام جراحت وارد بر تاندون خم کننده نیاز دارد. با این حال، برای پیوند با کمبود مواد مناسب مواجه هستیم و علاوه‌براین، هنوز اینکه استفاده از چه موادی برای التیام تاندون خم کننده بهتر است، مورد بحث می‌باشد، هم‌چنین، بیشتر روش‌های پیوند منجر به بروز چسبندگی می‌شوند و این مسئله حرکت مفصل را با محدودیت مواجه می‌کند.

به تازگی، پلاسمای غنی از پلاکت (PRP) که غلظت اتولوگ از پلاکت‌های خون است، به‌عنوان درمان احتمالی برای جراحت تاندون معرفی شده است.

PRP پتانسیل افزایش به کار گیری و تکثیر تنوسیت‌ها، سلول‌های بنیادی و سلول‌های اندوتلیال را دارد. یکی از عوامل رشد که در PRP وجود دارد، فاکتور رشد تغییر دهنده b (TGF-b) است. این فاکتور رشد، یک سایتوکاین کاملاً شناخته شده‌ای است که فرآیندهای مختلفی را در التیام تاندون تنظیم می‌کند. TGF-b پاسخ‌های التهابی را در مراحل اولیه التیام تنظیم می‌کند، در کنترل پیچیده آنژیوژنز شرکت می‌کند، رسوب پروتئوگلیکان را تنظیم و تولید کلاژن را با فیبروبلاست تاندون تحریک می‌کند.

استفاده از سلول‌های مزانشیمی اتولوگ از مغز استخوان برای ترمیم جراحاتی که به شکل تجربی بر تاندون‌ها و لیگامنت‌ها وارد شده است، به‌طور کامل در مدل‌های حیوانی توصیف شده است.

هدف استفاده از سلول‌های بنیادی، طراحی بافت جدید تاندون با استفاده از دستگاه ساخت سلول می‌باشد. این هدف یا از طریق مشارکت مستقیم تمایز فنوتیپ‌های سلولی اختصاصی بافت بدست می‌آید و یا از طریق تولید بافت متناسب با محصولات ماتریکس خارج سلولی و یا به شکل غیر مستقیم با اثرات تروفیک از طریق تولید پروتئین‌های بیواکتیو همچون فاکتورهای رشد، فاکتورهای ضد آپوپتوز و عوامل کموتاکتیک حاصل می‌شود. علاوه‌براین، مطالعات اخیر نقش ضد التهابی سلول‌های بنیادی جایگذاری شده را نیز مشخص می‌سازند.

۱-۲- هدف از مطالعه

هدف از این مطالعه شناسایی تاندون آلوژنیکی بود که بتواند با موفقیت جایگزین SDFT شود و هم‌چنین هدف دیگر، ارزیابی ترمیم تاندون آلوگرافت با PRP و MSCs مغز استخوان با استفاده از بررسی‌های بافت شناسی، تصاویر سونوگرافی، آزمایش ارزیابی قدرت کششی و آزمایش هیدروکسی پرولین بود.

۱-۳- ضرورت انجام مطالعه

تاندون‌ها به دلایلی چون تعدد زیاد، سطحی و بزرگ بودن، غالباً در حین جراحی و یا پارگی ناحیه پا دچار جراحت می‌شوند. از این رو، بررسی ما بر روی تاندون خم کننده سطحی صورت پذیرفت. در این مطالعه ما از PRP و MSCs برای بهبود سریع فرآیند التیام در آلوگرافت تاندون استفاده کردیم. استفاده از آلوگرافت تاندون به‌عنوان جایگزین SDFT از ارزش بالینی فوق‌العاده‌ای برخوردار است.

۱-۴- فرضیات

- تاندون منجمد با نگه‌داری ساختار آن، پیوند کاملاً مناسبی برای جایگذاری به جای SDFT است.
- استفاده از PRP و MSC پتانسیل التیام سریع پیوند به بافت تاندون میزبان را دارا می‌باشد.
- تاندون منجمد پل کاملاً مناسبی است برای انتقال نیرو از عضلات به استخوان.

مواد و روش کار

۳-۱- حیوانات

مطالعه بر روی ۱۵ بره نژاد شال (shal) با وزن بین ۱۸ تا ۲۰ کیلوگرم و با سن ۴ ماه انجام شد. سپس آیورمکتین ۱٪ با دز ۲ mg/kg به شکل زیر جلدی و لوامیزول ۲/۵ mg/kg به شکل خوراکی تجویز شد. این بره‌ها در شرایط یکسانی از لحاظ جایگاه، مدیریت و خوراک، نگه‌داری شدند.

حیوانات مذکور به شکل تصادفی در سه گروه پنج‌تایی تقسیم شدند.

۱. گروه کنترل
۲. گروه دریافت کننده پلاسمای غنی از پلاکت
۳. گروه دریافت کننده سلول‌های بنیادی مزانشیمی

مشاهدات تا دو ماه پس از ترمیم تاندون ادامه یافت.

پارامترهای مختلف مطالعه صورت گرفته شامل موارد زیر بودند:

۱. مشاهدات بالینی
۲. سونوگرافی
۳. قدرت کششی
۴. آزمایش هیدروکسی پرولین
۵. هیستوپاتولوژی

۳-۲- آلوگرافت تاندون

آلوگرافت SDFT در شرایط آسپتیک از کشتارگاه تهیه شد. تمامی تاندون‌ها به‌طور جداگانه با استفاده از نرمال سالین شست‌شو داده شدند و درآلومینیوم سیل شده با۰/۵ میکرومتر پلی اتیلن و کیسه‌های پلاستیکی بسته بندی شدند (سه لایه محافظ)، سپس نمونه‌ها در کنار یخ منتقل و در دمای ۲۰- درجه سانتی گراد به مدت ۴۵ روز نگه‌داری شدند.

در روز انجام پیوند، تاندون با نگه‌داری به مدت ۶ ساعت در دمای اتاق، از حالت انجماد خارج و برای ۳۰ دقیقه در داخل نرمال سالین (۰/۹ ٪ NaCl) غوطه‌ور شد (Robertson et al., 2006) (تصویر ۲).

۳-۳- آماده سازی مغز استخوان از سلول‌های بنیادی مزانشیمی

سرنگ جدا و برای اطمینان از مخلوط شدن کامل، چند مرتبه تکان داده شد. به میزان ۵ میلی‌لیتر از آسپیره مغز استخوان تیغه لگن (حدود ۲۰۰ واحد در هر میلی‌لیتر) در داخل هپارین قرار گرفت (تصویر ۴). ناحیه برش خورده با روش ساده منقطع بخیه شد (تصویر ۵).

نمونه‌های مغز استخوان جمع آوری و در دمای ۴ درجه سانتی‌گراد بلافاصله به آزمایشگاه محل منتقل شدند و تا انجام کشت سلول، یک شبانه روز در این شرایط قرار گرفتند.

۳-۴- آماده سازی پیش از عمل

تمامی حیوانات یک هفته قبل آیورمکتین ۵۰ mg /kg دریافت کردند و قبل از شروع مطالعه، از غذا محروم شدند. ناحیه پالمار استخوان متاکارپ تراشیده و شسته شد.

۳-۵- بی‌هوشی

ما از زایلازین (۰/۲ mg/kg) برای ایجاد خواب سبک و عمیق و سپس از کتالار (کتامین ۵ mg/kg IV- ۲) برای القاء بی‌هوشی استفاده کردیم. لوله داخل نایی (ET) کار گذاشته شد. بی‌هوشی عمومی به شکل ایمنی از طریق استنشاقی اعمال گردید (تصویر ۶). پس از القاء تزریقی، از ایزوفلوران استفاده شد. نحوه قرار دادن حیوانات به شکلی بود که سر حیوان بالاتر از بینی قرار می‌گرفت تا بزاق و یا استفراغ از طریق دهان بتواند خارج شود.

۳-۶- عملیات جراحی برای پیوند

جراحی تحت بی‌هوشی عمومی بر روی هر دو اندام قدامی انجام گرفت. ناحیه جراحی شده در موقعیتی که حیوان به پهلو قرار داشت به همان شکلی که پیشتر توضیح داده شد گچ‌گیری شد. برشی با طول ۸-۷ سانتی‌متر در ناحیه پالمار استخوان متاکارپ و سپس در ناحیه زیر جلدی ایجاد گردید. بدین ترتیب، تاندون خم کننده سطحی به راحتی در معرض قرار گرفت. بخیه‌های نگه‌دارنده در هر دو انتهای قدامی و خلفی تاندون قرار داده شد (تصویر ۷). ۵ سانتی‌متر از SDF را برداشت و ۵ سانتی‌متر تاندون آلوگرافت را جایگزین آن کردیم (تصاویر ۸، ۹، ۱۰). از الگوی بخیه لاکینگ لوپ (کسلر اصلاح شده) با نخ بخیه غیر قابل جذب (نایلون) برای بخیه کردن تاندون استفاده شد (Strickland, 1985). برای کاهش آسیب به بافت در حین بخیه، از سوزن قلاب‌دار استفاده نمودیم، سپس برش ایجاد شده را با روش معمول بستیم (تصاویر ۱۱، ۱۲، ۱۳).

گروه ۱ (کنترل)

در این گروه، گوسفندانی که تاندون آلوگرافت داشتند، MSCs و PRP را دریافت نکردند.

۳-۶-۲- گروه ۲ (MSCs)

کشت‌های اولیه MSCs با PBS شسته و سپس با تریپسین/EDTA تریپسینه شدند. سلول‌ها با تراکم 10×10^6/ml داخل نرمال سالین غوطه‌ور و به داخل سرنگ استریل ۱۰ میلی‌لیتری کشیده شدند. برای هر حیوان، ما حدود $20\text{-}50\times10^6$ سلول را آماده نموده و سپس آن‌ها را در شرایط سرد (دمای ۴-۰ درجه سانتی گراد) به بیمارستان منتقل کردیم. هر یک از حیوانات تعلیق MSC را در ناحیه آلوگرافت دریافت کردند (تصویر ۱۵). محلول MSCs را در ناحیه تاندون آلوگرافت و در ناحیه قدامی و خلفی تاندون در محل بخیه (تصویر ۱۴)، تزریق کردیم و سپس محل برش را به روش معمول بستیم.

۳-۶-۳- گروه ۳ (PRP)

PRP از نمونه‌های خون حیوانات که در زمان جراحی جمع آوری می‌شد، تهیه گردید. نمونه‌های خون جمع آوری شده در لوله‌های ACD، باید برای ۵ تا ۱۰ مرتبه با حرکات معکوس تکان داده می‌شدند تا ماده ضد انعقاد به خوبی با خون مخلوط شود و از فعالیت پلاکت‌ها قبل از استفاده از آن‌ها جلوگیری کند.

تا زمان انجام فرآیندها، نمونه‌های خون در دمای ۶-۱ درجه سانتی‌گراد نگه‌داری شدند، سپس بلافاصله به محل آزمایشگاه منتقل شدند. برای تزریق در داخل جراحت، PRP با ACS سرم اتولوگ ترکیب شد.

در روش PRP، ابتدا یک سانتریفوژ اولیه برای جداسازی گلبول‌های قرمز خون (RBC) انجام شد و به دنبال آن سانتریفوژ بعدی برای تغلیظ پلاکت‌ها که در کم‌ترین حجم نهایی پلاسما تعلیق می‌شوند، صورت پذیرفت.

برای جدا کردن گلبول‌های قرمز از پلاسما، نمونه خون سانتریفوژ شد. پلاسمای حاصل حاوی پلاکت و تعدادی گلبول سفید بود، سپس پلاکت‌ها در حجم کم پلاسما بیشتر تغلیظ شدند تا در نهایت PRP بدست آید.

ما PRP را در محل تاندون آلوگرافت و در قسمت قدامی و خلفی محل بخیه تاندون تزریق کردیم و سپس محل برش را با روش معمول بستیم.

پس از جراحی، با استفاده از سونوگرافی بافت، تصاویر ویژه‌ای برای مقایسه کمی ارزیابی صحت کامل ساختار تاندون تهیه شد.

۳-۷- مراقبت‌های پس از عمل

بعد از جراحی در هر گروه جهت جلوگیری از حرکت، گچ فایبر گلاس برای ۳ هفته کار گذاشته شد (تصویر ۱۵). در طی این مدت، هر ۵ روز یک بار گچ را تعویض و زخم جراحی را معاینه کردیم. سپس از بانداژ Robert Jones برای توانبخشی تدریجی حرکت اندام به مدت ۱ هفته استفاده نمودیم (که امکان بازسازی تدریجی تاندون را فراهم می‌کند).

حیوانات به جایگاه خود منتقل و زمانی که به‌طور کامل به‌هوش آمدند، بر روی جناغ قرار داده شدند. سپس به آن‌ها کمک شد تا بتوانند از جای خود بلند شوند. آب و غذا در اختیارشان قرار داده شد و تا بازگشت به حالت تعادل خود، تحت نظر قرار گرفتند.

به حیوانات آنتی‌بیوتیک تزریق شد. ترکیبی از آنتی بیوتیک‌های پروکائین بنزیل پنی‌سیلین (۸ mg/ kg)، دی هیدرواسترپتومایسین (۱۰ mg/ kg)، جنتامایسین و فلونکسین مگلومین ۵٪ (۲/۲ mg/ kg) به همراه ویتامین B complex برای مدت ۷ روز پس از جراحی تزریق شد و حیوانات به مدت ۸ روز مورد معاینه بالینی بعد از جراحی قرار گرفتند.

برداشت تاندون‌ها

بعد از گذشت ۲ ماه از جراحی، تاندون‌ها بلافاصله پس از ذبح دام‌ها برداشته شدند. از هر حیوان یک نمونه اخذ و برای بررسی‌های هیستوپاتولوژی در فرمالدئید ۱۰٪ قرار داده شد. باقی تاندون در گاز پانسمان آغشته به نمک پیچیده و برای ارزیابی قدرت کششی و هیدروکسی پرولین در کیسه‌های پلاستیکی برچسب‌دار در دمای ۲۰- درجه سانتی‌گراد نگه‌داری شد.

۳-۹- پارامترهای مورد ارزیابی

۳-۹-۱- مشاهدات بالینی

شرایط عمومی و توانایی حرکت کردن همه دام‌ها مورد مشاهده قرار گرفت. درجه حرارت مقعدی تا ۱ هفته پس از عمل ثبت می‌شد. حیوانات از لحاظ وضعیت و موقعیت نشستن، ایستادن و تعادل در هنگام راه رفتن تحت نظر قرار داشتند. تحمل وزن، جهت قرار گیری اندام‌ها، توازن و گام برداشتن حیوانات روزانه ارزیابی می‌شد.

۳-۹-۲- سونوگرافی

پس از جراحی و بعد از گذشت ۲ ماه، سونوگرافی بافت هر دو اندام قدامی جهت ارزیابی کمی و مقایسه‌ای صحت ساختاری تاندون و التیام هر دو اندام قدامی انجام شد (جدول ۳). تصاویر عرضی و طولی سونوگرافی

با استفاده از دستگاه Sonosite Micromaxx Ultrasonographic (SonositeInc, USA) و ترانسدیوسر خطی (Micromaxx HFL/ 13-6 MHz,SonositeInc, USA) انجام شد.

۳-۹-۳- هیستوپاتولوژی

پس از تثبیت در فرمالین بافر خنثی، تمامی بافت‌ها از طریق شیب الکلی، قرار داده شدن در بلوک‌های پارافین، تکه تکه شدن و رنگ شدن با هماتوکسیلین و ائوزین و رنگ ماسون تری کروم، فرآوری شدند.

برای رنگ آمیزی محلول‌های زیر آماده شدند:

- محلول Hematoxylin Wiegert : حجم یکسان از محلول‌های A و B Hematoxylin Wiegert با یکدیگر مخلوط شدند. محلول حاصل را پس از استفاده دور ریختیم.
- محلول‌های Phosphomolybdic – phosphotungstic acid: حجم‌های یکسان از محلول‌های Phosphomolybdic – phosphotungstic acid با یکدیگر مخلوط شدند. محلول حاصل را پس از استفاده دور ریختیم.

روش رنگ آمیزی:

۱. پارافین لام‌ها زدوده شد و مقاطع با استفاده از آب مقطر خیس شدند.
۲. لام‌ها در محلول بیون به مدت ۱ ساعت در دمای ۵۶ درجه سانتی گراد قرار داده شدند. سپس برای زدودن رنگ زرد از مقاطع، با جریان آب شسته شدند.
۳. لام‌ها با آب مقطر شسته شدند.
۴. رنگ آمیزی با محلول Hematoxylin Wiegert برای ۱۰ دقیقه صورت گرفت.
۵. مقاطع با جریان ملایم آب برای ۱۰ دقیقه شسته شدند و سپس با آب مقطر شست و شو شدند.
۶. لام‌ها با محلول Biebrich Scarlet-Acid Fucshin به مدت ۲ دقیقه رنگ آمیزی شدند.
۷. مقاطع با آب مقطر شست و شو شدند.
۸. محلول‌های Phosphomolybdic – phosphotungstic acid برای ۱۵-۱۰ دقیقه بر روی لام‌ها ریخته شد.
۹. محلول Aniline Blue برای ۵ دقیقه بر روی لام‌ها قرار داده شد و سپس لام‌ها با آب مقطر شسته شدند.
۱۰. اسید استیک ۱٪ برای ۵-۳ دقیقه بر روی لام‌ها قرار داده شد.
۱۱. مقاطع با الکل ۹۵٪ آب زدایی و با xylene تمیز شدند (سه مرتبه).

نتایج:

- کلاژن: آبی
- سیتوپلاسم، کراتین، عضله و فیبرهای داخل سلولی: قرمز
- هسته: سیاه

۳-۹-۴- غلظت هیدروکسی پرولین

کلاژن و اتصالات متقاطع با تعیین هیدروکسی پرولین آنالیز شدند. نمونه‌ها به همان شکل که پیشتر اشاره شد جمع‌آوری شدند و غلظت هیدروکسی پرولین آن‌ها با روش اسپکتروفوتومتری اصلاح شده اندازه گیری شد.

نمونه‌ها برای ۱۶-۱۴ ساعت در دمای ۱۰۵ درجه سانتی‌گراد در HCl ۶ مولار هیدرولیز شدند و هیدروکسی پرولین با کلروآمین T اکسیده شد. سپس با استفاده از ارلیخ و گرمخانه گذاری در دمای ۶۰ درجه سانتی گراد یک کروموفورم تشکیل شد. برای حذف کروموفورهای مداخله کننده، محصول هیدروکسی پرولین در محیط قلیایی در داخل تولوئن و سپس در داخل فاز اسیدی استخراج شد.

جذب فاز اسیدی در طول موج ۵۴۳ نانومتر قرائت شد و محتویات هیدروکسی پرولین از کالیبراسیون منحنی براساس محلول‌های استاندارد (که همانند نمونه‌ها قرائت شدند) محاسبه شد. جهت تشخیص درصد ماده خشک (DM) در هر نمونه تاندون، هم‌زمان با نمونه‌گیری ۱۰۰-۵۰ میلی گرم از هر نمونه برای آنالیز هیدروکسی پرولین در یک پلیت قرار داده شده و با قرار دادن در آون ۱۰۰ درجه سانتی‌گراد برای ۳ ساعت خشک شد. در نهایت محتوای هیدروکسی پرولین نمونه‌های تاندون در واحد mg/g ماده خشک بیان شدند (Sharifi et al., 2007).

۳-۹-۵- قدرت کششی

در همه حیوانات تمام طول SDFT با استفاده از دستگاه Zwick/Roell MDTL جمع‌آوری شد. آزمون قدرت کششی با استفاده از دستگاه Zwick/Roell MDTL (آنالیزور بیومکانیکال) با سرعت ۰/۰۷ میلی‌متر در ثانیه و کنترل کننده دیفرانسیل انتگرالی تناسبی (PID) صورت گرفت به شکلی که هر دو انتهای برش هر تاندون گرفته شده و با تسهیلات دندانپزشکی محکم نگه‌داری می‌شدند (Sharifi et al., 2009).

ویژگی‌های مکانیکی و ساختاری به ترتیب از طریق نیروی کششی و منحنی تنش- کشش ارزیابی شدند. سختی و همبستگی خطی به ترتیب در ناحیه خطی نیروی جابجایی و منحنی تنش- کشش محاسبه شد. تانژانت سختی به عنوان شیب منحنی نیروی جابجایی محاسبه شد که همگی نرمال بوده و منحنی‌ها تا حداکثر نیرو در شرایط in vivo اصلاح شدند (تصویر ۱۷) (Juncosa-Melvin et al., 2006).

تجزیه و تحلیل آماری

نتایج به شکل ارزش خطای استاندارد میانگین بیان شدند. داده‌ها از لحاظ آماری با استفاده از ANOVA یک طرفه با آزمون‌های مقایسه‌ای چندتایی و نرم افزار آماری (نرم افزار SPSS برای ویندوز ورژن ۲۰، USA) مورد تجزیه و تحلیل قرار گرفتند. $P \leq 0.05$ قابل توجه در نظر گرفته شد.

نتایج

ارزیابی بالینی

در طی انجام این مطالعه تجربی، وضعیت سلامت عمومی حیوانات هر روز بررسی می‌شد که در این مدت وضعیت خوبی مشاهده گردید. گوسفندان انتخاب شده مدتها قبل از مداخله جراحی، تجربه زمین‌گیری را نداشتند. بعد از برداشتن گچ در روز ۱۵ پس از جراحی، اندام‌های پیوند شده از لحاظ بالینی درجات مشابهی از لنگش را در همه بره‌ها نشان دادند. وضعیت لنگش حیوانات فقط بر روی سطح سفت بررسی شد. اما در گروه درمان، از شروع هفته سوم تا انتهای این هفته، وضعیت لنگش به تدریج بهبود پیدا کرد و حیوانات رفته رفته تحمل وزن طبیعی را پس از گذشت ۱ ماه بدست آوردند. در گروه درمان، تورم در ناحیه عمل شدت کم‌تری در انتهای هفته سوم داشت. در گروه‌های MSCs و PRP در هفته پنجم، حیوانات تحمل وزن بر روی هر چهار اندام را داشتند. زخم‌های پوست نیز در این زمان التیام پیدا کرد.

۴-۲- نتایج سونوگرافی

پس از گذشت ۶۰ روز از جراحی، در ناحیه مقطع عرضی افزایش قابل توجهی در تاندون گروهی که از MSCs استفاده شده بود وجود داشت، اما، در میزان ضخامت هیچ تفاوت قابل ملاحظه‌ای بین گروه PRP و MSCs مشاهده نشد. کاهش قابل توجهی در فیبر گروه PRP وجود داشت. با این حال، در عرض و اکوژنسیته تفاوت قابل ملاحظه‌ای در بین گروه‌ها وجود نداشت. در همه گروه‌ها (در گروه‌های کنترل، MSC و PRP)، پس از جراحی و بعد از گذشت ۶۰ روز از آن، تفاوت قابل توجهی در اکوژنسیتی و فیبر و هم‌چنین در بهبود ساختار تاندون مشاهده گردید ($P< 0.5$) (جداول ۳، ۴، ۵، ۶ و ۷)، (تصاویر ۵، ۶، ۷، ۸ و ۹)، (تصاویر سونوگرافی ۲۳-۱۰).

نتایج هیدروکسی پرولین

در گروه کنترل، استرس و Fmax برابر با 11.42 ± 3.44 N ثبت شد، در حالی که در گروه PRP برابر با 19.11 ± 6.20 و در گروه سوم برابر با 31.98 ± 5.06 N بود. ضریب الاستیسیته در گروه کنترل برابر با 1.89 ± 0.90 بود، در حالی که در گروه دوم (PRP) 3.36 ± 0.55 و در گروه سوم 4.20 ± 1.90 بود. تغییر شکل در گروه کنترل 7.00 ± 2.41 mm، در گروه دوم (PRP) 12.66 ± 4.90 و در گروه سوم (MSC) 16.64 ± 4.78mm بود. آخرین پارامتر مورد ارزیابی، انرژی جذب شده بود که در گروه کنترل 84.54 ± 38.32N.mm، در گروه دوم 90.62 ± 54.31 و در گروه سوم که از سلول‌های MSC استفاده شده بود 221.87 ± 48.44N.mm بود. در گروه سوم در مقایسه با گروه کنترل و گروه PRP تغییرات قابل توجهی مشاهده شد ($P<0.05$).

در مورد RMN و انرژی جذب شده، افزایش قابل توجهی در گروه MSC وجود داشت. RMN/nm^2 نیز در گروه PRP افزایش یافت. در مورد سایر پارامترها افزایش ملایمی در گروه MSC مشاهده شد که قابل توجه نبود.

یافته‌های هیستولوژی

از رنگ آمیزی تریکروم ماسون برای مشاهده تاندون SDF استفاده شد. رنگ آبی نشان‌دهنده کلاژن در تاندون بود. هسته‌های متعلق به فیبروبلاست‌های تاندون که رنگ سیاه را به خود گرفته بودند به شکل موازی و صاف بین فیبرهای کلاژن قرار داشتند. فیبریل‌های کلاژن (تصویر ۱) در جهت طولی قرار گرفته بودند. گروه MSCs فیبریل‌های بسیار سازمان یافته کلاژن را نشان داد، علاوه‌براین، در تنوسیت‌ها افزایش اندازه و طول، در انواع کلاژن. فرآیند بازسازی خوب، فعال و مشابه‌ای در گروه MSC (۵/۵) که در ۱۰۰٪ موارد بافت فیبروز تشکیل نشده بود، مشاهده گردید. سطح بافت بازسازی شده به نظر صاف و هموار و تقریباً نرمال می‌رسید. در گروه PRP فیبرهای کلاژن تقریباً در جهت طولی قرار داشته و افزایش اندازه، ضخامت و طول در تنوسیت‌ها و فیبرها مشاهده شد، اما فیبرهای کلاژن در برخی قسمت‌ها انقباض و پیچش و عروق خونی را نشان دادند، البته از لحاظ تعداد و اندازه کم و بدون برجستگی بودند (تصویر 1B). گروه PRP (۵/۵) نتایج دلگرم کننده‌ای را نشان داد که (۳/۵) ۶۰٪ مشابه گروه MSCs بود، اما فرآیند بازسازی شده (۲/۵) ۴۰٪ نتایج متغیری را از خوب تا متوسط بسته به ضخامت، آرایش و اندازه هسته نشان داد. تشکیل عروق خونی نیز به همین منوال بود. در گروه کنترل، جهت گیری طولی و صاف بود، اما فیبرها نازک و تنوسیت‌ها از لحاظ اندازه کوچک بودند، با این وجود، همانند گروه‌های PRP و MSCs خیلی قابل ملاحظه نبوده و علاوه‌براین، عروق خونی تازه تشکیل شده در ناحیه حضور نداشتند (تصویر 1C) و اگر هم بودند با تعداد کم مشاهده شدند.

طبق جدول ۴-۳ در شصتمین روز پس از جراحی، گروه کنترل ارزش کم‌تری در مقایسه با سایر گروه‌ها داشت، اما از لحاظ خصوصیات تشکیلات ماتریکس خارج سلولی، گروه کنترل وضعیت بهتری را در میان گروه‌های دیگر نشان داد، هم‌چنین، از لحاظ نسبت سلول به گروه ماتریکس سلول مزانشیمی، سلولاریتی این گروه در مقایسه با سایر گروه‌ها افزایش یافت. ماتریکس، تنظیم سلول و ... در MSCs بهتر از سایر گروه‌ها و در PRP بهتر از گروه کنترل بود، علاوه‌براین، میزان تشکیل عروق خونی در گروه PRP و گروه کنترل در نتایج ما بیشتر بود.

بحث:

جراحات وارد بر تاندون برای جراحان ارتوپد از اهمیت زیادی برخوردار هستند. در مسیر التیام جراحات این ناحیه موانعی وجود دارد. خونرسانی به تاندون تقریباً ضعیف بوده که باعث کاهش میزان متابولیسم آن می‌شود و در نتیجه روند التیام پس از وارد آمدن جراحت به آهستگی صورت می‌گیرد (Pastides and Khan, 2011). این التیام ساختار طبیعی تاندون را باز نمی‌گرداند و منجر به شکل گیری بافت اسکار

می‌شود که کیفیت کارایی آن را کم‌تر از تاندون طبیعی می‌کند، بنابراین، یک تاندون التیام یافته، فعالیت‌های طبیعی خود را همچون قبل به دست نخواهد آورد و ممکن است متحمل آسیب‌های مکرر شود (Sadegh et al., 2016).

تاندون‌های قوی (SDFT) مسئول برخی از فعالیت‌های مهم و مفید هستند که نیاز به مداخلات بهتری برای پیشرفت درمانی این تاندون‌ها وجود دارد. یک مدل جراحی از جراحت حاد وارد بر تاندون با موفقیت توسعه یافت و برای تاندون‌های قوی گوسفند و اسب به کار برده شد. این مدل پتانسیل استفاده برای جراحت تاندون در اسب و انسان را نیز دارد (Tyelor, 2013).

برای قضاوت امکان استفاده از MSC و PRP برای بهبود فرآیند بازسازی تاندون در انسان، تجربیات دقیق‌تر و قابل اعتمادتری با انجام روش‌هایی چون آزمایش‌های بیوشیمیایی و استفاده از یک مدل حیوانی بزرگ مانند گوسفند یا سگ مورد نیاز است (Li et al., 2007).

موارد زیادی از پارگی تاندون و انقباض اکتسابی- مادرزادی در تاندون‌های خم کننده در گله‌های گوسفند بیمارستان دامپزشکی دانشگاه بصره گزارش شده است. برای مطالعه مکانیسم پایه‌ای التیام تاندون آلوگرافت منجمد و عوامل درمانی که در واقع می‌تواند همان مراقبت‌های بالینی باشد، مدل بره انتخاب شده است (Sharma and Maffulli, 2006; Tomopoulos et al., 2015; Wu and Tang, 2013).

تاندون‌ها بافت‌های پر تحرکی هستند و آسیب به آن‌ها به‌طور قابل توجهی کیفیت زندگی را کاهش می‌دهد. در حال حاضر، هیچ درمان دارویی مؤثری برای درمان آسیب‌های تاندونی وجود ندارد، بنابراین، جایگزینی بافت آسیب دیده امری است ضروری (Youngstrom and Barrett, 2015). تاندون‌های آلوگرافت نقش مهمی را در بازسازی تاندون به‌خصوص در جایی که کمبود بافت موضعی در دسترس و مناسب وجود دارد، ایفا می‌کنند (Robertson et al., 2006).

بافت آلوگرافت تازه برای جایگزینی مناسب نیست، چراکه به شدت ایمونوژن است و دسته بندی بافتی را غیر ممکن می‌کند. برای حل این معضل، بافت آلوگرافت تازه را فریز می‌کنند و فرآیند انجماد به‌طور قابل توجهی ایمنی‌زایی بافت را کاهش می‌دهد (انجماد عمیق در دمای ۲۰- درجه سانتی‌گراد برای ۴۵ روز) به‌طوری‌که با ازبین بردن تنوسیت‌ها، میزان آنتی‌ژنیسیتی بافت را به حداقل می‌رساند (Robertson et al., 2006).

مطالعه حاضر جهت بررسی سونوگرافی، محتویات هیدروکسی پرولین، بیوشیمیایی و فرآیند بازسازی بافتی در تاندون آلوگرافت منجمد شده با روش جراحی و با جایگذاری ۵ سانتی‌متر تاندون SDF بره‌ها، صورت گرفته است. استفاده از MSC و PRP در طی جراحی توصیه شده است.

بیشترین MSCهای استفاده شده برای عملیات ارتوپدی از بافت مغز استخوان بدست آمده است، چراکه این سلول‌ها به نسبت سهل الوصول بوده و به نسبت تعداد زیادی MSC را در مقایسه با سایر منابع در

اختیار قرار می‌دهند. تیغه لگنی رایج‌ترین محل برای برداشت MSC است، اگرچه منابع دیگری نیز شناسایی شده‌اند (Chaudhury, 2012).

قبل از جراحی هیچ‌یک از گوسفندان لنگش نداشتند و در بررسی سونوگرافی علامتی از حضور جراحت تاندون در آن‌ها وجود نداشت.

پس از برداشتن گچ در روز ۱۵ بعد از جراحی، اندام‌هایی که در آن‌ها پیوند انجام شده بود از لحاظ بالینی در تمام بره‌ها درجات مشابهی از لنگش را نشان دادند که به دلیل شباهت جراحات، تقریباً یکسان بودند. لنگش بیشتر در طی شروع هفته سوم مشخص شد، ولی به تدریج در گروه درمان در اواخر هفته سوم بهبود پیدا کرد و حیوانات با گذشت ۱ ماه، به شکل طبیعی تحمل وزن بر روی اندام‌ها را داشتند. تورم در ناحیه جراحی گروه درمان، شدت کم‌تری در انتهای هفته سوم داشت. نتیجه تأثیر PRP و سلول‌های مزانشیمی در محل اتصال آلوگرافت با تاندون میزبان، از لحاظ بالینی کاهش فرآیند التهاب و لنگش را نشان داد و علاوه‌براین، باعث تسکین درد موضع از طریق رها سازی فاکتورهای رشد و تسریع تجمع تنوسیت‌ها در ناحیه اتصال شد (sharifi, 2007; sharifi, 2011; Bosch et al., 2010).

به نظر می‌رسد که در گروه‌های MSC و PRP، افزایش تکثیر تنوسیت‌ها و به‌دست آمدن تمامیت تاندون به تدریج منجر به بازگشت ویژگی‌های بیومکانیکی مرتبط با تحمل کامل وزن در هفته پنجم می‌گردد. یافته‌های بالینی در این مطالعه نشان می‌دهد که استفاده از MSC و PRP در موضع حتی با به کار بردن تاندون آلوگرافت، روند التیام را به‌طور غیر مستقیم با افزایش فعالیت تنوبلاست و فیبروبلاست تشویق می‌کند (Jackson et al., 1996).

سونوگرافی یکی از دقیق‌ترین، با دوام‌ترین و غیر تهاجمی‌ترین روش‌ها برای ارزیابی ساختار تاندون پس از جراحی و در طی فرآیند التیام است و می‌توان از آن در پیش آگهی وضعیت تاندون استفاده نمود (Mostafa et al., 2015).

یافته‌های سونوگرافی افزایش قابل توجهی در مقطع عرضی تاندون در گروه MSC را نشان داد که این نتیجه در توافق با یافته‌های Barreira و همکاران در سال ۲۰۰۸ بود. محققان مذکور پس از به کار بردن سلول‌های تک هسته‌ای مشتق از سلول‌های مغز استخوان، با استفاده از روش سونوگرافی و از نمای مقطع عرضی، تفاوت در میانگین درصد پارگی فیبرهای کلاژن را بین گروه‌های درمان و گروه کنترل اثبات نمودند.

اما نتایج ما با مطالعه انجام گرفته توسط Fortier و Smith در سال ۲۰۰۷ متفاوت بود که در بررسی خود با روش سونوگرافی نشان دادند جایگذاری MSCهای مشتق از مغز استخوان باعث تشدید جراحت و حتی واکنش تاندون نشده و هیچ افزایش قابل توجهی نیز در ناحیه تاندون ایجاد نمی‌شود.

در میزان ضخامت، تفاوت قابل توجهی بین گروه PRP و MSC وجود نداشت، اما، در میزان فیبر کاهش مشخصی در گروه PRP مشاهده شد. اندکی پس از انجام پیوند، فیبرهای کلاژن با تراکم بیشتری تکثیر

پیدا کردند و سپس بازسازی فیبرهای کلاژن و تشکیل بافت تقریباً نرمال تاندون در گروه PRP نسبت به سایر گروه‌ها زودتر اتفاق افتاد. بنابراین، کاربرد PRP باعث کوتاه شدن مرحله التهاب شده و التیام تاندون را در طی مرحله تکثیر بهبود می‌بخشد (Takamura et al., 2017).

با این وجود، در عرض و اکوژنیسیته، تفاوت قابل توجهی در میان گروه‌ها وجود نداشت و این مسئله در توافق با نتایج de Mattos Carvalho و همکاران در سال ۲۰۱۱ و Nixon و همکاران در سال ۲۰۰۸ است که نشان دادند در پارامترهای سونوگرافی بین اندام‌هایی که سل تراپی شده بودند و اندام‌های گروه کنترل در اسب، تفاوت قابل توجهی وجود ندارد.

در همان گروه‌ها، پس از جراحی و پس از روز ۶۰، بهبودی قابل توجهی در میزان اکوژنیسیته و فیبر مشاهده شد (Schnabel et al., 2009; Mostafa et al., 2015).

بهبود اکوژنیسیته و هم‌ترازی فیبرها در طی ترمیم تاندون نشان‌دهنده حضور بافت جوانه گوشتی نابالغ است که دارای فیبروژنزیز فعال و تولید کلاژن همراه با افزایش تراکم آکوستیک می‌باشد (Mostafa et al., 2015).

نتایج سونوگرافی نشان دهنده بهبود تاندون آلوگرافت منجمد در جایگذاری تاندون است و در گروه‌های MSC و PRP بازسازی آن تسریع می‌شود.

میانگین نرمال هیدروکسی پرولین 137.171±5.291 mg/g ماده خشک بود، درحالی‌که در گروه کنترل 87.694±6.502 و در گروه PRP 99.694±1.839 بود. با این وجود، در گروه MSC برابر با 134.322±2.123 mg/g بود ($p<0.05\%$).

تفاوت قابل توجهی بین تاندون گروه‌های کنترل و PRP با تاندون حیوان سالم وجود داشت. این تفاوت وقتی تاندون گروه درمان با اندام طبیعی مقایسه می‌شد کاهش بسیاری پیدا کرده بود. با این حال، افزایش قابل توجهی در محتویات هیدروکسی پرولین در گروه سوم که از سلول‌های مزانشیمی استفاده کرده بودند مشاهده گردید. این نتایج نشان دهنده قوت بیشتر گروه MSC نسبت به گروه‌های کنترل و PRP می‌باشد. بهبود روند ترمیم تاندون در مواردی که MSC به کار برده شده است، با استفاده از فاکتورهای رشد افزایش می‌یابد. استفاده از MSC در تاندون‌ها منجر به غلظت بیشتر کلاژن و سرعت بیشتر بازسازی می‌گردد (Huo et al., 2009).

MSC مشتق از مغز استخوان به دنبال تطابق با شرایط محیطی ویژه‌ای در تاندون، می‌تواند به سلول‌های خاصی در آلوگرافت التیامی تمایز پیدا کند، همانطور که می‌تواند بازسازی و بلوغ پیوند را تشویق نماید. زمانی که MSC در سطح پیوند تاندون به کار برده می‌شود، ممکن است برای بازگشت جمعیت سلولی تاندون، به‌عنوان منبع دیگری از سلول‌های شبه فیبروبلاست عمل کند یا ممکن است در فعالیت به‌کارگیری تشکیلات فیبروبلاست شرکت کنند (Li et al., 2007).

محتویات هیدروکسی پرولین PRP، افزایش قابل توجهی در مقایسه با گروه کنترل نداشت، در حالی که Allahverdi و همکاران در سال ۲۰۱۵، نشان دادند که PRP باعث افزایش محتویات هیدروکسی پرولین در تاندون‌های تنوتومیزه خرگوش می‌شود.

در ارزیابی قدرت کششی، RMN و انرژی جذب شده، افزایش قابل توجهی در گروه MSC مشاهده شد. RMN/nm^2 در گروه PRP نیز افزایش پیدا کرد، اما خیلی قابل توجه نبود. در مورد سایر پارامترها افزایش ملایمی در گروه MSC و افزایش کم‌تری در گروه PRP مشاهده شد که قابل توجه نبود.

افزایش خصوصیات بیومکانیکی در تاندون‌های گروه MSC و مقدار نسبی این نتایج در مقایسه با گروه کنترل و PRP، نشان می‌دهد که گروه MSC دارای پتانسیل بیشتری برای بازگرداندن سریع‌تر فعالیت‌های طبیعی تاندون می‌باشد و مشخص می‌سازد که استفاده از MSC برای ترمیم تاندون آلوگرافت، باعث بهبود تاندون می‌شود. بخش قابل توجهی از مقاطع عرضی سلول‌ها، به ترمیم کمک می‌کنند و افزایش میزان ویژگی‌های ساختاری، می‌تواند توجیهی برای این نتیجه باشد (young et al., 1998).

TGF-beta1 آزاد شده از فیبروبلاست‌ها که از سلول‌های MSC متمایز می‌شوند، می‌تواند قدرت مکانیکی در التیام تاندون‌های آلوگرافت را با تنظیم ساخت کلاژن‌های تیپ III و I، تشکیل اتصالات متقاطع و بازسازی ماتریکس افزایش دهد (Hou et al., 2009). در این مطالعه، نتایج بافت شناسی، یافته‌های بیومکانیکی را تأیید می‌کنند.

نتایج بیومکانیکی PRP در مطالعه حاضر، با بررسی Kraus و همکاران در سال ۲۰۱۶ هم‌خوانی نداشت. نتایج بیومکانیکی و ایمونوهیستولوژی این محققان، تأثیرات مثبت گروه MSC را در بازسازی تاندون موش‌هایی که دارای مشکل در تاندون آشیل بودند، نشان نداد و پس از گذشت ۱۲ هفته، سلول‌های بنیادی اثر قابل توجهی بر روی نتایج بیومکانیکی نداشتند.

در ارزیابی هیستوپاتولوژی، از رنگ آمیزی تریکروم ماسون برای مشاهده تاندون SDF استفاده شد. رنگ آبی نشان‌دهنده کلاژن در تاندون بود. هسته‌های فیبروبلاست تاندون که رنگ سیاه را به خود گرفته بودند، در ردیف‌های موازی و صاف بین فیبرهای کلاژن قرار داشتند.

اگرچه گروه کنترل از ارزش کم‌تری در میان سایر گروه‌ها برخوردار بود ولی وضعیت بهتری را در میان گروه‌های درمان از لحاظ خصوصیات سازماندهی ماتریکس خارج سلولی نشان داد. توضیح احتمالی برای این روند التیام آهسته تاندون و در نتیجه تشکیل ماتریکس خارج سلولی نامرغوب از جهت مکانیکی، میزان اندک خونرسانی به بافت تاندون، حضور سلول‌هایی که نشان‌دهنده کاهش فعالیت تقسیم میتوز هستند و حضور تعداد کمی سلول‌های پیشرو در بافت می‌باشد (de MattosCarvalho et al., 2011).

جهت گیری فیبرهای کلاژن در گروه‌های MSC و PRP ، در مقایسه با گروه کنترل سازمان‌یافته‌تر بود. تعداد عروق تازه تشکیل شده در گروه MSC در مقایسه با گروه‌های کنترل و PRP کاهش یافت و این امر

نشان می‌دهد که PRP ممکن است دوره فرآیند التیام را کوتاه کند (Lyras et al., 2009). فاکتورهای رشد مشتق از پلاکت، باعث افزایش تکثیر و مهاجرت سلول‌ها و تولید کلاژن شده و بازسازی تاندون آلوگرافت را افزایش می‌دهد (Bosch et al., 2010; Li et al., 2007).

با این حال، از لحاظ نسبت سلول‌ها به ماتریکس سلول‌های مزانشیمی، سلولاریتی در مقایسه با سایر گروه‌ها افزایش یافت. از لحاظ ماتریکس، هم‌ترازی سلولی و غیره، گروه MSC از سایر گروه‌ها و گروه PRP از گروه کنترل بهتر بود. علاوه‌براین، نتایج حاکی از عروق‌دار شدن بیشتر در گروه‌های PRP و کنترل بودند.

Bosch و همکاران در سال ۲۰۱۰، تأثیر اصلی PRP بر روی فرآیند التیام تاندون را یک تأثیر ماندگار بر روی تکثیر و مهاجرت سلول‌ها دانستند و بنابراین تأثیر آن را بر روی فعالیت کلی متابولیک مشاهده نمودند. این نتایج با یافته‌های بافت شناسی مطابقت دارد. در تاندون‌هایی که از PRP استفاده شده است، تراکم ناحیه‌ای بیشتری از سلول‌ها و همینطور عروق‌دار شدن بیشتر مشاهده شد که نشان‌دهنده فعالیت متابولیکی بالاتر می‌باشد.

علی‌رغم فواید استفاده از PRP، باید به استفاده بهینه از فاکتورهای رشد توجه شود. یکی از نگرانی‌های مهم، طول عمر کوتاه این عوامل بیواکتیو است که تأثیر آن‌ها را محدود می‌سازد (Kiapour and Murray, 2014).

رگ‌زایی (آنژیوژنز) کمتری در گروه MSC در مقایسه با گروه‌های PRP و کنترل مشاهده گردید که نشان می‌دهد MSC منجر به تسریع روند التیام می‌شود. رگ‌زایی مرحله‌ای ضروری برای فرآیند التیام تاندون و بازسازی تاندون پیوندی بوده و تشکیل عروق جدید و رسیدن سلول‌های التهابی، فیبروبلاست‌ها و فاکتورهای رشد به محل زخم را افزایش می‌دهد، بنابراین، رگ‌زایی می‌تواند منجر به تسریع بازسازی تاندون پیوندی شده (Li et al., 2007)

تراکم بیشتر سلولی و رگ‌زایی بیشتر، نشانه فعالیت متابولیکی بیشتر می‌باشد (Li et al., 2007; Pastides and Khan, 2011).

TGF-β که از سلول‌های فیبروبلاست متمایز از سلول‌های بنیادی مزانشیمی MSC ترشح می‌شود، دارای نقش کلیدی در ترمیم بافت‌های مختلف است. TGF-β می‌تواند تولید فاکتور رشد اندوتلیوم عروق (VEGF) را افزایش دهد. TGF-β برخی از فرآیندهای بیولوژی شامل تکثیر، مهاجرت، تمایز، آپوپتوز و رسوب ماتریکس خارج سلولی را تنظیم می‌کند. TGF-β به‌خصوص تکثیر و ساخت ماتریکس تاندون و فیبروبلاست‌های لیگامنت را تسریع می‌کند. علاوه‌براین، TGF-β رسوب پروتئوگلیکان را تنظیم و تولید کلاژن را با فیبروبلاست‌ها تحریک می‌کند. VEGF یک محرک قوی برای رگ‌زایی است و به‌خصوص در مراحل تکثیر و بازسازی از اهمیت زیادی برخوردار است (Kushida and Lida, 2014).

میزان التهاب در گروه PRP در مقایسه با گروه کنترل و MSC کاهش پیدا کرد. تزریق PRP ممکن است باعث سرکوب شدن التهاب و در نتیجه کاهش درد تاندون و بنابراین بهبود فعالیت آن در حیوانات شود. درواقع فاکتور رشد هپاتوسیت (HGF) در PRP فعالیت ضد التهابی دارد (Zhang et al., 2013; Wang and Nirmala, 2016).

نتایج گروه PRP با مطالعه Parafioriti و همکاران در سال ۲۰۱۱ در تضاد بود. این محققان نشان دادند تزریق PRP به تنهایی در ترمیم پارگی تاندون آشیل موش مؤثر نیست. PRP یک ادجوانت نیست که در مدل پارگی تاندون آشیل موش، فعالیت تاندون را تکمیل کند.

ارزیابی هیستوپاتولوژی نشان داد که در گروه MSC و سپس در گروه PRP، سازمان دهی فیبرهای تاندون باعث کاهش بیشتر التهاب نسبت به گروه کنترل می‌شود.

در مدل سلول درمانی، التهاب اطراف عروقی، تراکم فیبروبلاست‌ها و تشکیل عروق جدید افزایش یافته و استفاده از آن منجر به بهبود کیفی التیام ماتریکس خارج سلولی تاندون از لحاظ جهت‌گیری فیبر و نسبت کلاژن I و III می‌شود (de MattosCarvalho et al., 2011).

نتیجه‌گیری:

در مطالعه ما مشخص شد که کاربرد MSC و PRP برای آناستوموز و آلوگرافت تاندون، در حفظ ویژگی-های خاص ساختاری در طی مرحله ابتدایی بازسازی تاندون مؤثر است. استفاده از MSC باعث القاء که در نتیجه بازسازی بافت تاندون و سپس رسوب کلاژن را در ناحیه پیوند افزایش می‌دهد، بنابراین، این تکنیک ابزار مناسبی برای بهبود ترمیم تاندون آلوگرافت و بازگردادن قدرت تاندون می‌باشد.

کاربرد PRP روش امیدوار کننده‌ای برای تسریع التیام تاندون است، علاوه‌براین، کم هزینه بودن PRP در مقایسه با استفاده از MSC و همینطور ذات خوش‌خیم PRP، باعث اولویت داشتن آن در استفاده در مطالعات بالینی شده است.

شماره ثبت: ۷۸۹

بدین وسیله تایید می گردد این پایان نامه با عنوان: " **ارزیابی پیوند تاندون منجمد پوشش داده شده با سلول‌های بنیادی مزانشیمی مشتق شده از مغز استخوان خودی و پلاسمای غنی از پلاکت جهت حفظ طول تاندون در بره گوسفند**" توسط آقای **رافد مجید نعیم الخلیفه** دانشجوی دکتری تخصص رشته جراحی دامپزشکی تهیه وتدوین شده و در جلسه هیات محترم داوران متشکل از اساتید محترم:

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در تاریخ۱۳۹۷/۰۴/۰۶ ارائه گردید و طبق صورت جلسه جداگانه با درجه **عالی** ونمره **۱۹/۳۰** مورد پذیرش هیأت محترم داوران قرار گرفت.

دکتر سید مصطفی پیغمبری
معاون آموزشی و تحصیلات تکمیلی

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دانشکده دامپزشکی دانشگاه تهران
اداره آموزش و تحصیلات تکمیلی

University of Tehran
Faculty of Veterinary Medicine

Thesis Title

Evaluation of the Frozen Tendon Graft Covered with Mesenchymal Cell Derived from Bone Marrow and Platelet Rich Plasma (PRP) in Maintaining the Normal Length of Tendon in Sheep Lamb

By:

Rafid Majeed Naeem Alkhalifa

Under supervisor of:

Dr. Davood Sharifi

Thesis submitted to the office of graduate studies
in partial fulfillment of the requirements for
the degree of Doctorate in Veterinary Surgery

June, 2018

APPROVAL SHEET

Evaluation of the Frozen Tendon Graft Covered with Mesenchymal Cell Derived from Bone Marrow and Platelet Rich Plasma (PRP) in Maintaining the Normal Length of Tendon in Sheep Lamb

Doctoral Thesis
In:
Veterinary Surgery

Rafid Majeed Naeem Alkhalifa

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Certification

This is to certify that this thesis entitled: **"Evaluation of the Frozen Tendon Graft Covered with Mesenchymal Cell Derived From Bone Marrow and Platelet Rich Plasma (PRP) in Maintaining the Normal Length of Tendon in Sheep Lamb"** was prepared by **Rafid Majeed Naeem Alkhalifa** and presented on June, 2018 to the following Committee of Referees including:

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Author's Declaration

At no time during the registration of the degree of Doctor of Philosophy (Ph.D) has the author been registered for any other university award.

The Ministry of Higher Education and Scientific Research, Iraq Government funded this study.

All experimental work was carried at the Large & Small Animal Veterinary Hospital, according to the Experts Research Committee in the Faculty of Veterinary medicine University of Tehran. MSCs and PRP was prepared at Royan Institute, Tehran. The studies were conducted according to Tehran Home Office Regulation under the home office project license.

Signed.....

Date.....

Dedication

- To Almighty God who owns the kingdom of heaven and earth ... The Lord of Glory Allah, most gracious most merciful.
- To the one who guided and reunited us.... Prophet Mustafa peace be upon him.
- To the Lights of guidance and the ships of survival...Ahla-Albait, to them peace and blessings.
- To the land for which thousands of people bled seas of blood.... Iraq
- To those who live wishing a day to live in peace.... My beloved people of Iraq.
- To our martyrs the righteous ... Glory and honor to them
- To the flower which beautifies my life with her tenderness.... My mother
- To the sail of my life and the joy of my heart and my destiny, whom we lost with grief and sadness, we ask God to put them in paradise ... My father and my elder brother.
- To those who were candles enlightening my path.... My brothers, sisters, my wife and my children.

Rafid Majeed Naeem Alkhalifa

Acknowledgment:

All thanks for the most gracious most merciful Allah firstly and finally

I would like to thank the deanery of Veterinary of Medicine Colleges /University of Basra and Tehran and for support to accomplish the research and I'd like to express my deep thanks to **the all teaching and non-teaching staffs of Department of Veterinary Surgery and Radiology; Faculty of Veterinary Medicine University of Tehran**, I would forever be thankful and grateful.

I am deeply and extremely grateful to my supervisor **Prof. Davood Sharifi** for his supervision, support, constant interest, encouragement and advices.

I would like to thank Co-advisors **Dr. Abbas Tavasoli** and **Dr.Majid Masoudifard** deeply and extremely for their help and advices throughout this research.

My sincere thanks to my close friends **Dr. Ala'a A. Ebrahim, Dr. Ali Reza Bashiri, Dr. Reza Javadi, Dr. Saeed Ferzaad, Dr. Esmaeel Nazhad, Dr. Tarkumani, Dr. Banna Sadeq, Dr. Farnoosh Bagheri, prof. Saleh K. Majeed, Mr.Reza Samani, Mr. Rouhoulah Esanejad, Mr. Gholam Reza Shams, Mr. Hussein Shad, Dr. Hassan Khalaf, Dr. Hussam Aldeen, Dr. Ashraff Abdulrazaaq, Ebrahim Bijari and Davood Faskhoudi** for their continuous support and help throughout this research.

Special acknowledgment goes to **staff of Royan laboratory** for segregation of Mesenchymal stem cell and isolation platelet rich plasma.

I would like to express my deep thanks to everyone who gave me a favor and helped me in my study.

Rafid Majeed Naeem Alkhalifa

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Student: Rafid Alkhalifa

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Abstract:

Background: Tendon grafting is commonly required to repair an injury to flexor tendon and because of the importance of this tendon; it needs methods to speed up the re-vitality and strength of the tendon allograft. **Objective:** The aim of this study was to determine the effectiveness of using Platelet Rich Blasma (PRP) and bone marrow derived mesenchymal cells in improving regeneration process after grafting superficial digital flexor tendon (SDFT) with frozen allograft tendon in lamb. Fifteen "four months" lambs were selected for replacement a 5 cm SDFT with frozen allograft tendon of the same age. **Methods:** Under strict a septic condition a 5 cm mid portion of SDFT were removed from both forelimbs in all 15 lambs, and were immediately replaced with frozen allograft tendon being harvested from slaughterhouse and frozen under -20°C for 45 days. Subsequently, the animals were divided into 3 subgroups of 5 lambs each. The 1st group was act as a control, whereas the 2nd group 1 ml PRP was injected at the site of anastomosis and in the 3rd group 1 ml of mesenchymal cell (10^{10}) was infiltrated at the site of anastomosis. 2 months post-surgery ultrasonographic evaluations, hydoxyproline content, tensile strength, and histopathological evaluation were performed. **Results:** Clinically all lambs showed the same degree of lameness, which was almost identical due to similarity of the lesions. The lameness was mostly apparent after removal of cast on day 15 of surgery but gradually improved in the treated group at the end of third week, which showed to have normal weight bearing after month. In ultrasonographic evaluation, there is significant increase in Cross Sectional Area (CSA) in MSCs treated group tendons, but in thickness, there is no significant difference between PRP and MSCs groups. In fiber score there is significant decrease in PRP group. However, in Width and Echogenicity score, there is no significant difference between all groups. Hydroxyproline test showed the positive effect of MSCs in third group with 134.322±2.123 mg/g dry matter as compared to normal 137.171±5.291 mg/g dry matter these two groups indicated the high amount of collagen for fiber orientation in early tendon union. Hydroxyproline content was very low in the control group 87.694±6.502 mg/g and PRP group 99.116±1.839 mg/g dry matter.

Biomechanical study showed in control group stress (Rm N) was recorded 11.42±3.44 N, whereas in PRP group was 19.11±6.20 and in the third group it was 31.98±5.06. Stiffness or Elastic modulus (N/mm) was recorded 1.89±0.90 in control group whereas in second group (PRP) 3.36±0.55 and in the third group 4.20±1.90. Deformation (εFmax mm) in control group was recorded 7.00±2.41 mm and 12.66±4.90 in second group (PRP) and the third group (MSc) 16.64±4.78mm, and the last parameter recorded absorbed energy (w upto Fmax Nmm), in control group was 84.54±38.32 and in PRP group 90.62±54.31 and 221.87±48.44 in the third group, which used MSCs. In Stress RMN and Absorbed energy, there was a significant increase were observed in third group ($P<0.05$) compared with that of PRP and control groups. Histopathological study showed that MSCs could enhance cell proliferation and collagen organization etc., the PRP group better than control histopathologically but MSC group was superior. **Conclusion:** the results of this study showed that MSCs accelerate and enhance allograft tendon regeneration and improve its quality (tensile strength, collagen content etc.). A single administration of PRP could have a little improvement in tendon allograft healing process.

Key words: mesenchymal stem cells, platelet rich plasma, frozen allograft tendon, lamb.

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Chapter One:
Preface

1.1 Introduction

Tendon is collagenous structure, which transmits tensile force from muscle to bone. It is brilliant white in color and fibro-elastic in texture, showing high resistance to mechanical loads.

Tendon tissue has low blood supply, cell turnover, and metabolism, similar to menisci or articular cartilage. For this reason, the healing of tendon injuries is prolonged and often results in the formation of inferior scar or remaining lesions. In orthopedics, both the healing of a cute tendon ruptures and degenerative changes of the tendons represent a serious problem for the clinicians. New approaches have been investigated to improve healing of tendon tissue and to develop new, biological therapies for tendon alignment.

Tendon possesses a highly organized fibrillar matrix, consisting of type I collagen and various "minor" collagens, proteoglycan and glycoprotein. The tendon matrix is maintained by the resident tenocytes. Tendon injuries are predominantly degenerative in nature and are often recalcitrant to treatment, being slow to heal, and rarely regaining their original strength and elasticity.

Healing process occurs due to scar tissue formation leading to poor outcomes for patients. Current surgical and nonsurgical treatments modalities lead to sub optimal tendon healing and each of them have complications. Therefore, to repair and restore tendon structure and function, a tendon graft is needed; therefore, autografts, allografts and xenografts have been used. Each of these grafts is associated with several limitations such as donor site morbidity and pain, poor biocompatibility, disease transmission and immune reaction, which lead to graft rejection and failure.

Many experimental studies were performed, focusing on flexor tendon healing, suture technique, suture material and postoperative rehabilitation, the evolving interest in primary flexor tendon repair stimulated research, which rise further questions regarding what can be done to improve healing process.

Tendon grafting is commonly required to repair an injury to flexor tendon. However there is lack of suitable graft material and controversy remains as to which materials are best for flexor tendon repair. Additionally most grafting procedures lead to adhesion, which limits joint mobility.

Recently Platelet rich plasma (PRP), an autologous concentrate of blood platelets has been introduced as a possible new therapy for the treatment of tendon injury.

PRP can potentially enhance the recruitment and proliferation of tenocytes, stem cells and endothelial cells. One of the growth factor included in PRP is transforming growth factor b (TGF-b); it is a well-known cytokine that regulates various processes in tendon healing. It modulates the inflammatory responses in early healing stages, participates in the intricate control of angiogenesis, regulates the proteoglycan deposition and stimulates production of collagens by tendon fibroblast.

The use of autologous mesenchymal cells from bone marrow to repair experimental injuries of tendons and ligaments has been amply described in experimental animals.

The goal of using stem cells is to engineer new tendon tissue using cellular synthetic machinery. This goal can be achieved either via a direct contribution through differentiation into tissue specific cell phenotypes and via the production of tissue appropriate extracellular matrix products or indirectly by trophic effects through the production of bioactive

proteins, such as growth factors, anti-apoptotic factors and chemotactic agents. In addition, recent studies have suggested an anti-inflammatory role of implanted stem cells.

1.2 The aim of study

This study aims to determine whether an allogenic tendon can be successfully used to replace SDFT, and also aims to evaluate tendon allograft repair with PRP and bone marrow MSCs by histological examination, sonographic picture, tensile strength and hydroxyproline test.

1.3 The necessity of the study

Tendon are frequently encountered during surgery or laceration injury of the foot and leg since they are numerous, superficial, and at time quite large. For these reason our investigation on superficial flexor tendon. In this study, we use PRP and MSCs for improvement and speed tendon allograft healing process. The use of tendon allograft as SDFT substitute will be a great clinical value.

1.4 Hypothesis

- Frozen tendon by maintaining its ultrastructure is quite suitable replaced graft for SDFT
- Addition of MSc & PRP can potentiate healing speed of the graft to Host's tendon tissue
- The frozen tendon is quite suitable bridge for transmitting forces from muscles to bone

Chapter Two

Literature Review

2.1 Tendon

2.1.1 Anatomy of tendon

The tendon has a multi-unit hierarchical structure composed of collagen molecules, fibrils, fiber bundles, fascicles and tendon units that run parallel to the tendon's long axis. The fibril is the smallest tendon structural unit; it consists largely of rod-like collagen molecules aligned end-to-end in a quarter staggered arrays. (Wang, 2006)

Tendons are unique forms of connective tissue that connect and transmit forces from muscle to bone. They are able to store elastic energy and withstand the high tensile forces upon which locomotion are entirely dependent. (Docheva et al., 2015)

Flexor tendons, by definition, flex the digit when the limb is in the swing phase. In the stance phase they are under very high tensile stress as, depending on the gait, they may be challenged by loads of more than two times body weight. (Cadby, 2013)

Superficial digital flexure tendon produces highly to resist body weight on limb during the stance phase and flexes the digit during the swing phase of locomotion. It is an energy strong structure that significantly improve locomotor efficacy at high speed gait due to expandable elastic crimp with the collagen fascicles SDFT is one of the top ten most common injury of the stander bred race horse (Tyelor, 2013)

Tendons transmit force from muscle to bone, thereby permitting movement of the whole body. Consequently, any damage to tendons will rapidly have an invalidating effect. (Wilson and Lichtwark, 2011; Cadby, 2013)

2.1.2 The extracellular tendon matrix (ECM)

The extracellular matrix (ECM) of tendons is composed of the collagen fibers, elastic fibers, the ground substance, and the anorganic components.It is composed of collagen and a smaller fraction of elastin embedded in a hydrated proteoglycan matrix. The principal role of the collagen fibers is to resist to tension, whereas proteoglycans are primarily responsible for the viscoelastic properties of the tendon (Kannus, 2000).

The smallest structural unit is the collagen fibril. Each fibril is built from soluble tropocollagen molecules forming cross-links to create insoluble collagen molecules which then aggregate progressively into microfibrils, fibrils and finally into fibers (fig 2-1). (Benjamin et al., 2008; Kannus, 2000; Dowling et al., 2000)

Bundles of fibers are bound together by thin layers of loose connective tissues known as the epi- and endotenon, which allow the fiber groups to glide on each other in an almost frictionless manner; they also carry blood vessels, nerves and lymphatics to the deeper portion of the tendon (Benjamin et al., 2008).

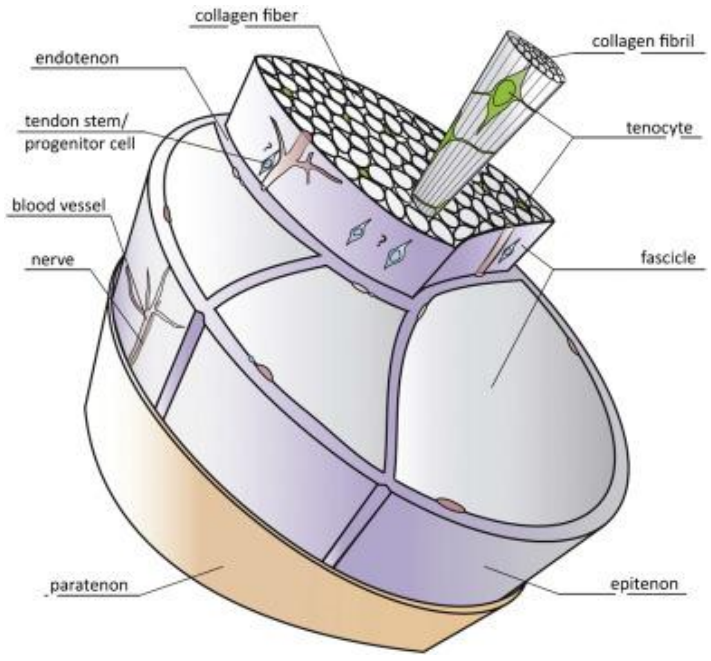

Figure 2-1: A schematic drawing of basic tendon structure. The collagen molecules are organized hierarchically in fibrils, fibers and fascicles. The cellular content is dominated by the tenocytes, which are terminally differentiated cells. Tendons contain stem and progenitor cell populations, whose exact location is still debated.
Different sheets, endotenon and epitenon (loose connective tissues), and paratenon (fatty areolar tissue) are shown as well as blood vessels and nerves (Docheva et al., 2015)

The smooth gliding of tendons as they move is aided by the lubricating molecule, lubricin (sun et al., 2006). Altogether this complex, threedimensional, internal ultrastructure endows the tendon with high tensile force and resilience, while preventing damage and separation of the fibers under mechanical stress (fig 2-1) (Docheva et al., 2015).

A fine sheath of connective tissue called endotenon invests each collagen fiber and binds fibers together. A bunch of collagen fibers forms a primary fiber bundle, and a group of primary fiber bundles forms a secondary fiber bundle. A group of secondary fiber bundles, in turn, forms a tertiary bundle, and the tertiary bundles make up the tendon. The entire tendon is surrounded by a fine connective tissue sheath called epitenon**.** (Kannus, 2000)

Tendons present a hierarchical structure with their collagen bundles predominantly ordered in a longitudinal direction. The basic structural unit is tropocollagen, a triple helix polypeptide chain. Five tropocollagen molecules form each collagen filament. These are then organized into the larger structural units of the tendon: subfibrils, fibrils, fibres, sub-fascicles, fascicles, tertiary fibre bundles and the tendon itself (Screen et al., 2004 & Cadby, 2013)

Tendons have a typical white glistening appearance due to collagen and water being their main components. Collagen type I is the most abundant molecule in the ECM. Type III is the next most abundant collagen (Kannus, 2000). Type III collagen is found in considerable amounts in immature tendons and injured tendons (sun et al., 2006; Kannus, 2000).

Normally, collagen type III is restricted to the tendon sheets; however, it is found abundantly in pathological tendons and it is also the first collagen to be produced in high quantity during tendon healing (Riley, 2004).

Other collagens in tendon include types V, VI, XII, XIV and XV are also present but in small quantities (Liu et al., 2011; Docheva et al., 2015)

The mechanical properties of tendon collagen depend largely on intra- and intermolecular cross-linking, which is essential for the stability of collagen fibrils (Parry, 1988; Tsuzaki et al., 1993; Cadby, 2013).

Another important constituent of the tendon extracellular matrix (ECM) is formed by proteoglycans and their associated glycosaminoglycan (GAG) side chains such as 8 dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS). Proteoglycans form bridges and bonds between the collagen fibrils in longitudinal as well as transverse directions (Cribb and Scott, 1995; Docheva et al., 2015).

Besides collagen fibers, the tendon ECM is composed of many other components including elastic fibers, the ground substance and inorganic components. In general, elastic fibers ensure tissue flexibility and extensibility, permitting long-range deformability and passive recoil without energy input. Furthermore, they are thought to be involved in the recovery of the crimp pattern of the collagen fibers after tendon stretching. (Docheva et al., 2015)

Proteoglycans are also believed to influence many important physiological processes, such as ion transport, the diffusion of nutrients and water retention (Xu and Murrell, 2008). Further, they play a role in fibrillogenesis and matrix architecture (Birk et al., 1989; Weber et al., 1996). Changes in the glycosaminoglycan and proteoglycan composition have been described as occurring during maturation and ageing, after immobilization and exercise in response to mechanical cues and also after tendon injury (Riley and Harrall, 1994).

2.1.3 Paratenon, epitenon and endotenon

Many of the tendons are surrounded by loose areolar connective tissue called paratenon. true tendon sheaths can only be found in the areas where a change of direction and increase in friction necessitate very efficient lubrication (Docheva et al., 2015).

The collagenous fiber system of the paratenon is well defined. The main components of the paratenon are the type I and type III collagen fibrils and the elastic fibrils (Kvist et al., 1985), and the paratenon is lined on its inner surface by synovial cells (Williams, 1986).

Paratenon functions as an elastic sleeve (although probably not so effectively as a true tendon sheath), permitting free movement of the tendon against the surrounding tissues (Hess et al., 1989).

Under the paratenon, the entire tendon is surrounded by a fine connective tissue sheath called epitenon (Figure 2-1). On its outer surface, the epitenon is contiguous with paratenon and on its inner surface with the endotenon. Inside the tendon, the endotenon invests each tendon fiber and binds individual fibers, and in larger units fiber bundles, together (Kannus, 2000).

The endotenon is a thin reticular network of connective tissue inside the tendon that has a well-developed crisscross pattern of collagen fibrils. The endotenon fibrils invest tendon fibers and bind fibers together (Figure 2-1) (Kannus, 2000).

To improve binding, there is a high degree of hydralation of proteoglycan components between the endotenon and the surface of the tendon fascicles (Rowe, 1985). Along with its important function of binding, the endotenon network allows the fiber groups to glide on each

other and carries blood vessels, nerves, and lymphatics to the deeper portion of the tendon (Hess et al., 1989; Kannus, 2000)

Tendons consist of collagen (mostly type I collagen) and elastin embedded in a proteoglycan-water matrix with collagen accounting for 65–80% and elastin approximately 1–2% of the dry mass of the tendon (Kannus, 2000).

These elements are produced by tenoblasts and tenocytes, which are the elongated fibroblasts and fibrocytes that lie between the collagen fibers, and are organized in a complex hierarchical scheme to form the tendon proper Soluble tropocollagen molecules form cross-links to create insoluble collagen molecules which then aggregate progressively into microfibrils and then into electronmicroscopically clearly visible units, the collagen fibrils. A bunch of collagen fibrils forms a collagen fiber, which is the basic unit of a tendon (Dowling et al., 2000).

2.1.4 The surrounding structures of the tendons can be divided into five categories:

1. The fibrous sheaths or retinacula: are the canals through which the (usually long) tendons glide during their course. Without these bony grooves and notches friction could considerably impair tendon gliding. The grooves and notches are usually lined with a fibro cartilaginous floor and covered with the fibrous sheath or retinaculum. Characteristic examples are the retinacula of the extensors and flexors of the hand and feet.
2. The reflection pulleys: are the anatomic reinforcements of the above-mentioned fibrous sheaths located in places where there are curves along the course of the tendon. Their task is to keep the tendon inside its sliding bed.

3. The synovial sheaths: are access tunnels for tendons at bone surfaces or other anatomic structures that might cause friction. Most frequently, they can be found around the tendons of the hand and feet. Under a fibrous layer, there are two thin and serous sheets, the parietal and visceral sheets. These sheets form a closed duct including peritendinous fluid for lubrication.
4. In some tendons, i.e., tendons which do not have a true synovial sheath, there can be a peritendinous sheet: (paratenon) to reduce friction. It is composed of loose fibrillar tissue and functions as an elastic sleeve permitting free movement of the tendon against surrounding tissues. Characteristically, the Achilles tendon has a well identifiable paratenon with thin gliding membranes
5. The tendon bursae: are the fifth extratendinous structure playing an important role in the reduction of friction. They are located in those anatomic sites where a bony prominence might otherwise compress and wear the gliding tendon. Typical examples are the subacromial, deep trochanteric, pes anserinus, infrapatellar, and retrocalcaneal bursae (Kannus, 2000).

2.1.5 Tendon cells

The tendon cells, the tenoblasts and tenocytes, comprise about 90–95% of the cellular elements of the tendon. Tenoblasts are immature tendon cells. They are spindle-shaped and have numerous cytoplasmic organelles, reflecting their high metabolic activity. As they mature, tenoblasts become elongated and transform into tenocytes. Tenocytes have a lower nucleus-to-cytoplasm ratio than tenoblasts, with decreased metabolic activity (Kannus, 2000).

The other 5–10% includes the chondrocytes at the pressure and insertion sites, the synovial cells of the tendon sheath on the tendon

surface, and the vascular cells, such as capillary endothelial cells and smooth muscle cells of the arterioles, in the endo and epitenon. In pathological conditions, many other types of cells, such as inflammatory cells, macrophages, myofibroblasts, can be observed in the tendon tissue (Jozsa and Kannus, 1997).

The cells are key players in tendon healing.Tendon cells are able to remodel the tendon matrix, synthesize new collagen and improve overall structural integrity of the tendon after injury, leading to better mechanical properties. The cells residing in the tendon or around it in the peritenon have enormous potential to adapt to different situations. These residing cells are playing key roles, both in maintaining normal tendon homeostasis and in the normal and sometimes abnormal healing processes of tendons. The structure, composition and mechanical properties of the ECM are also important in the overall function of the tendon (Cadby, 2013).

Tenocytes are active in energy generation through the aerobic Krebs cycle, anaerobic glycolysis, and the pentose phosphate shunt, and they synthesize collagen and all components of the extracellular matrix network (O'Brien, 1997).

2.1.6 Blood Supply

Tendons receive their blood supply from three regions. It comes form the intrinsic vascular system at

(1) Musculotendinous junction and

(2) Osteotendinous junction and from the extrinsic segmental vascular system

(3) Through the paratenon which surrounding the tendon (Paavola et al., 2002; Fenwick et al., 2002).

Blood vessels enter tendon at the myotendinous junction, which then supply the rest of the tendon. They run parallel to the fascicles as well as

within the endotenon. It is still controversial if enthesis is an important site for the entry of blood vessels (Benjamin et al., 2008).

2.1.7 Innervation

Tendon innervation originates from cutaneous, muscular and peritendinous nerve trunks. At the MTJ, nerve fibers cross and enter the endotenon septa. Nerve fibers from rich plexuses in the paratenon, and branches penetrate the epitinon. Most nerve fibers do not actually enter the main body of the tendon of the tendon, but terminate as nerve ending on its surface. (Barr & Kiernan, 1988; Sharma & Maffulli, 2006)

2.2 Tendon Injury

Tendon injury is associated with high morbidity, pain, and long-term suffering for the patient. Due to the low cellularity and vascularity of tendon tissue, once damage has occurred, the repair process is slow and inefficient, resulting in mechanically, structurally, and functionally inferior tissue (Walden et al., 2017).

2.2.1 causes of tendon injury

Tendon injuries can be acute or chronic and are caused by intrinsic or extrinsic factors, either alone or in combination. In acute trauma, extrinsic factors predominate. (Sharma and Maffulli, 2005).

Overuse injuries generally have a multifactorial origin. Interaction between intrinsic and extrinsic factors is common in chronic tendon disorders (Williams, 1986; Sharma and Maffulli, 2005).

In case of acute trauma, extrinsic factors predominate while chronic injuries are generally multifactorial (Paavola et al., 2002). Extrinsic factors are for example trauma, repetitive movement or excessive loading. The list of intrinsic factors that are known to influence tendon disorders is long.

Among others, they include: mal-alignment, diabetes, age, hypertension and genetics (Cadby, 2013).

Tendon rupture is an acute injury in which extrinsic factors predominate, although intrinsic factors are also important. Degenerative tendinopathy is the most common histological finding in spontaneous tendon ruptures. Tendon degeneration may lead to reduced tensile strength and a predisposition to rupture (Sharma and Maffulli, 2005). Tendon degeneration may lead to reduced tensile strength and a predisposition to rupture (Tallon et al., 2001; Sharma and Maffulli, 2005).

Tendinopathy, as opposed to tendinitis or tendinosis, is the best generic descriptive term for the clinical conditions in and around tendons arising from overuse (Sharma and Maffulli, 2006).

In 1995, Aström and Rausing reported the surgical findings and the histopathology for 163 human patients with chronic Achilles tendinopathy. They found Degenerative changes (tendinosis) characterized by abnormal fiber structure, focal hypercellularity, and vascular proliferation were noted in 90% of biopsy specimens from symptomatic parts of the tendons. Partial tendon ruptures were present in 19% of the patients and always occurred in areas afflicted with tendinosis. The paratenon was mostly normal or revealed only slight changes. Increasing age and male gender were associated with more pronounced histopathologic changes. Tendinosis, sometimes complicated by partial rupture, appears to be the major lesion in chronic Achilles tendinopathy; the paratenon is rarely involved. Important features are a lack of inflammatory cells and a poor healing response.

Tendon injuries are a common cause of physical disability. They present a clinical challenge to orthopedic surgeons because injured tendons respond poorly to current treatments without tissue regeneration

and the time required for rehabilitation is long (Sharma and Maffulli, 2005).

Excessive loading of tendons during vigorous physical training is regarded as the main pathological stimulus for degeneration (Selvanetti et al., 1997) and there may be a greater risk of excessive loading inducing tendinopathy in the presence of intrinsic risk factors. Tendons respond to repetitive overload beyond the physiological threshold with either inflammation of their sheath or degeneration of their body, or both (Benazzo and Maffulli, 2000).

Different stresses induce different responses. Unless fatigue damage is actively repaired, tendons will weaken and eventually rupture (Ker, 2002). The repair mechanism is probably mediated by resident tenocytes, which maintain a fine balance between ECM production and degradation. (Sharma and Maffulli, 2005; Sharma and Maffulli, 2006)

Tendon damage may even occur from stresses within physiological limits, as frequent cumulative microtrauma that lead to local tissue damage in the form of cellular and extracellular degeneration and may not allow enough time for repair. Microtrauma can also result from non-uniform stress within tendons, producing abnormal load concentrations and frictional forces between the fibrils, resulting in localised fibre damage. (Wilder and Sethi, 2004; Arndt et al., 1998; Sharma and Maffulli, 2006)

The etiology of tendinopathy remains unclear, and many causes have been theorised. Hypoxia, ischaemic damage, oxidative stress, hyperthermia, impaired apoptosis inflammatory mediators, fluoroquinolones, and matrix metalloproteinase imbalance have all been implicated as mechanisms of tendon degeneration (Goodship et al., 1994; Sharma and Maffulli, 2006)

Primary disorders of tendons (tendinopathies), due to overuse or age-related degeneration, are widely distributed clinical problems in

society, possibly resulting in acute or chronic tendon injuries (Docheva et al., 2015).

The most commonly accepted cause for tendinopathy is overuse in possible combination with intrinsic and extrinsic factors leading to what may be seen as progressive failing of the innate healing response. The clinical symptoms of tendinopathy in horses and humans differ slightly. In both species the damaged tendon is swollen and tender upon palpation. However, whereas pain is a hallmark of the initial phase in both species, persistent pain is common in humans but not in horses. In the equine species the loading capacity of affected tendons is severely reduced and the risk of reinjury is high, but horses generally become relatively quickly free of lameness (Cadby, 2013).

Under light microscopy, the pathological aspects of tendinopathy are similar in both species and are characterized by disrupted collagen, increased proteoglycan content, more prominent and numerous tenocytes and neovascularization. Lipoid degeneration as well as calcification has also been observed in some cases of tendinopathy (Astrom and Rausing, 1995).

The mechanical theory of tendon overuse injury proposes that, when a tendon is repeatedly stretched by submaximal loads, it is unable to endure further tension, whereupon injury occurs. The tenocytes are overcharged by the repetitive micro-traumatic process and are unable to repair the tendon accordingly. The collagen fibres start to slide past one another, breaking their crosslinks and, in the worst case scenario; the collagen fibrils themselves rupture (Paavola et al., 2002)

In 1997, Selavetti et al. stated that overuse tendon injury are often the result of a failed cell-matrix adaptation to the repetitive load or use, which outpace the basal tendon capacities to repair itself. They may occure

from cyclic exogenous or endogenous microtraumas as well as from application of an unexpected overload, which weakens a healthy tendon making it less able to sustain subsequent loading.

2.2.2 Treatment of tendon injury

The cellularity of tendon tissue is low (as opposed to epithelial tissue which has high cellularity), explaining the low turnover and poor self-healing capacity of the tissue. Tendons contain blood vessels though it is considerably less than that in the other tissues such as muscles. The blood supply is particularly low in tendon regions that wrap around the bony pulleys (Walden et al., 2017; Petersen et al., 2002). Such areas of diminished or absent blood supply are commonly the sites of tendon degeneration and/or rupture, suggesting that blood supply is important for tendon repair after injury. (Benjamin et al., 2008) Important features are a lack of inflammatory cells and a poor healing response (Åström and Rousing, 1995)

Tendon injury is currently managed by two approaches:

1) Conservative treatment which aims to relieve pain and

2) Surgical excision and repair.

Irrespective of the approaches used, the treated tendon heals slowly and fails to regain its full function due to the formation of mechanically inferior scar tissue, ectopic bone, and adhesion or the lack of regeneration of fibrocartilage at the tendon to bone junction (TBJ). Repeated ruptures, joint stiffness, and restricted movement are common problems encountered even after repair (Lui, 2015)

Therapeutic options used to repair ruptured tendons have consisted of suture, autografts, allografts, and synthetic prostheses. To date, none of these alternatives has provided a successful long-term solution, and often

the restored tendons do not recover their complete strength and functionality (Docheva et al., 2015).

2.2.3 Tendon healing

Tendon injuries heal slowly and the repair tissue is often functionally inferior to normal tendon tissue, leading to a high re-injury rate. The intrinsic repair process of injured tendons relies on the ability of tenocytes to proliferate and produce extracellular matrix (ECM), which is composed mainly of collagen and proteoglycans. The poor healing tendency of tendons has been attributed to the high ECM–cell ratio (Raikin et al., 2013; Cadby, 2013).

Two cellular mechanisms of tendon healing, known as extrinsic and intrinsic healing, have been suggested. The hypothesis is that first fibroblasts and inflammatory cells from the tendon periphery, blood vessels and circulation are attracted to the injured site contributing to cell infiltration and the formation of adhesions. Thereafter, intrinsic cells from the endotenon are activated as they migrate and proliferate at the injury site, reorganizing the ECM and giving support to the internal vascular networking (Docheva et al., 2015).

The process of tendon healing follows a pattern similar to that of other healing tissues (table 2-1). The three main stages of tendon healing are inflammation, repair or proliferation, and remodeling, which can be further divided into consolidation and maturation. (Thomopoulos et al., 2015 ; Sharma and Maffulli, 2006 ; Sharma and Maffulli, 2005; Kushida and Lida, 2014).

Upon tissue damage, blood vessels will rupture and signaling molecules released by intrinsic cells will trigger a coagulation cascade that will coordinate the formation of a clot around the injured area. The clot will

contain cells and platelets that will immediately begin to release a variety of molecules, most notably growth factors, causing acute local inflammation. During this **inflammatory phase**, there is an invasion by extrinsic cells such as neutrophils and macrophages which clean up

Table 2-1: Summary of the healing process in tendons (molloy et al., 2003)

Time (days)	Phase	Process
0	Immediately post-injury	Clot formation around the wound
0-1	Inflammatory	First battery of growth factors and inflammatory molecules produced by cells within the blood clot
1-2	Inflammatory	Invasion by extrinsic cells, phagocytosis
2-4	Proliferation	Further invasion by extrinsic cells, followed by a second battery of growth factors that stimulate fibroblast proliferation
4-7	Reparative	Collagen deposition; granulation tissue formation; revascularisation
7-14	Reparative	Injury site becomes more organised; extracellular matrix is produced in large amounts
14-21	Remodelling	Decreases in cellular and vascular content; increases in collagen type I
21+	Remodelling	Collagen continues to become more organised and cross-linked with healthy matrix outside the injury area. Collagen ratios, water content and cellularity begin to approach normal levels

necrotic debris by phagocytosis, and together with intrinsic cells (such as endotenon and epitenon cells) produce a second battery of cytokines to initiate the reparative phase. This stage sees collagen deposition and granulation tissue formation, as well as neovascularisation, extrinsic fibroblast migration and intrinsic fibroblast proliferation. These fibroblast

are responsible for synthesis the new extracellular matrix, consisting largely of collagens and glycosaminoglycan (Molloy et al., 2003; Ramdass and Koka, 2015). In this stage, the tenocytes are involved in the synthesis of large amounts of collagen and proteoglycans at the site of injury, and the levels of GAG and water are high (Wang, 2006).

Finally, a remodelling phase begins, which sees decreases in the cellular and vascular content of the callus tissue, and increases in collagen type I content and density. Eventually, the collagen will become more organised and is orientated and cross-linked with the healthy matrix outside the injury area. After the healing process is complete, cellularity, vascularity, and collagen makeup will return to something approximating that of the normal tendon, although the diameters and cross-linking of the collagen fibrils often remain inferior after healing (Molloy et al., 2003; Sharma and Maffulli, 2005).

In most patients, especially aged individuals, the healed tendon usually does not regain the mechanical properties of the uninjured tissue. The reduced strength of the repaired tissue compared to the native tendon results from reduced integration of collagen fibers with a higher ratio of collagen type III to collagen type I. As a consequence, the tendon thickens and stiffens to overcome the lower unit mechanical strength; thus the tendon quality and its functional activity are inferior to that of healthy tendon. (Docheva et al., 2015).

The final maturation stage occurs after ten weeks, and during this time there is an increase in crosslinking of the collagen fibrils, which causes the tissue to become stiffer (Wang, 2006).

Matrix metalloproteinases or MMPs have a very important role in the degradation and remodeling of the ECM during the healing process after a tendon injury. Certain MMPs including MMP-1, MMP-2, MMP-8, MMP-13,

and MMP-14 have collagenase activity, meaning that, unlike many other enzymes, they are capable of degrading collagen I fibrils. The degradation of the collagen fibrils by MMP-1 along with the presence of denatured collagen are factors that are believed to cause weakening of the tendon ECM and an increase in the potential for another rupture to occur (Riley et al., 2002)

Connective tissue injuries, specifically tendon sprain, may require long-term rehabilitation. Although the early soft tissue healing phase requires seven to ten days, com-plete tendon healing can take several weeks or months (Araújo et al., 2007).

stretching can disrupt healing during the initial inflammatory phase, it has been shown that controlled movement of the tendons after about one week following an acute injury can help to promote the synthesis of collagen by the tenocytes, leading to increased tensile strength and diameter of the healed tendons and fewer adhesions than tendons that are immobilized. In chronic tendon injuries, mechanical loading has also been shown to stimulate fibroblast proliferation and collagen synthesis along with collagen realignment, all of which promote repair and remodeling (Wang, 2006).

To further support the theory that movement and activity assist in tendon healing, it has been shown that immobilization of the tendons after injury often has a negative effect on healing. In rabbits, collagen fascicles that are immobilized have shown decreased tensile strength, and immobilization also results in lower amounts of water, proteoglycans, and collagen crosslinks in the tendons (Sharma and Maffulli, 2005).

2.3. Growth factors

Tendon healing is a complex and highly regulated process that is initiated, sustained and eventually terminated by a large number and

variety of molecules. Growth factors represent one of the most important of molecular families involved in healing, and a considerable number of studies have been undertaken in an effort to elucidate their many functions (Table 2-2). A truly practical and effective treatment based on the application or regulation of growth factors in vivo may still be some time away, the future cytokine based therapy is promising (Molloy et al., 2003).

The interaction of cells, matrix and biomolecules such as growth factors, play an important rule that can potentially modulate, enhance, or impede the healing response in allografts (Jackson et al., 1996).

In response to repeated mechanical loading or injury, cytokines may be released by tenocytes and can induce the release of MMPs, causing degradation of the ECM and leading to recurring injury and chronic tendinopathies (Sharma and Maffulli, 2006).

There are five growth factors that have been shown to be significantly upregulated and active during tendon healing: insulin-like growth factor 1 (**IGF-I**), platelet-derived growth factor (**PDGF**), vascular endothelial growth factor (**VEGF**), basic fibroblast growth factor (**bFGF**), and transforming growth factor beta (**TGF-β**). These growth factors all have different roles during the healing process. **IGF-I** has been shown to be highly expressed during the early inflammatory phase in a number of animal tendon healing models, and appears to aid in the proliferation and migration of fibroblasts and to subsequently increase collagen production. **TGFβ** is also active during inflammation, and has a variety of effects including the regulation of cellular migration and proliferation, and fibronectin binding interactions. (Wang, 2006; Molloy et al., 2003).

The three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) are known to play a role in wound healing and scar formation (Moulin et al., 2001). Bone

morphogenetic proteins (BMPs) are a subgroup of TGF-β superfamily that can induce bone and cartilage formation as well as tissue differentiation,

Table 2-2: Summary of the roles of five growth factors during tendon healing (Molloy et al., 2003)

Growth factor	Phase in which growth factor is most active	Roles
IGF-I	Inflammation, proliferation	Promotes the proliferation and migration of cells, stimulates matrix production
TGFβ	Inflammation	Regulates cell migration, proteinase expression, fibronectin binding interactions, termination of cell proliferation, and stimulation of collagen production
VEGF	Proliferation, remodelling	Promotes angiogenesis
PDGF	Proliferation, remodelling	Regulates protein and DNA synthesis at the injury site, regulates the expression of other growth factors
bFGF	Proliferation, remodelling	Promotes cellular migration, angiogenesis

bFGF = basic fibroblast growth factor; **IGF-I** = insulin-like growth factor-I; **PDGF** = platelet-derived growth factor; **TGFβ** = transforming growth factor; **VEGF** = vascular endothelial growth factor.

and BMP-12 specifically has been shown to influence formation and differentiation of tendon tissue and to promote fibrogenesis (Boyer et al., 2001; Moulin et al., 2001).

VEGF is produced at its highest levels only after the inflammatory phase, at which time it is a powerful stimulator of angiogenesis and to induce endothelial cell proliferation and migration. PDGF is produced shortly after tendon damage and helps to stimulate the production of other growth factors, including IGF-I, and has roles in tissue remodeling (Molloy et al., 2003).

2.4. Tendon graft

In 2017 Wu et al. reported Repair of flexor tendon injuries requires adhesion-free healing with smooth tendon surfaces and good gliding ability to restore hand function, which remains a great challenge for hand surgeons. Tendon graft repair is therapeutically indicated when the direct repair fails primarily due to severe adhesions and ruptures of the repaired tendon.

The main surgical repair techniques aim to re-establish tendon alignment by suturing the ruptured ends together, which requires a non-degenerate tendon with healing potential. The reconstructions are limited by the tendon's biology. Sometimes an autograft is used to bridge certain defects, while use of allografts has increased in recent years (Docheva et al., 2015).

Therapeutic options used to repair ruptured tendons have consisted of suture, autografts, allografts, and synthetic prostheses. To date, none of these alternatives has provided a successful long-term solution, and often the restored tendons do not recover their complete strength and functionality (Docheva et al., 2015).

Tendon allografts play an important role in tendon and ligament reconstruction in a number of anatomical sites, particularly where there is a shortage of suitable available local tissue. Surgeons using tendon allografts must be aware of the major issues surrounding their procurement, processing, and use. Adequate screening and processing can reduce, but will not eliminate, the risk of disease transmission (Robertson et al., 2006).

The advantages of using allograft tissue include a lack of donor site morbidity, high tensile strength, decreased surgical time, a faster immediate post-operative recovery, less post-operative pain, no need for

graft harvest, smaller surgical incisions, larger variety of graft sizes and shapes available, and improved cosmesis and a low risk of arthrofibrosis (Bonasia and Amendola, 2012; Robertson et al., 2006).

The disadvantages include their limited availability, susceptibility to rejection due to immuno-incompatibility between the donor and recipient and potential risk for bacterial, viral and prion disease transmission (Harner et al., 1996; Robertson et al., 2006).

Graft processing requires sterile laboratory conditions and aims to ensure that maximum use is made of donor tissue by trimming, sizing and splitting if required. It's also seek to minimize antigenicity within the tissue and to ensure that the graft material is free from bacterial and viral contamination, while minimizing damage to its structural integrity (Robertson et al., 2006; Alva et al., 2013).

In 1984, shino et al. studied the revascularization and remodeling of allografts used to replace the interior cruciate ligament in the canine knees. 26 allografts were obtained from the patellar tendons of other dogs and were stored by deep freezing. In control study a strip of patellar tendon from the same leg was used as an autologous free graft. Microangiography showed that the allograft had been revascularized from the sixth postoperative week and had later develop an intrinsic vascular pattern similar to that of a normal interior cruciate ligament, the allograft regained a fibrous frame work similar to that a normal ligament and showed no evidence of immunological rejection, and they found There were no significant differences between the mechanical properties of the allografts and the autografts.

Synthetic materials such as nylon, carbon fibers and silicone have also been employed but they can cause an inflammatory reaction or an

antigenic response leading to failure at fixation sites and deficiencies in long-term biocompatibility (Alva et al., 2013)

Clinical conditions may preclude such autogenous tendon grafting due to:

1. Patients do not want to sacrifice their own normally functioning tendons.
2. Multiple tendon defects require more donor tendons than a patient can offer (Xie and Tang, 2012)

All tendon grafts, whether autogenous or allogenic, undergo a similar process of integration with graft necrosis, revascularisation, cell repopulation and remodeling (Jackson et al., 1996).

2.5 Platelet-rich plasma (PRP)

Platelet-rich plasma (PRP)—a component of whole blood containing powerful, transforming growth factors—has garnered worldwide attention as a possible biologic method to treat tendinosis and tendon tears, as well as many other challenging conditions. (Kajikawa et al., 2008)

PRP is defined as a platelet concentration higher than the physiologic platelet concentration found in healthy whole blood (Foster et al., 2009) . Some authors have adopted a more objective definition of five times the platelet concentration of whole blood (Marx et al., 1998). The ability to concentrate platelets allows a higher concentration of the bioactive growth factors reported to promote healing (Lee et al., 2011)

Platelets are small anucleated cytoplasmic fragments of megakaryocytes that are commonly thought of as the responsible agents for hemostasis. Although the platelet is central to the coagulation cascade, it is also essential to tissue healing. The first step of the healing process is clot formation and platelet activation (Wroblewski et al., 2010).

After platelet activation, many growth and differentiation factors are released from the α-granules, which are storage units found in platelets (Blair and Flaumenhaft, 2009). Ninety-five percent of the existing factors are released within 10 minutes of clot formation, whereas the rest of the growth factors are released as they are formed over several days (Marx, 2004; lee et al., 2011).

PRP is an autologous blood product centrifuged to produce a concentrated platelet product with numerous bioactive molecules and growth factors, including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), fibroblast growth factor, platelet-derived growth factor (PDGF), transforming growth factor (TGF) b, and epidermal growth factor (EGF) (Baksh, et al., 2013; Tohidnezhad et al., 2011; Everts et al., 2006).

The complex interactions of these growth and differentiation factors, along with adhesive protein factors such as fibronectin and vitronectin, are what is responsible for the healing response; promoting the long regenerative process of chemotaxis, cell proliferation, removal of tissue debris, angiogenesis, extracellular matrix formation, osteoid production, and collagen synthesis (lee et al., 2011).

Wound healing involves an intricate process that is often categorized into three overlapping phases: inflammation, proliferation, and remodeling (Fig. 2-1). Once tissue injury occurs, a hematoma forms at the site of tissue damage, platelets adhere to exposed collagen creating a clot, and the inflammatory phase begins with activation of platelets resulting in release of growth, bioactive, and hemostatic factors (Everts et al., 2006; lee et al., 2011)

In 2009, Mc Carrel et al. hypothesized that the growth factors and other bioactive molecules present in PRP have the potential to augment

cellular migration, proliferation, angiogenesis, and matrix deposition in tendon healing.

Chronic tendon injury, such as tendinopathy, frequently has histopathological features of collagen fiber disruption, mucoid degeneration, neovascularization, and absence of inflammation (Paoloni et al., 2011).

Numerous animal models have demonstrated the positive effects of these growth factors, both individual and synergistic, in enhancing cellular migration and proliferation, angiogenesis, and matrix deposition in tendon and wound healing (McCarrel and Fortier, 2008).

In general, PRP is avoided when there are signs of local inflammation or infection or if there is a history of malignancy. The patient is also educated with regard to shortterm and long-term expectations. Because the injection of PRP induces local inflammation, pain should be expected after the procedure. Nonsteroidal antiinflammatory drugs are also avoided 2 weeks prior and at least 2 weeks after the procedure so as to not inhibit the effects of growth factors and the healing response. Although the follow-up protocol may vary, physical therapy at least 2 weeks after the procedure is often considered (Lee et al., 2011).

The working definition of PRP is 1,000,000 per μL platelet count, which is five times the normal concentration found in whole blood (Marx, 2001; Weibrich et al., 2002).

In 2008, Kajikawa et al. hypothesized that Circulation-derived cells play a crucial role in the healing processes of tissue. In early phases of tendon healing processes, circulation-derived cells temporarily exist in the wounded area to initiate the healing process and decrease in number with time. They assumed that a delay of time-dependent decrease in circulation-derived cells could improve the healing of tendons. They injected platelet-

rich plasma (PRP) containing various kinds of growth factors into the wounded area of the patellar tendon, and compared the effects on activation of circulation-derived cells and enhancement of tendon healing with a control group (no PRP injection). The findings suggest that locally injected PRP is useful as an activator of circulation-derived cells for enhancement of the initial tendon healing process.

For acute ligament and tendon injuries, there may be an argument for the use of PRP to synergistically assist the inflammatory cascade and regenerative processes in healing injured tissue. With chronic injuries, Particularly tendon injuries with no, or minimal, inflammatory component, the rationale for use of autologous blood products, including PRP, is less clear. However, as is recognized in treating ligament and tendon injuries, the healing pathways are extremely complex and not fully understood with regard to stimulatory, inhibitory, and regulatory influences on healing (Kajikawa et al., 2008).

In acute tendon injury models in animals, there is evidence that circulation-derived cells, such as macrophages and fibroblasts, are present in the early stages of tendon healing and decrease with time. Plateletrich plasma enhanced the number of fibroblasts in tendon, and the amount of collagen synthesis. Platelet-rich plasma has been demonstrated to increase the synthesis of VEGF and HGF within cultured human tendon cells and as such may provide both an angiogenic response and a cellular proliferative response (Paoloni et al., 2011).

Platelet-derived growth factor contributes to cell proliferation, whereas transforming growth factor-b1 increases collagen synthesis but inhibits cellular proliferation possibly through the increase in VEGF and reciprocal decrease in HGF in cultured human tendon cells (Anitua et al., 2007).

There is evidence that single PRP injection significantly increased tendon force to failure, stiffness, and ultimate stress in the early stages of tendon healing in rats; however, this effect seems to abate with time in both patellar tendon and Achilles tendon (Paoloni et al., 2011; Aspenberg and Virchenko, 2004)

Fresh allograft tissue is unsuitable for implantation because it is highly immunogenic and tissue typing is impractical. The processes of fresh-freezing, freeze-drying or cryopreserving allograft tissue significantly reduce the immunogenicity of the tissue by killing fibroblasts within it. This removes the loci for the major histocompatibility antigens, allowing allografts to be used in immunologically incompatible hosts without provoking a significant immune response (Arnoczky et al., 1986; Robertson et al., 2006).

Cadaver tissue (carcass) has the advantage that multiple allografts may be obtained at any time within the first 24 hours following death and that clean rather than sterile conditions are required. The main disadvantages are the need for additional tissue processing because of an increased risk of disease transmission, and that any significant delay in harvest of the graft may increase the risk of clostridial infection (Malinin et al., 2003; Robertson et al., 2006).

In 2015, Wong et al. reported the use of tendon graft has diminished as regimes repairs and rehabilitation have improve, but they remain important in secondary reconstruction, and they discuss some of the future directions in tendon reconstruction by grafting, biomaterials and cell based therapies.

2.6. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in the bone marrow and having capacity to differentiate towards osteoblasts, chondroblasts, adipocytes and some other cell types. The potential plasticity and self-renewal capacity of MSC offer a huge potential

for clinical tissue regeneration. Some clinical trials using MSC have given encouraging results (Väänänen, 2005).

Bone marrow mesenchymal stem cells are the first reported and mostly used stem cell type to help tendon repair (Chen et al., 2013).

Source of MSCs is not only bone marrow, but also other adult tissues such as fat, hair follicles and scalp subcutaneous tissue, periodontal ligament, thymus and spleen, as well as pre-natal tissues, such as placenta, umbilical cord blood, fetal bone marrow, blood, lung, liver, spleen (Krampera et al., 2006).

Tendon disorders have traditionally been treated by reducing inflammation, restoring flexibility, and, if necessary, performing surgical repair. When inflammation (tendinitis) and degeneration (tendinosis) cannot be resolved, primary repairs, autografts, allografts, xenografts, resorbable biomaterials, and even synthetic polymers have been attempted, but with varying success. (Juncosa-Melvin et al., 2005)

Failure of tendon regeneration following degeneration and damage has encouraged the search for biological, mechanical and surgical therapies to improve the strength and structure of healing tendons. There is increasing interest in the application of stem cells to enhance tendon healing (Chaudhury, 2012).

The inability of tendon to self-repair and the inefficiency of current treatment regimens used clinically have sparked the exploration of alternative treatment strategies. The use of stem cells to repair tendon is particularly exciting and promising as stem cells have the potential to differentiate into tenocytes, show high proliferative and synthetic activities, and can secrete paracrine factors and exhibit immunomodulatory effects to promote tendon regeneration. However, a number of challenges have to be

overcome before they can be used as a safe and effective therapeutic option for promoting tendon repair (lui, 2015).

Stem cells can simply be defined as a cellular population with the ability to self-replicate through mitosis to form daughter cell lines, which have the potential to terminally differentiate into a number of different cell lineages. The most common stem cell sources are embryonic, perinatal (from the umbilical cord or amniotic tissue) or somatic adult cells (Chaudhury, 2012).

A more novel source of stem cells are induced pluripotent stem cells (IPSCs) which are initially mature adult cells that have undergone *in-vitro* modulation and obtained the characteristics of adult stem cells, such as pluripotency (Takahashi et al., 2007; Chaudhury, 2012).

The majority of orthopaedic related stem cell research to date has focused upon adult stem cells rather than embryonic or perinatal stem cells, as the latter are associated with numerous regulatory and ethical constraints. Induced pluripotent stem cells (IPSCs) are a relatively new field that has generated a great deal of interest. Adult stem cells are predominantly either mesenchymal stem cells or haematopoietic stem cells. Distinction between these different stem cell populations is based upon their surface markers, although some overlap has been reported (Chaudhury, 2012).

Mesenchymal stem cells (MSCs, which are sometimes also referred to as mesenchymal stromal cells) are defined by three specific characteristics. Firstly they are multipotent cells that are capable of differentiating into a number of daughter cell lines such as chondrocytes, osteocytes and adipocytes. Secondly, MSCs are able to adhere to plastic. Thirdly, they present stem cell specific antigens on their surface (Chaudhury, 2012).

The majority of MSCs utilized for orthopaedic applications are obtained from bone marrow tissue as these cells are relatively easy to access and provide relatively high numbers of MSCs compared to other sources. The iliac crest is the most common site for MSC harvesting, although a number of other sources have been identified. MSCs can be successfully aspirated while reaming long bones using a reamer-irrigation-aspirator (RIA), with comparable differentiation potential to iliac crest derived MSCs and superior numbers of total cells and colony forming units (Cox et al., 2011; Chaudhury, 2012).

Sources of autologous adult stem cells include iliac crest bone marrow aspirate and fat cells. Stem cell transplantation from these sources requires a two-stage procedure. In the first stage the marrow is aspirated for processing in the laboratory. The cells are isolated and then passaged several times to increase the number of cells to the required 2 to 4 million cells needed for clinical transplantation. Cells are usually used after two passages. This processing takes about two weeks to complete in the laboratory (Cox et al., 2011).

The transplantation of cells can only be done in a second stage after the first stage produces the "cell pellet". It is pertinent to note that the passaging of cells can lead to bizarre cell formation and apoptosis.[5,6] Since fresh cells could be obtained from RIA without the need for cell expansion, these stem cells are less likely to develop bizarre cell formation and apoptosis – a distinct advantage of RIA (Cox et al., 2011).

Cell suspensions derived from bone marrow or other tissues are the normal sources of MSCs. Cells may be expanded with complete medium in culture plates or flasks, where they adhere, start proliferating and form fibroblastic-like cell clusters (fibroblast colony forming units, CFU-F),

whose number depends on MSC clonogenic potential of the sample . Before cells become confluent, they are split and expanded in larger flasks, thus becoming a more and more homogenous adherent cell population that may proliferate without differentiating up to 40 generations (Krampera et al., 2006).

MSCs have been initially identified in bone marrow as nonhematopoietic stem cells that may differentiate into tissues of mesodermal origin, such as adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes and visceral stromal cells (Krampera et al., 2006).

Therapeutic application of adult mesenchymal cells are approaching clinical use also in several fields other than bone and cartilage regeneration. Promising new tissue targets among other seem to be, for instance tendon, ligament, menisci and other connective tissues (Väänänen, 2005; Krampera et al., 2006).

Different signals can direct MSCs to mobilize and differentiate into cells of connective tissue lineages. Such signals might include damage in the tissue including trauma, fracture, inflammation, necrosis and tumors. Chemotaxis and local microenvironment can also play a role in the fate of MSCs (Pountos and Giannoudis, 2005).

A number of studies have suggested that MSC treatment may improve both the volume and quality of regenerated tendons, with a greater propensity to heal via the generation of fibrocartilagenous tissue rather than scar tissue. MSCs treatment for lower limb tendons have resulted in improvements in a number of studies (Chaudhury, 2012)

Induction of MSC differentiation into connective tissues other than bone and cartilage, such as tendons and ligaments, has been investigated for a potential clinical application. Several animal studies and human

clinical trials have been carried out to evaluate the efficiency of MSC local injection in tendon repair (Ringe et al., 2002).

BM-MSCs are an excellent candidate for tissue engineering and cell therapy due to its high proliferation, multilineage differentiation, tissue homing and immunomodulatory properties, hence they are being explored to regenerate damaged tissue instead of just repairing them. (Mohapatra et al., 2015)

Chapter Three

Materials and Methods

3. Materials and methods

3.1 animals

The study was conducted on 15 apparently healthy shall (lamb), weighting between 18-20 kg, and aging 4 months. These were administrated Ivermectin(1) 1% 2mg/kg subcutaneously and Levamezol(2) (2.5 mg/kg) orally, and were maintained under similar housing, management, feeding and watering conditions

Animals were randomly divided in to 3 groups, 5 slamb each.

1. Control group
2. Platelet rich plasma treated group
3. Mesenchymal stem cells treated group

The observations were made up to 2 months after tendon repair. Various aspects studied were:

1. Clinical observations;
2. Ultrasonography;
3. Tensile strength;
4. Hydroxylproline test
5. Histopathology

3.2 Tendon allograft

The allograft SDFT were collected from Slaughter house in restricted aseptically conditions. All tendons were separately rinse in normal saline and aluminum folly packaged in sealed 0.5 micrometer polyethylene and nylon bags (triple protection) and were transferred in ice-box to Refrigerator and were kept cryopreserved at -20°C for 45 days .

On the day of grafting, the tendons were Thawed after kept 6 hours

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(1) . Razak laboratories Co. Tehran Iran.

(2). Shanghai Tongren Pharmaceutical Co.,Ltd. China (tablet 150 mg)

in room temperature and immerse in Normal Saline in 0.9 %NaCl solution for 30 minutes (figure 3-2 (1))(Robertson et al., 2006).

3.3 Autologous bone marrow derived mesenchymal stem cell preparation:

Bone marrow of lambs was collected After light anesthesia had been induced; anterior iliac crests were prepared with use of sterile technique (Mareschi, et al 2006).

1 cm stab incision was made in the skin facilitate passing the needle through the skin. A bone marrow aspiration needle was advanced into the intramedullary cavity at a site directly at the greatest prominence of the cranial iliac crest, parallel to the long axis of the wing of the ilium. The obturator was removed, and a ten-milliliter syringe containing one milliliter of heparinized (1000 units per milliliter) normal saline solution was fixed to the needle. Negative pressure was established by drawing the plunger back to approximately the six-milliliter marker until marrow began flowing into the syringe; the pressure then was reduced, and the appropriate volume of marrow was collected for three to six seconds (figure 3-2 (2)) .

The syringe was detached and was inverted several times to ensure complete mixing. Five-mL marrow aspirates were harvested in heparin (about 200 units/mL final) from the iliac crest. Incision was sutured with 1 simple interrupted suture (figure 3-2 (4)).

Aspirates were pooled (figure 3-2 (3)) and shipped at 4°C in thermic containers via overnight delivery to the cell culture facility and processed the following morning. The bone marrow aspirate was immediately transported to on-site laboratory.

3.4 Pre-operative preparations

All animal were injected a week before with ivermectin 50 mg /kg body weight and kept off feed before starting the study. The palmar aspect of the metacarpus was clipped, shaved and washed.

3.5 Anesthesia

We used Xylazine(1) (0.2 mg/kg) to achieve light to heavy sedation, Followed by Ketalar (Ketamine(2) 2-5 mg/kg IV) for induction. The endotracheal tube (ET) is inserted, secured and the cuff inflated. General anesthesia was safely maintained with inhalants. Isoflurane(3) was used after injectable induction. The patient was placed as its poll is higher than its nose so that excess saliva and regurgitated material drain out of the mouth(figure 3-2 (5)).

3.6 Surgical operation for grafting

Surgery was performed under general anaesthesia in both forelimbs. Animals were cast in lateral recumbency as described earlier. Surgical incision was made in the palmar aspect of the metacarpus 7-8 cm, then sub cutaneous tissue. The superficial flexor tendon was exposed easily. Stay sutures were placed on both proximal and distal end of the tendon (figure 3-2(6)). We removed a 5 cm of SDFT and replaced by allograft 5cm tendon allograft (figure 3-3 (1, 2)). The locking loop suture pattern (modified Kessler) was used; Non-absorbable suture material (nylon) was used for tendon repair (figure 3-3 (3, 4)) (Strickland, 1985). Swaged-on needles were helpful in limiting tissue trauma during suturing, and then we closed the incision with usual manner (figure 3-3 (6)).

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(1). Xylazin 2 %, Interchemie werken "De Adelaar" B. V. metaalweg 8 venray, Holland.

(2). Ketamine HCL 10 %, alfasan WOERDEN- Holland.

(3). Isoflurane, Aesica Queenborough L td., Queenborough L td., Kent, ME 115 EL, UK.

3.6.1 Group 1 (control)

Sheep in this group were not given MSCs or PRP in allograft tendon.

3.6.2 Group 2 (MSCs)

Primary cultures of MSCs were washed with PBS and trypsinized with trypsin/EDTA. The cells were suspended in normal saline at a density of 10×10^6 /ml medium and loaded into 10 ml sterile syringes. For each animal, we prepared approximately $20\text{-}50\times10^6$ cells which were subsequently taken to the hospital in a cold (0-4˚C) box in 1-2 ml of normal saline. Each animal received the MSC suspension at the allograft site. We injected MSCs solution in the site of allograft tendon and in proximal and distal of tendon suturing site (figure 3-4 (1)), and then we closed the incision with usual manner.

3.6.3 Group 3 (PRP)

PRP was obtained from samples of animal's blood drawn at the time of surgery. Blood samples collected in Anticoagulant Citrate Dextrose (ACD) tubes and should be inverted 5-10 times for proper mixing of the anticoagulant and blood. The blood draw occurred with the addition of an anticoagulant, to prevent platelet activation prior to its use.

Blood were stored at 1 - 6° C until processing was done. Blood samples were immediately transported to process on-site in laboratory. The PRP was returned in combination with autologous conditioned serum ACS for intra-lesional injection.

In the PRP method, an initial centrifugation to separate red blood cells (RBC) is followed by a second centrifugation to concentrate platelets, which are suspended in the smallest final plasma volume.

A sample of the animal’s blood was withdrawn and processed in a centrifuge to separate the red blood cells from the plasma. The plasma

contains the platelets and some of the white blood cells. The platelets were then further concentrated into a smaller volume of plasma and the end result is PRP.

We injected PRP in allograft tendon site and in proximal and distal suture sites of the tendon (figure 3-4 (1)) and then we closed the incision with usual manner.

Post-surgery, ultrasonographic tissue characterisation images were made for comparative quantitative evaluation of tendon structural integrity.

3.7 Postoperative care

In each group, Fiber glass cast was used for immobilization after surgery for 3 weeks (figure 3-4 (2)). During this period we changed the cast and examined the surgical wound every 5 days. Then we applied a Robert Jones bandage for a week to rehabilitate moving the leg gradually (allows gradual loading of the tendon).

Animals were transferred to the animal shed. When the animals completely recovered from anesthesia, they were made to sit in sternal recumbency. Later, they were helped to stand up. Food and water were offered. Watch was kept till they got themselves balanced (figure 3-4 (3)).

Animals were injected antibiotics combination antimicrobial (Procaine Benzylpenicillin 8 mg/ kg and Dihydrostreptomycin 10mg/kg)(1), Gentamycin(2) Flunixin Meglumine 5% (3) 2.2 mg/kg B. W. and Vitamin B Complex(4) for 7 days after surgery and examined clinically 8 weeks post-surgery.

……

(1). Norbrook Laboratories limited. Ireland

(2). Razak Laboratories Co., Km 10, Karaj, Makhsous Rd., Tehran.

(3). Aburaihan Pharma Co. Veterinary Division. Tehran. Iran.

(4). Razak laboratories Co. Tehran Iran.

3.8 Harvesting of tendons

After 2 month of surgery; tendons were immediately harvested after sacrifice, each animal one specimen was taken in formaldehyde10% for histopathology and the rest of tendons were wrapped with saline-soaked gauze, and frozen in labeled plastic bags at _20°C. for tensile strength and hydroxylproline.

3.9 Parameter investigated:

3.9.1 Clinical observations

In all animals general condition and mobility were observed (table 3-1)). Rectal temperature was recorded for a week postoperatively. The animals were observed for the posture and position they adopted while sitting, standing, balancing or walking. Weight bearing, direction of the limbs, coordination, and the gait of animals were noticed daily.

Table 3-1: Scoring system for lameness (Angell et al., 2015)

Score No.	Name of score	Description
0	Sound	Bears weight evenly on all four feet and walks with an even rhythm.
1	Mildly lame	Steps are uneven but it is not clear which limb or limbs are affected
2	Moderately lame	Steps are uneven and the stride may be shortened; the affected limb or limbs are identifiable
3	Severely lame	Mobility is severely compromised such that the sheep frequently stops walking or lies down due to obvious discomfort. The affected limb or limbs are clearly identifiable and may be held off the ground while walking or standing.

3.9.2 Ultrasonography

Computerised ultrasonographic tissue characterization was performed on both forelimbs Post-surgery and after 2 months for comparative quantitative evaluation of tendon structural integrity and healing for both forelimbs in all animals (table 3-2).

Table 3-2: Variation in sonography for comparative quantitative evaluation of tendon

Fiber score	(Score 0-4) or (0, 25%, 50%, 75%, 100%) best-worse
Echogenicity score	(Score 0-4) or (0, 25%, 50%, 75%, 100%) best-worse
Cross sectional area	millimeter
Width	millimeter
Thickness	millimeter
Peri tendinous edema	**+** or – (exist or not)

Transverse and longitudinal ultrasonographic imaging planes were done by using Sonosite Micromaxx Ultrasonographic Machine (Sonosite Inc, USA) and linear trasducer (Micromaxx HFL/ 13-6 MHz, Sonosite Inc, USA).

3.9.3 Histopathology

After fixation in 10% neutral buffered formalin, all tissues were processed through a gradient of alcohols, embedded in paraffin blocks, sectioned, and stained with

1. Hematoxylin and Eosin
2. Masson Trichrome Stain

The following solutions were prepared for coloring

- Hematoxylin Wiegert`s solution: the same volumes of solution Hematoxylin Wiegert`s A and B were mixed before use, we discarded the used solution

Table 3-3: Histopathological score of superficial digital flexor tendon allograft (Bürgisser et al., 2016; Stoll et al., 2011)

Pathologic characteristics	Score 2	Score 1	Score 0
ECM (extracellular matrix)organization of whole tendon	Wavy, compact and parallel arranged collagen fibers	In part compact, in part loose or not orderly	Loosely composed, not orderly (granulation tissue)
Cellularity/cellmatrix-ratio	Physiological	Locally increased cell density	Increased cell density and decreased ECM content
Cell alignment	Uniaxial	Areas of irregularly arranged cells (10–50%)	More than 50% of cells with no uniaxial alignment
Cell distribution	Homogeneous, physiological	In some areas increased cell density	Focal areas of elevated cell density (cell clustering)
Cell nucleus morphology	Predominantly elongated, heterochromatic cell nuclei (tenocytes)	10–30% of the cells possess large, oval, euchromatic or polymorph heterochromatic nuclei	Predominantly larger, oval, euchromatic or polymorph heterochromatic nuclei
Organization of repair tissue of the tendon callus	Homogeneous (whole tissue with similar composition)	In some areas its heterogeneous tissue composition	Whole tissue composition has completely changed
Vascularisation in the defect area	Hypo-vascularized, (small capillaries) 40-10 pcs	Hyper-vascularized (Increase angiogenesis (70- 41)	Hyper-vascularized (increased small or larger capillaries)(71-100)
Inflammation	Inflammatory cell infiltration	Increase inflammatory cell penetration	Severe infiltration of various types of inflammatory cells, including neutrophils, macrophages, lymphocytes, etc.

- Phosphomolybdic – phosphotungstic acid solutions: the same volumes of solutions, Phosphomolybdic–phosphotungstic acid solutions were mixed before use and we discarded the used solution.

Staining method

1. Slides are Deparaffinized and hydrated to distal water.
2. Slides were put in a Bouin's Solution for 1 hour at 56 ° C. and, were washed in running tap water for removing yellow color from sections.
3. The sections were rinsed in distal water.
4. staining was done with 10 minutes of Hematoxylin Wiegert`s solution.
5. Sections were washed in slow-moving tap water for 10 minutes, and were rinsed in distal water.
6. staining was done with 2 minutes of Biebrich Scarlet-Acid Fucshin
7. The sections were rinsed in distal water.
8. In Phosphomolybdic – phosphotungstic acid solutions for 10-15 minutes.
9. Slides were placed in Aniline Blue Solution 5 minutes, and were rinsed in distal water.
10. In Acetic Acid 1% for 3-5 minutes.
11. Sections were dehydrated through alcohol 95% (absolute), and were cleared in xylene (three times)

Results of Masson Trichrome staining:

- Collagen: Blue
- Cytoplasm, Keratin, Muscle and intracellular fibers: Red
- Nuclei: Black (Martinello et al., 2015)

3.9.4 hydroxyproline concentration

Collagen and cross-links were analyzed by determination of hydroxyproline Samples were collected as mentioned before and their hydroxyproline concentration was measured by a modified spectrophotometer method.

The samples were hydrolyzed in 6 molar HCl at 105°C for 14-16 hr. and hydroxyproline was oxidized by chloramines T, then by using Ehrlich's reagent and incubating at 60°C, a chromophore was formed. To remove

interfering chromophores, hydroxyproline product in alkaline media was extracted into toluene and then into acid phase.

The absorbance of acid phase was read at 543 nm and hydroxyproline content was calculated from calibration curve based on standard solutions run as the same as samples. In order to determine the percentage of the dry matter (DM) in each tendon sample, 50-100 mg of each sample, and concurrent with sampling for hydroxyproline analysis were placed on a plate and dried at 100°C in an oven for 3 hr. Finally hydroxproline content of tendon samples were expressed in mg/g DM (Sharifi et al 2007).

3.9.5 Tensile strength:

In all animals, the full length of SDFT was collected to be subjected to test of tensile strength using Zwick/Roell MDTL Machine (biomechanical analyzer)(figure 3-4 (4)) speed of 0.07 mm sec−1 and Proportional Integral Deferential(PID)controller. The both cut ends of each tendon were grasped and tightly secured with dentition compansment (Sharifi et al., 2009).

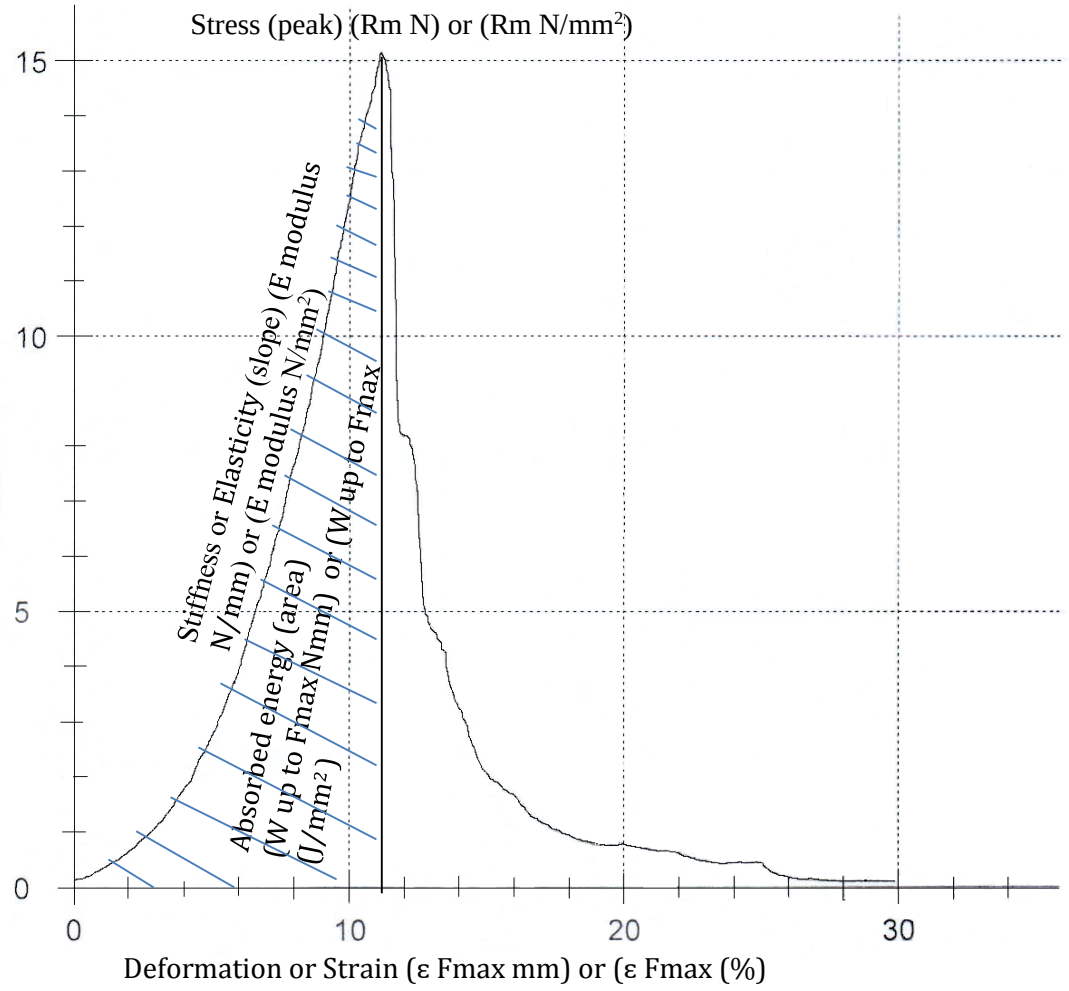
Table 3-4: biomechanical study parameter for evaluatiing tensile strength of tendon

Parameter of tensile strength	
Stress (peak)	Rm (N) Rm (N/mm^2)
Stiffness (slope) (elasticity)	E modulus (N/mm) E modulus (N/mm^2)
Deformation (strain)	ε Fmax (mm) ε Fmax (%)
Absorbed energy (area)	W up to Fmax (Nmm) W up to Fmax (J/mm^2)

Structural and mechanical properties were determined from the force-elongation and stress-strain curves, respectively. Linear stiffness and modulus were calculated in the linear region of the force-displacement and stress-strain curves, respectively. Tangent stiffness was computed as the slope of the force-displacement curve all along the normal and repair curves up to peak *in vivo* forces (figure 3-1) (table 3-4) (Juncosa-Melvin et al., 2005).

3.10 Statistical analysis

Results were expressed as mean values _ standard errors. Data was statistically analyzed by One-Way ANOVA with multiple comparison tests using statistical software program (SPSS for windows version 20, USA) Differences were considered significant at ($P \leq 0.05$).



Nr	σ Low N/mm²	σ Low N	σ High N/mm²	σ High N	E-Modulus N/mm²	E-Modulus N/mm	RB N/mm²	RB N	ε Fmax. %	ε Fmax. mm	ε Break %
1	0.99	6.06	1.48	9.09	4.30	2.05	-	-	86.69	11.15	-

Nr	ε Break mm	W up to Fmax. Nmm	W up to Fmax. J/mm²	W up to break Nmm	W up to break J/mm²	Rm N	Rm N/mm²
1	-	55.77	0.01	-	-	15.15	2.47

Figure 3-1: showing tensile strength test parameters have to analyze

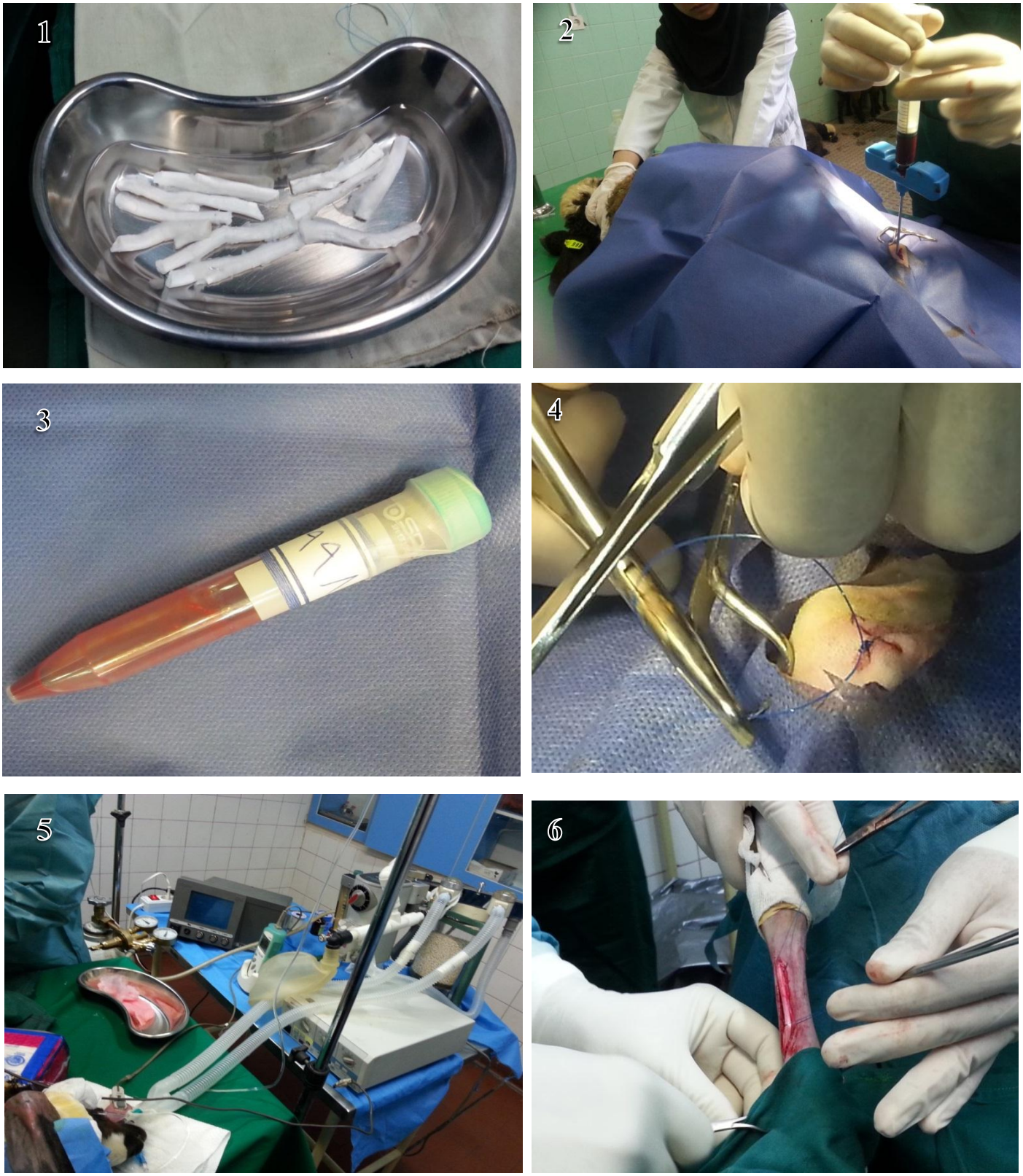


Figure 3-2: (1) Allograft tendons were immersed in Normal Saline in 0.9 %NaCl solution for 30 minutes before grafting. (2) Bone marrow aspirate from the iliac crest of a sheep. (3) Bone marrow aspiration. (4) Incision suture. (5) Lamb under control of inhalation anesthesia. (6) SDFT exposed in surgical incision in the palmar aspect of metacarpus.

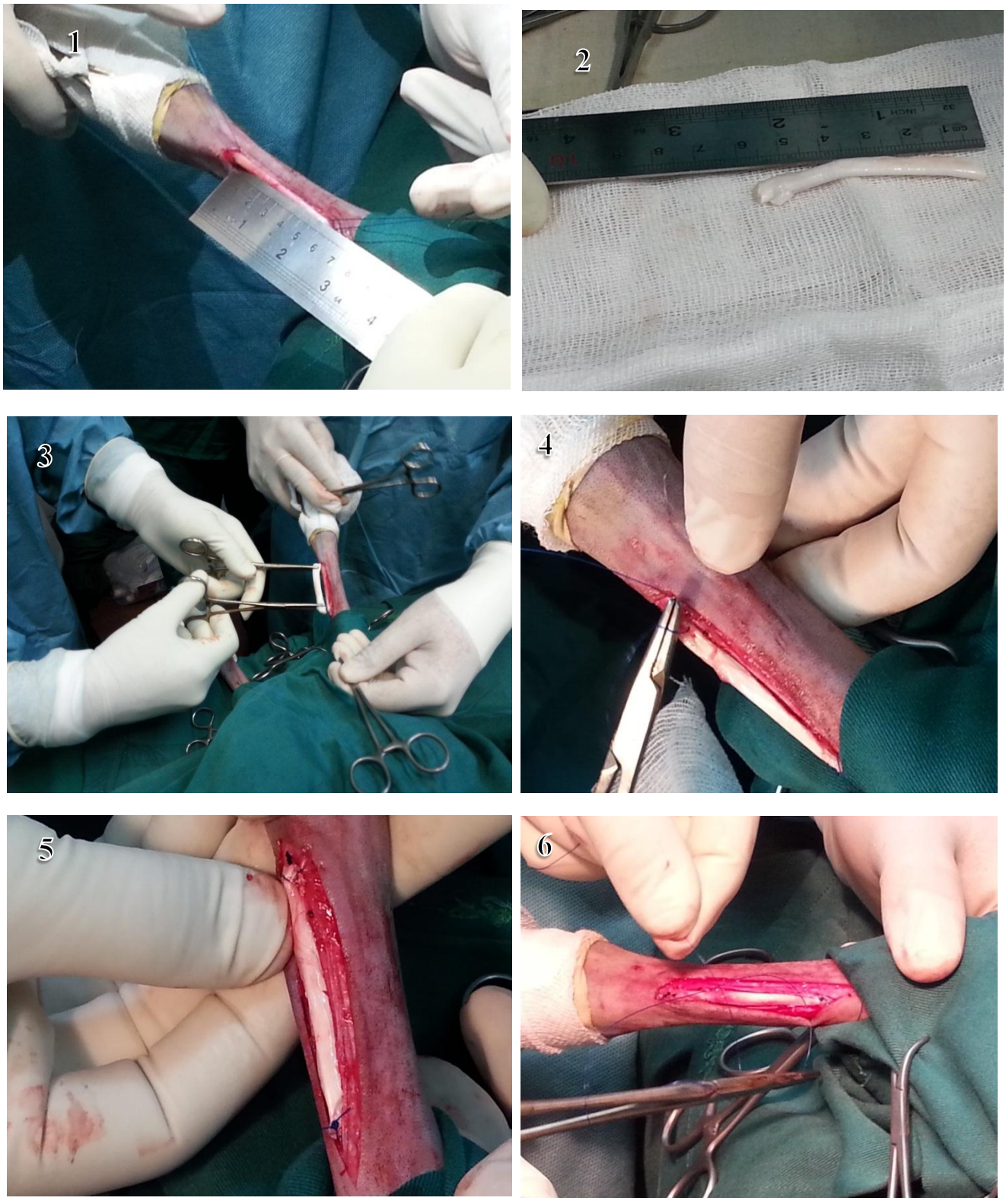
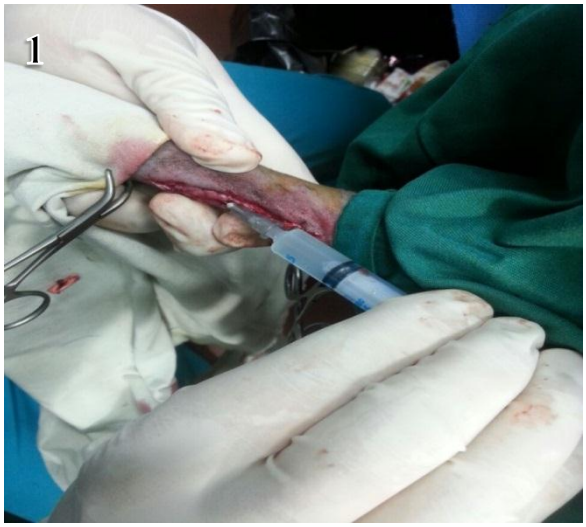
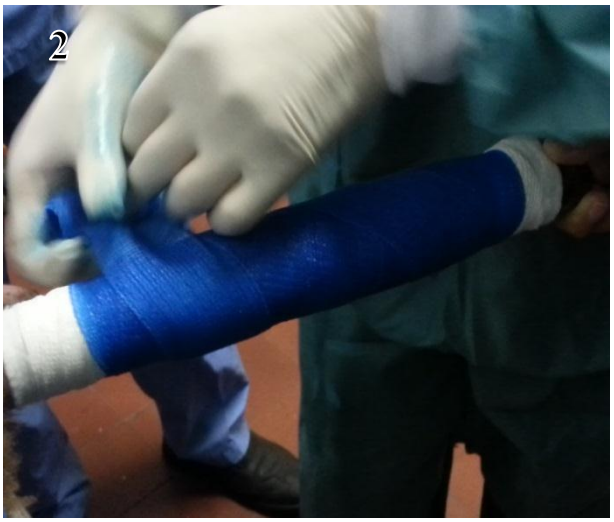


Figure 3-3: (1) Measuring 5 cm of SDFT before removal. (2) Measuring 5cm of allograft tendon for replacement. (3) Parallel measure the allograft tendonbefore amastomosis. (4) Modified kessler suture pattern was used for anastomosis. (5) Complete covering SDFT gap with allograft tendon. (6) Subcutanuous tissue was sutured in continuous paterrn.





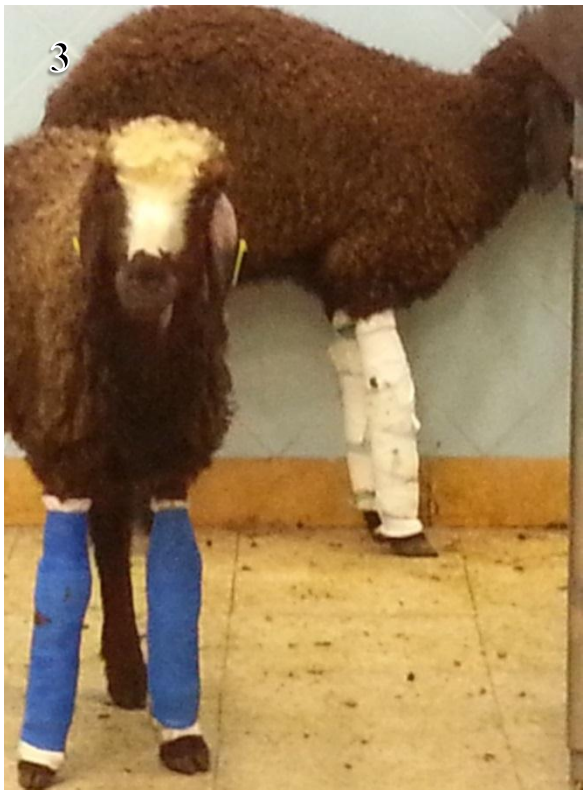


figure 3-4: (1) Infiltration of MSCs solution in allograft and site of anastomosis of the grafted tendon. (2) Application of fiberglass cast for full limb treated. (3) Full-limb cast for better stability of grafted tendon. (4) Biomechanical device used for assessment tensile strength of tendon. Machine data: 2.5 H1S WN: 150888/ Cross head travel monitor WN: 150888/ Force Sensor ID: 0 WN: 150889 2 kN. Tarbiat Modares University, Faculty of Medical Science, physical therapy department, Tehran. “Dr. Tarkumani and Dr. Banna Sadeq”

Chapter Four
The results

4.1 Clinical evaluation

During period of this experiment, general health status was assessed daily, and remained well. Sheep were not recumbent for longer durations than before the surgical intervention. After removal of the cast on day 15 after surgery, the grafted limbs in all the lambs clinically showed the same degree of lameness. Lameness was assessed on a hard surface only. During beginning of third weeks but gradually improved in the treated group at the end of third week, which showed to have normal weight bearing after month (figure 4-1, table 4-1). The swelling at the site of operation was less severe in the treated group by end of 3rd weeks. Animals had full limbs weight bearing in MSCs and PRP groups in being of 5th weeks. The skin wounds had healed by that time.

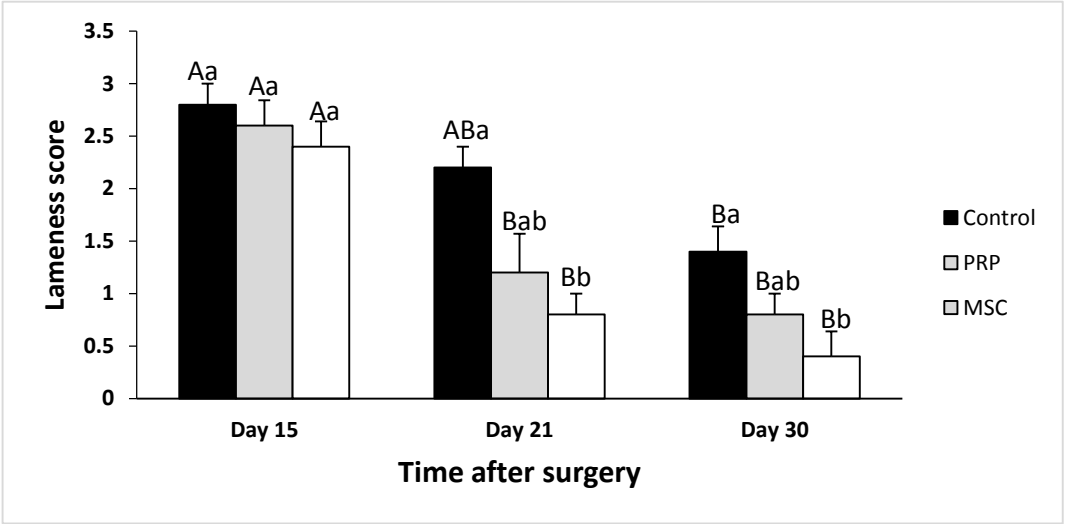


Figure 4-1: Lameness scoring study between the 3 groups (control, PRP and MSC group) after surgery in the days 15, 21, 30. ^{AB}Different letters within group indicates significant differences ($P<0.05$). ^{ab}Different letters among groups indicates significant differences ($P< 0.05$).

Table 4-1: Lameness scoring study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

groups	Day 15	Day21	Day 30
control	2.8 ± 0.20^{Aa}	2.2 ± 0.12^{ABa}	1.40 ± 0.24^{Ba}
PRP	2.6 ± 0.24^{Aa}	1.2 ± 0.37^{Bab}	0.80 ± 0.20^{Bab}
MSCs	2.4 ± 0.24^{Aa}	0.8 ± 0.2^{Bb}	0.40 ± 0.24^{Ba}

^{ABC}Different letters within each row indicates significant differences ($P<0.05$). ^{ab}Different letters within each column indicates significant differences ($P<0.05$).

4.2 Ultrasonography results:

After 60 days of surgery, there is significant increase in Cross sectional area in MSCs treated group tendons, but in thickness, there is no significant difference between PRP and MSCs groups. In fiber score there is significant decrease in PRP group. However, in Width and Echogenicity score, there is no significant difference between all groups. In same group; after surgery and after 60 days of surgery, there is significant difference in Echogenicity and fiber score(figures 4-2, 3, 4, 5, 6, 7) (tables4-2, 3, 4, 5, 6, 7) ($P< 0.5$), Figures sonography 4-8, 9, 10 in control, PRP and MSC groups, Improvement of the tendon structure.

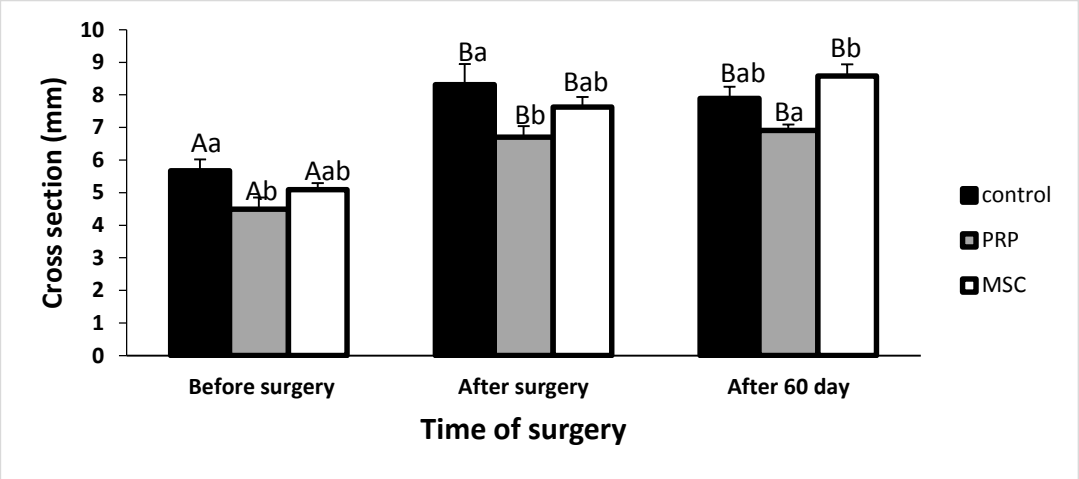


Figure 4-2: ultrasonography study, cross sectional area for control, PRP and MSCs groups, before, after surgery and after 60 days of surgery. ^{AB}Different letters within group indicates significant differences ($P<0.05$). ^{ab}Different letters among groups indicates significant differences ($P<0.05$).

Table 4-2: CSA study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

Type of group (CSA)	Before surgery	After surgery	After 60 dayes
Control group	5.67 ± 0.34^{Aa}	8.31 ± 0.36^{Ba}	7.88 ± 0.36^{Bab}
PRP group	4.49 ± 0.35^{Aa}	6.70 ± 0.33^{Bb}	6.90 ± 0.17^{Ba}
Mesenchymal group	5.09 ± 0.19^{Aab}	7.62 ± 0.31^{Bab}	8.57 ± 0.35^{Bb}

^{AB}Different letters within each row indicates significant differences ($P< 0.05$). ^{,ab}Different letters within each column indicates significant differences ($P< 0.05$).

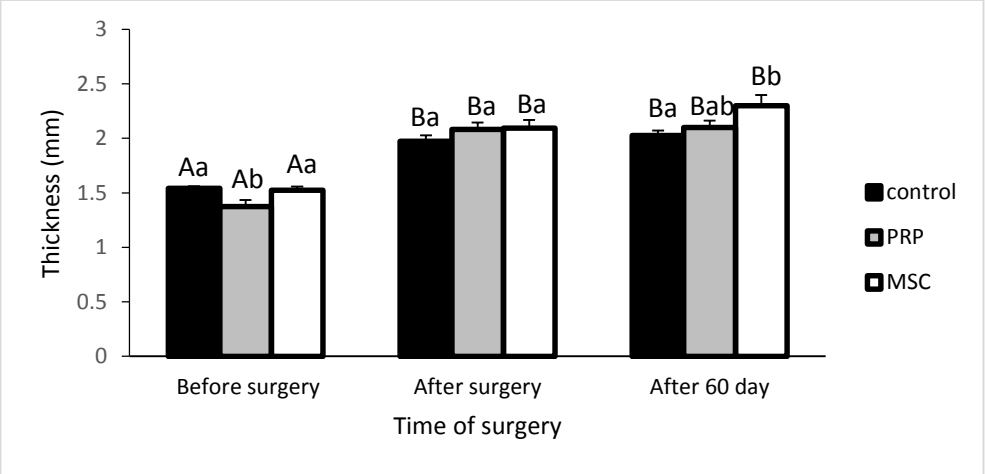


Figure 4-3: ultrasonography study, thickness of tendon for control, PRP and MSCs groups, before, after surgery and after 60 days of surgery. ^{AB}Different letters within group indicates significant differences (P< 0.05). ^{ab}Different letters among groups indicates significant differences (P< 0.05).

Table 4-3: thickness of tendon study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

Type of group	Before surgery	After surgery	After60 dayes
Control group	1.54±0.01^{Aa}	1.97±0.05^{Ba}	2.02±0.04^{Ba}
PRP group	1.37±0.06^{Aa}	2.08±0.06^{Ba}	2.10±0.06^{Bab}
Mesenchymal group	1.52±0.03^{Aa}	2.09±0.07^{Ba}	2.29±.09^{Bb}

^{AB}Different letters within each row indicates significant differences (P< 0.05). ^{ab}Different letters within each column indicates significant differences (P< 0.05).

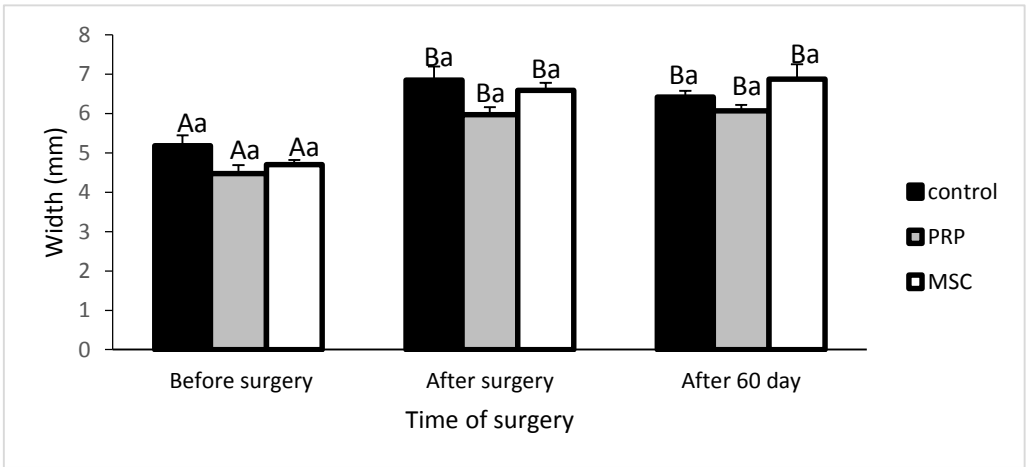


Figure 4-4: ultrasonography study, width of tendon for control, PRP and MSCs groups, before, after surgery and after 60 days of surgery. ^{AB}Different letters within group indicates significant differences (P< 0.05). ^{ab}Different letters among groups indicates significant differences (P< 0.05).

Table 4-4: width of tendon study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

Type of group	Before surgery	After surgery	After 60 days
Control group	5.18±0.25^{Aa}	6.84±0.34^{Ba}	6.41±0.16^{Ba}
PRP group	4.47±0.20^{Aa}	5.97±0.18^{Ba}	6.06±0.15^{Ba}
Mesenchymal group	4.69±0.12^{Aa}	6.58±0.18^{Ba}	6.87±0.37^{Ba}

^{AB}Different letters within each row indicates significant differences (P< 0.05).
^{ab}Different letters within each column indicates significant differences (P< 0.05).

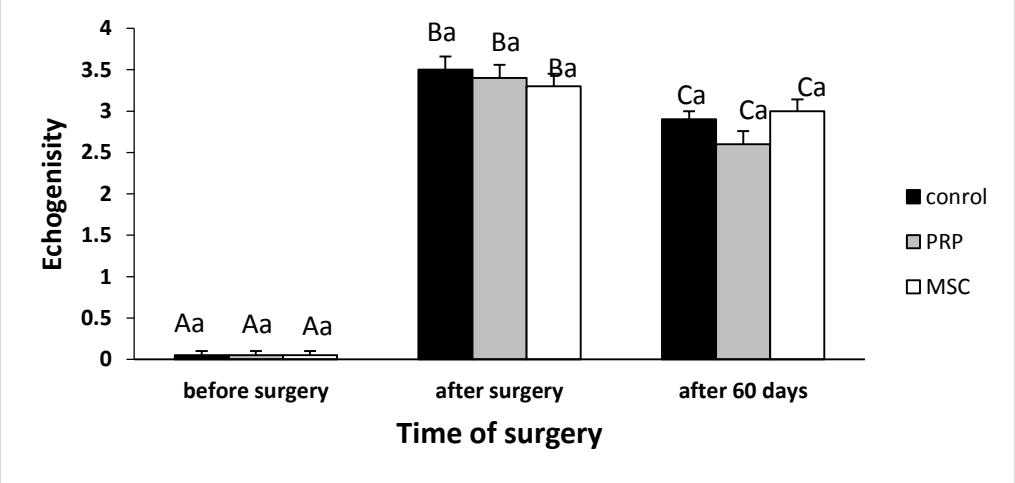


Figure 4-5: ultrasonography study, Echogenicity of tendon for control, PRP and MSCs groups, after surgery and after 60 days of surgery. ^{AB}Different letters within group indicates significant differences (P< 0.05). ^{ab}Different letters among groups indicates significant differences (P< 0.05)

Table 4-5: Echogenicity of tendon study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

group	Before surgery	After surgery	After 60 days
control	0.00	3.50±0.16^{Ba}	2.90±0.10^{Ca}
PRP	0.00	3.40±0.16^{Ba}	2.60±0.16^{Ca}
MSCs	0.00	3.30±0.15^{Ba}	3.00±0.14^{Ca}

^{AB}Different letters within each row indicates significant differences (P<0.05).
^{ab}Different letters within each column indicates significant differences (P<0.05).

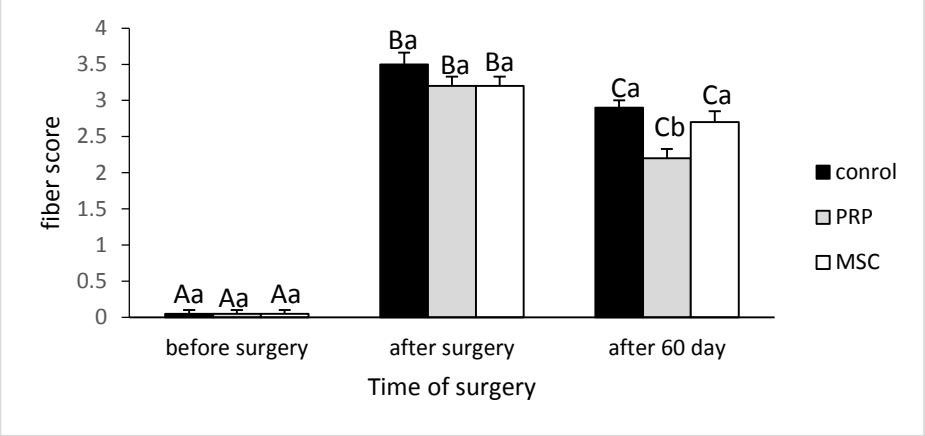


Figure 4-6: ultrasonography study, Fiber scores of tendon for control, PRP and MSCs groups, after surgery and after 60 days of surgery. ^{ABC}Different letters within group indicates significant differences (P< 0.05). ^{ab}Different letters among groups indicates significant differences (P< 0.05)

Table 4-6: fiber score of tendon study for control, PRP and MSCs groups, in different time of surgery (means and standard errors)

Group	Before surgery	After surgery	After 60 days
Control	0.00	3.50 ± 0.16^{Aa}	2.90 ± 0.10^{Ba}
PRP	0.00	3.20 ± 0.13^{Aa}	2.20 ± 0.13^{Bb}
MSCs	0.00	3.20 ± 0.13^{Aa}	2.70 ± 0.15^{Ba}

^{AB}Different letters within each row indicates significant differences (P< 0.05). ^{,ab}Different letters within each column indicates significant differences (P< 0.05).

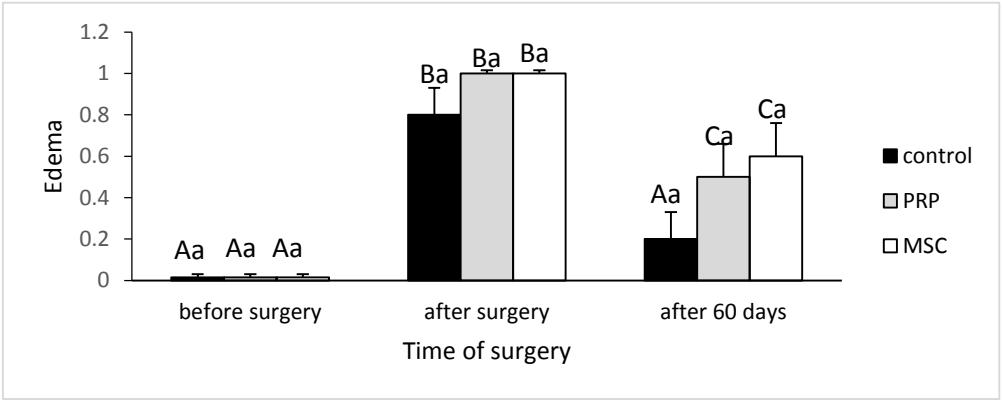


Figure 4-7: ultrasonography study, edema of tendon for control, PRP and MSCs groups, before, after surgery, and after 60 days of surgery. ^{ABC}Different letters within group indicates significant differences (P< 0.05). ^{ab}Different letters among groups indicates significant differences (P< 0.05).

Table 4-7: edema of tendon study for control, PRP and MSCs groups, in different time of surgery (means and standard errors).

groups	Before surgery	After surgery	After 60 days
control	0.00^{Aa}	0.80 ± 0.13^{Ba}	0.20 ± 0.13^{Aa}
PRP	0.00^{Aa}	1.00 ± 0.00^{Ba}	0.50 ± 0.16^{Ca}
MSCs	0.00^{Aa}	1.00 ± 0.00^{Ba}	0.60 ± 0.16^{Ca}

^{ABC}Different letters within each row indicates significant differences (P<0.05). ^{ab}Different letters within each column indicates significant differences (P< 0.05).

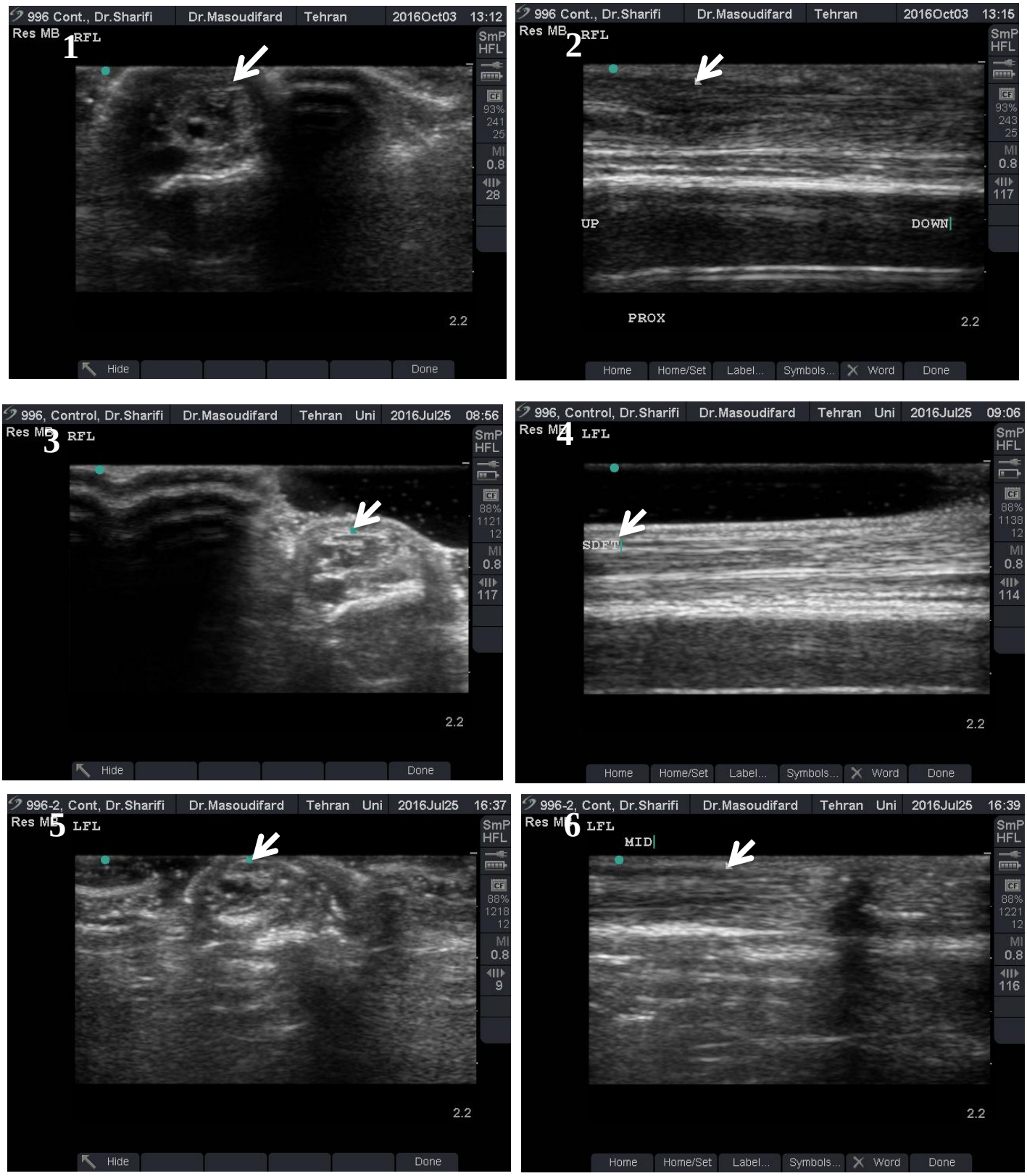


Figure 4-8: (1, 2): sonography figures for control group (sagittal and transverse) before surgery. Figure (3, 4): sonography figures right after surgery. Figure (5, 6): sonography figures at the 60th day after surgery.

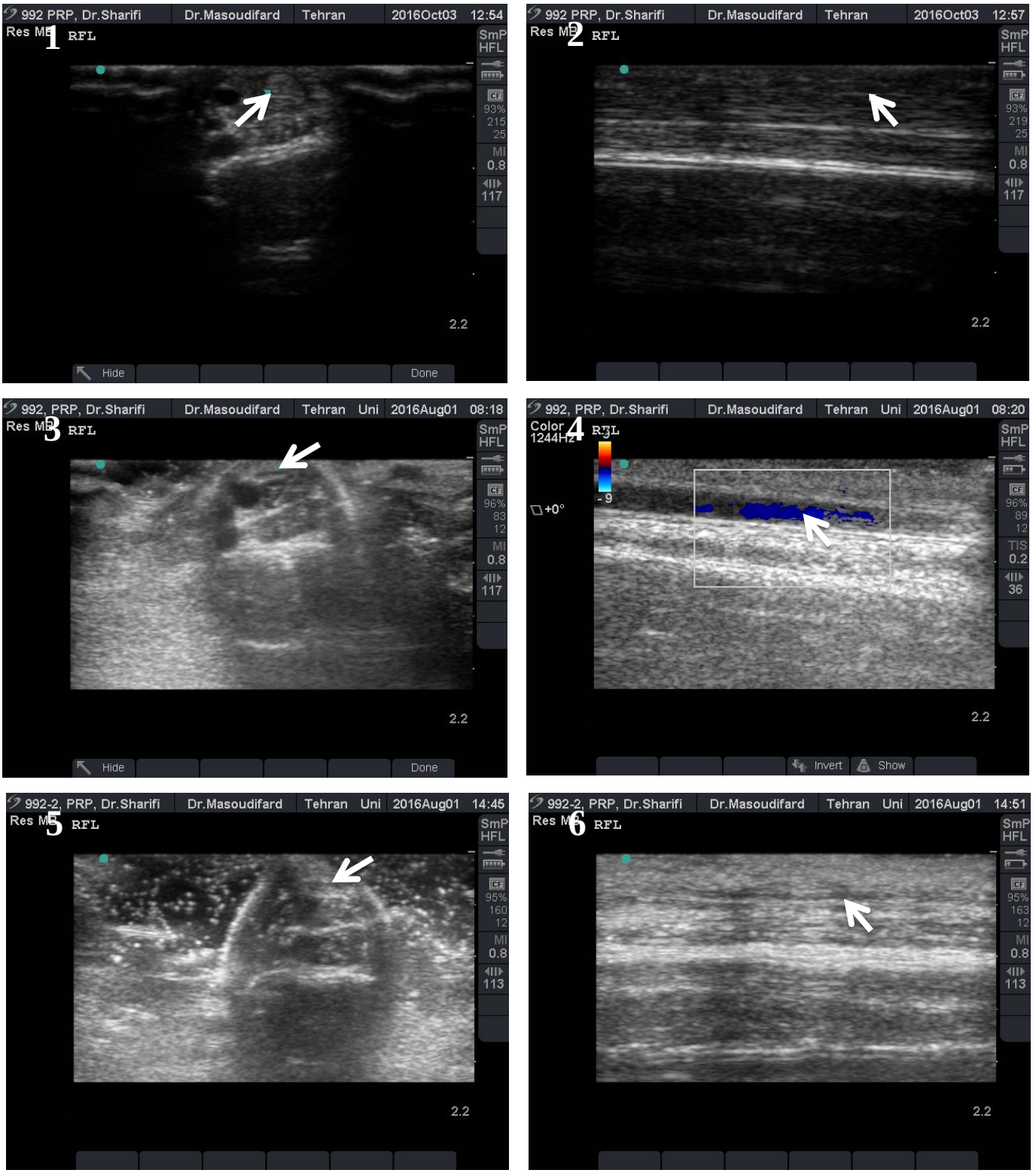


Figure 4-9: (1, 2): sonography figures for PRP group (sagittal and transverse) before surgery. Figure (3, 4): sonography figures right after surgery. Figure (5, 6): sonography figures at the 60th day after surgery.

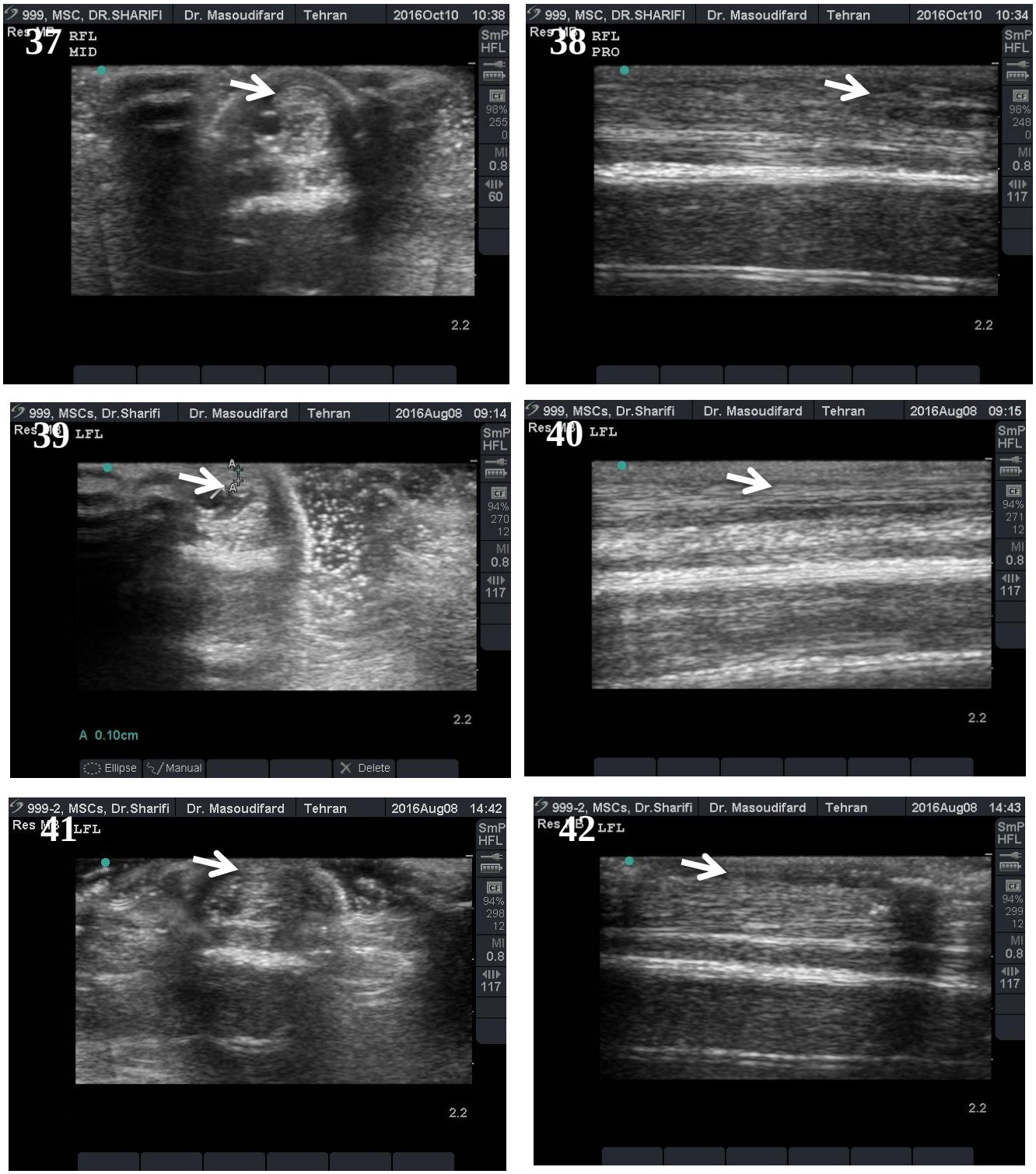


Figure 4-10: (1, 2): sonography figures for MSCs group (sagittal and transverse) before surgery. Figure (3, 4): sonography figures right after surgery. Figure (5, 6): sonography figures at the 60th day after surgery.

4-3Hydroxyproline Results: The main results of the study are summarized in table (4-8) and figure (4-11). The positive effect of MSc in third group with 134.322±2.123 mg/g dry matter As compared to normal 137.171±5.291 mg/g dry matter these two groups indicated the high amount of collagen for fiber orientation in early tendon union. Hydroxyproline content was very low in the control group 87.694±6.502 mg/g and up to some extend acceptable range in PRP group 99.116±1.839 mg/g dry matter.

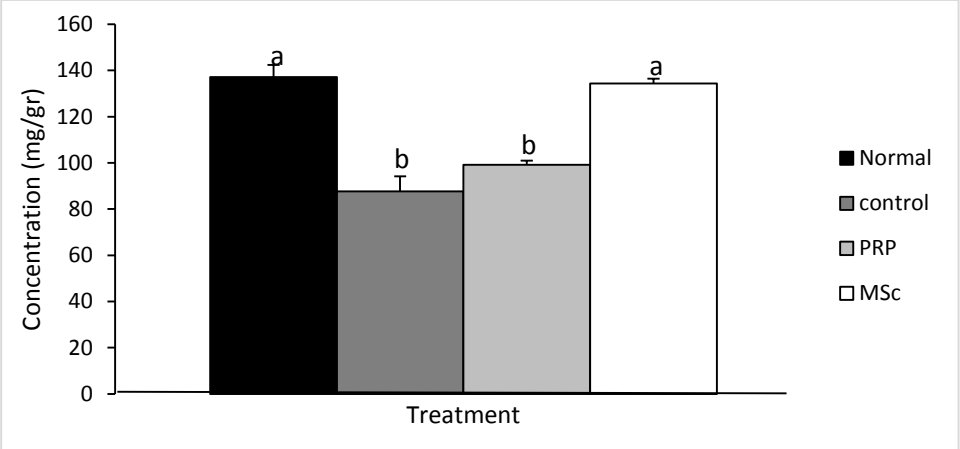


Figure 4-11: Hydroxyproline study of tendon for control, PRP and MSCs groups, after 60 days of surgery. [ab]Different letters among groups indicates significant differences (P< 0.05)

Table 4-8: Hydroxyproline study of tendon for control, PRP and MSCs groups, after 60 days of surgery (means and standard errors)

Type of group	After 60 days means and S.Er
Normal group	137.17 ±5.29 a
Control group	87.69 ±6.50 b
PRP group	99.11 ±1.83 b
Mesenchymal group	134.32 ±2.12 a

[ab]Different letters within each column indicates significant differences (P< 0.05).

4-4Biomechanical results:

In control group stress (Rm N) was recorded 11.42±3.44 N, whereas in PRP group was 19.11±6.20 and in the third group it was 31.98±5.06. Stiffness or Elastic modulus (N/mm) was recorded 1.89±0.90 in control group whereas in second group (PRP) 3.36±0.55 and in the third group 4.20±1.90. Deformation (εFmax mm) in control group was recorded 7.00±2.41 mm and 12.66±4.90 in second group (PRP) and the third group (MSc) 16.64±4.78mm, and the last parameter recorded absorbed energy (w upto Fmax Nmm), in control group was 84.54±38.32 and in PRP group 90.62±54.31 and 221.87±48.44 in the third group, which used MSCs.. Significant changes were observed in third group (P<0.05) compared with that of PRP and control groups (tables 4-9 to 4-16)

In Stress RMN and Absorbed energy, there significant increase in MSC group, but in RMN/nm^2, PRP group also increased. In the rest of parameters, there are slight increase values in MSC group but it is not significant (figures 4-12 to 4-19).

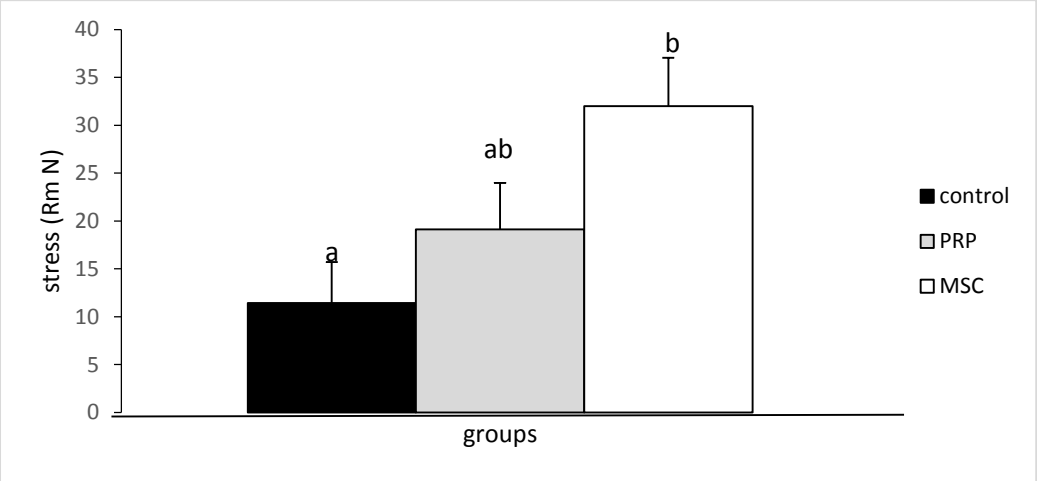


Figure 4-12: Tensile strength study showing stress (Rm N) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-9: Tensile strength study showing means and standard errors on stress (Rm N) test in control, PRP and MSC groups after 60 days of grafting

Group (stress Rm N)	Mean and standard error
Control	11.42±3.44[a]
PRP	19.11±6.20[ab]
MSC	31.98±5.06[b]

[ab]Different letters within column indicates significant differences (P< 0.05).

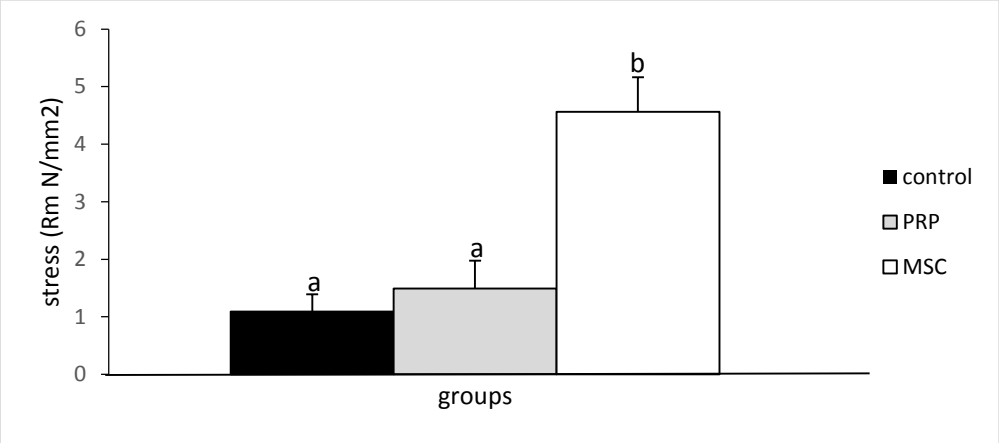


Figure 4-13: Tensile strength study showing stress (Rm N/mm^2) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-10: Tensile strength study showing means and standard errors on stress (Rm N/mm^2) test in control, PRP and MSC groups after 60 days of grafting

groups	Means and standard errors Rm N/mm^2
Control	1.09±0.30[a]
PRP	1.49±0.48[a]
MSC	4.56±0.60[b]

[ab]Different letters within column indicates significant differences (P< 0.05).

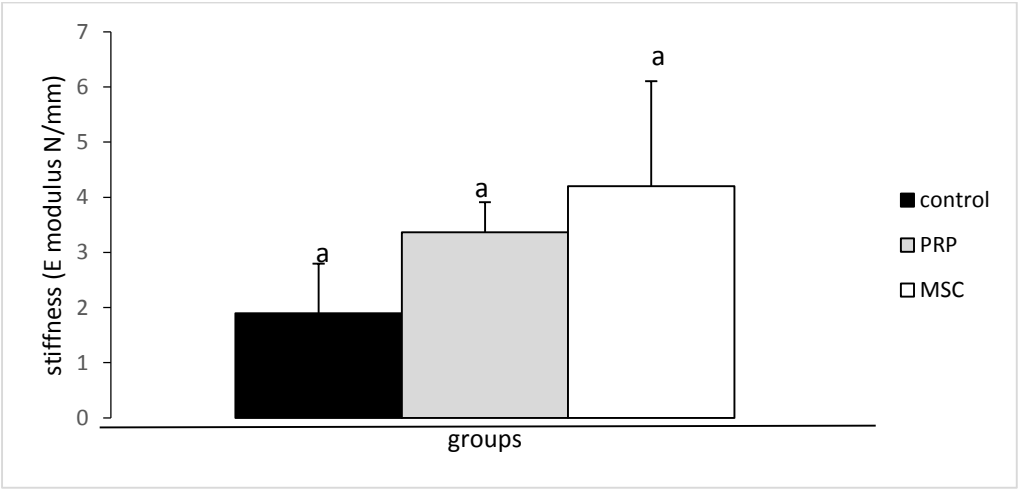


Figure 4-14: Tensile strength study showing stiffness (E modulus N/mm) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-11: Tensile strength study showing means and standard errors on stiffness (E modulus N/mm) test in control, PRP and MSC groups after 60 days of grafting

Group stiffness	Means and standard error N/mm
control	1.89±0.90[a]
PRP	3.36±0.55[a]
MSC	4.20±1.90[a]

[ab]Different letters within column indicates significant differences (P< 0.05).

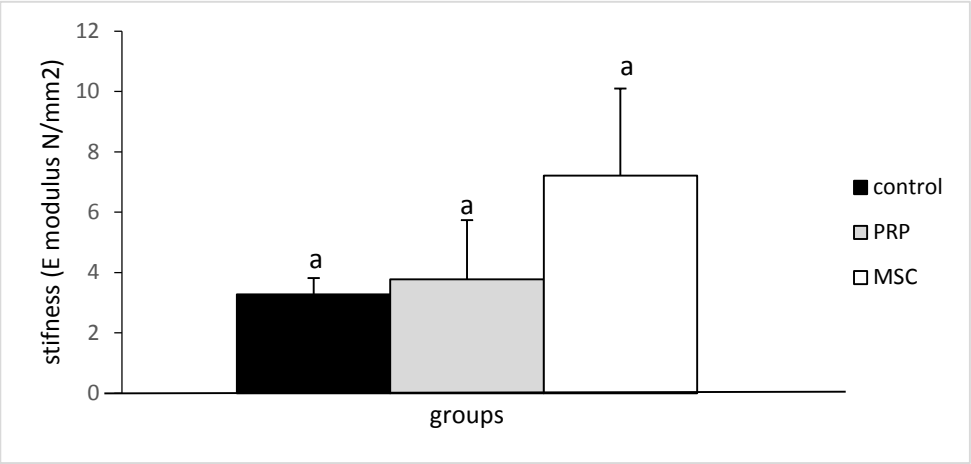


Figure 4-15: Tensile strength study showing stiffness or elasticity (E modulus N/mm^2) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-12: Tensile strength study showing means and standard errors on stiffness or elasticity (E modulus N/mm^2) test in control, PRP and MSC groups after 60 days of grafting

Group stiffness N/mm^2	Means and standard errors
control	3.27±0.54[a]
PRP	3.77±1.97[a]
MSC	7.21±2.89[a]

[ab]Different letters within column indicates significant differences (P< 0.05).

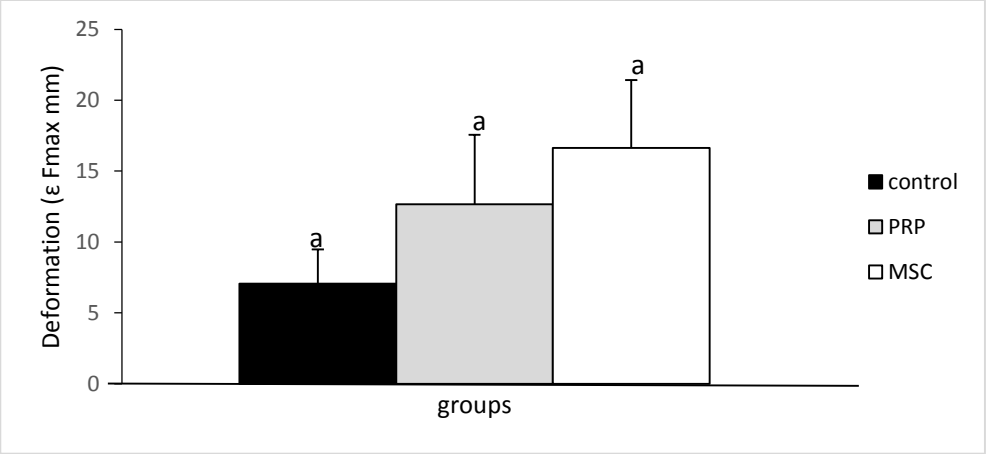


Figure 4-16: Tensile strength study showing deformation (εFmax mm) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-13: Tensile strength study showing means and standard errors on deformation (εFmax mm) test in control, PRP and MSC groups after 60 days of grafting

Group deformation ε Fmax mm	Means and standard errors
Control	7.00±2.41[a]
PRP	12.66±4.90[a]
MSC	16.64±4.78[a]

[ab]Different letters within column indicates significant differences (P< 0.05).

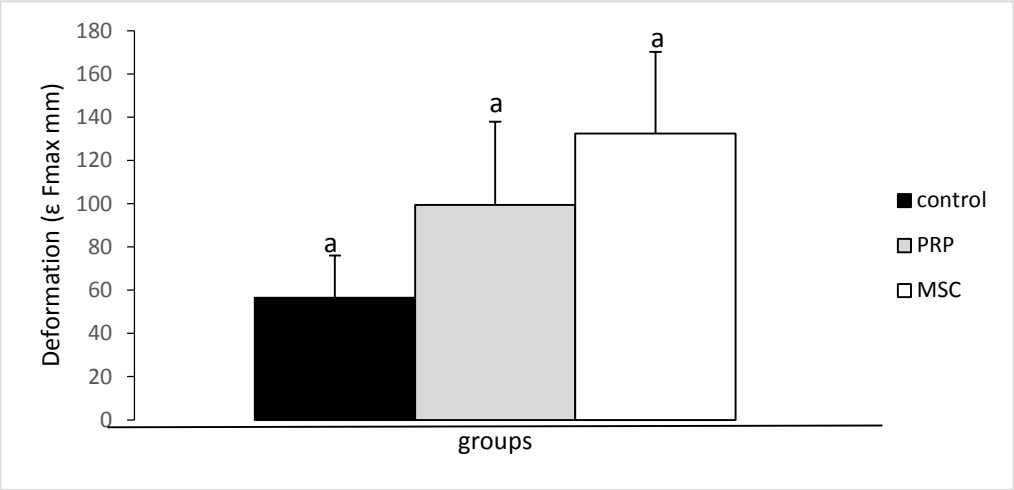


Figure 4-17: Tensile strength study showing deformation (εFmax %) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-14: Tensile strength study showing means and standard errors on deformation (εFmax %) test in control, PRP and MSC groups after 60 days of grafting

Group deformation ε Fmax %	Means and standard errors
Control	56.42±19.65[a]
PRP	99.47±38.50[a]
MSC	132.44±37.75[a]

[ab]Different letters within column indicates significant differences (P< 0.05

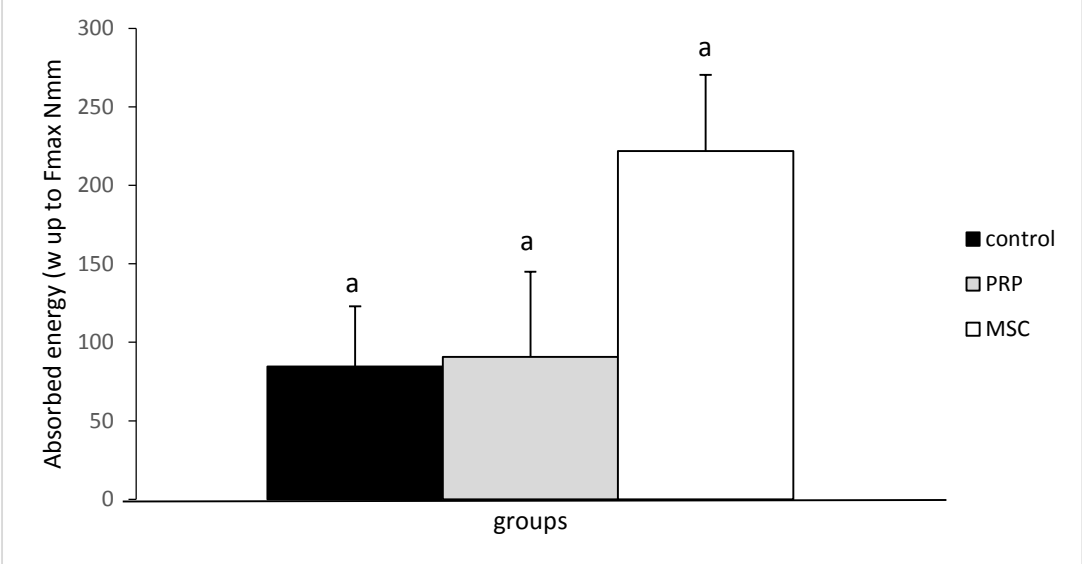


Figure 4-18: Tensile strength study showing absorbed energy (w upto Fmax Nmm) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-15: Tensile strength study showing means and standard errors on absorbed energy (w upto Fmax Nmm) test in control, PRP and MSC groups after 60 days of grafting

Group/absorbed energy Nmm	Means and standard errors
Control	84.54±38.32[a]
PRP	90.62±54.31[a]
MSC	221.87±48.44[a]

[ab]Different letters within column indicates significant differences (P< 0.05).

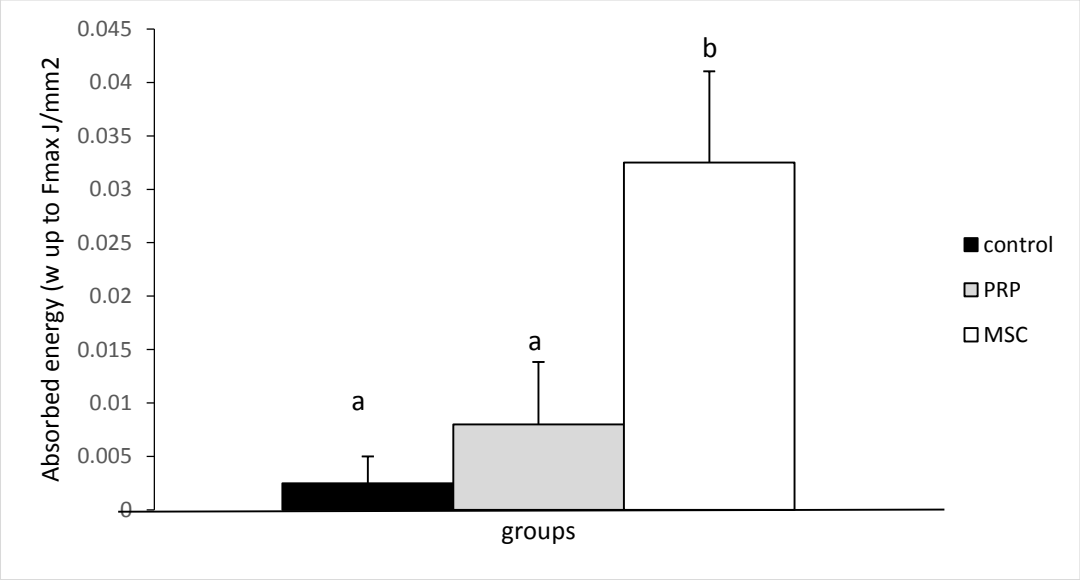


Figure 4-19: Tensile strength study showing absorbed energy (w upto Fmax J/mm^2) test in control, PRP and MSC groups after 60 days of grafting. [ab]Different letters among groups indicates significant differences (P<0.05).

Table 4-16: Tensile strength study showing means and standard errors on absorbed energy (w upto Fmax J/mm^2) test in control, PRP and MSC groups after 60 days of grafting

Group/absorbed energy J/mm^2	Means and standard errors
Control	0.0025±0.00250[a]
PRP	0.0080±0.00583[a]
MSC	0.0325±0.00854[b]

[ab]Different letters within column indicates significant differences (P< 0.05).

4-5 Histologic Findings:

Trichrome Masson staining was used for visualization of the SDFT tendon. Blue staining represents collagen in the tendon. The dark staining nuclei of the tendon fibroblasts are located in parallel rows flattened between collagen fibers.

The collagen fibrils (Figure 4-21, 4-24) were oriented longitudinally. The MSCs group showed very highly organized collagen fibrils and has been shown to increase the size and length of tenocytes, and increasing the proliferation rate and production of collagen types. There is presence numbers of blood vessels in the area. Furthermore. An active, good and similar regeneration and remodeling process was observed in MSC group (5/5) 100% cases without fibrous tissue formation. The surface of the regenerated tissue appeared smooth and regular, almost normal.

The PRP group showed almost longitudinal oriented collagen fibers as well as thick and increased size and length of tenocytes and fibers, but the collagen fibers in some parts showed crimps and abducted may be due to presence of edema and the newly formed blood vessels were seen but in few number and size without engorgement (figure 4-22, 4-25). The PRP group (5/5) showed encouraging results (3/5) 60% were similar to MSCs group but (2/5) 40% revealed fluctuate outcome from good to moderate regenerated and remodeling process depending on the thickness, arrangement, size of nuclei as well as formation of blood vessels.

The control group showed a smooth longitudinal orientation, but the fibers were thin and the tenocytes were small in size the nuclei were not so obvious like in the MSC and PRP groups as well as many blood vessels in the area (Figure 4-20, 4-23).

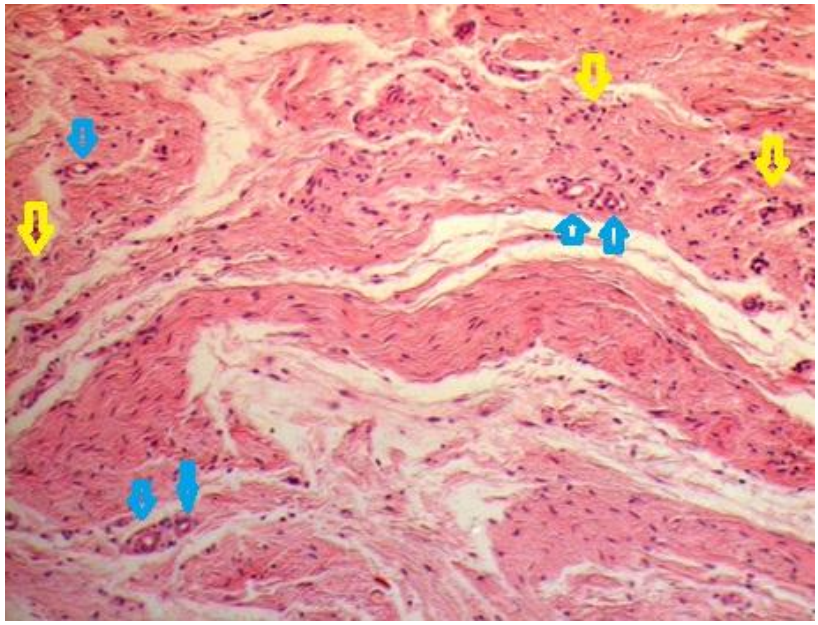


Figure 4-20: A histopathological section of control group showed intact fiber with black stained tenocyte fibroblast (yellow arrows) and formation of blood vessels (blue arrows). H. E. Stain 40X.

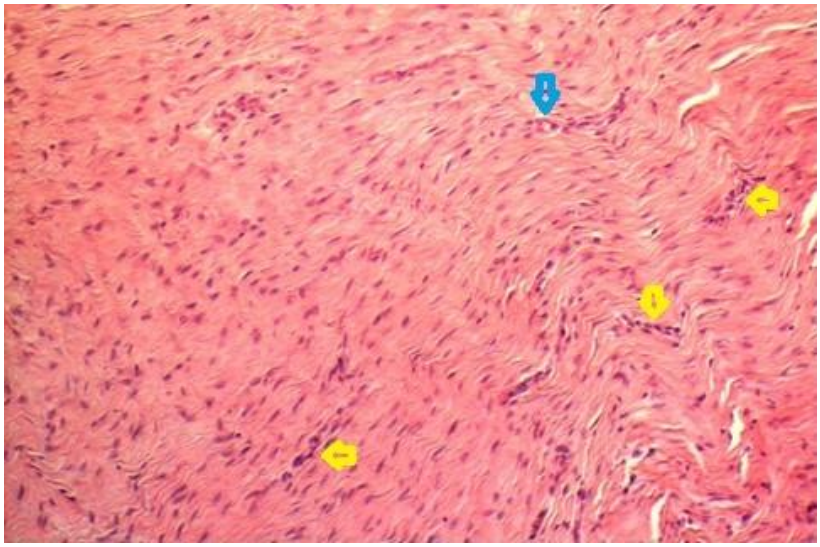


Figure 4-21: A histopathological section of MSCs group showed longitudinal oriented fibers and obvious black nuclei (yellow arrows) between fibers as well as the presence of some blood vessels (blue arrow). H. E. Stain 40 X.

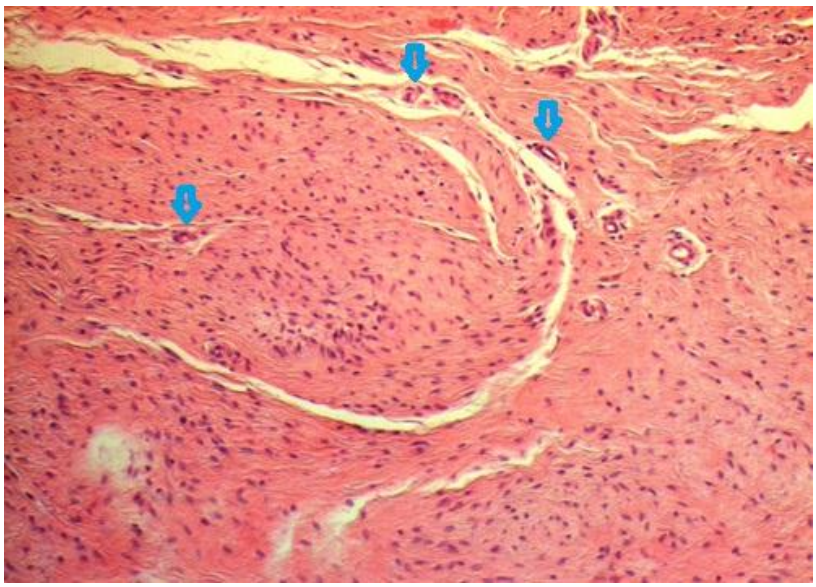


Figure 4-22: A histopathological section of PRP group showed similar histological characterization of MSC group, but newly formed blood vessels in the regenerated area (blue arrows). H. E. Stain 40X.

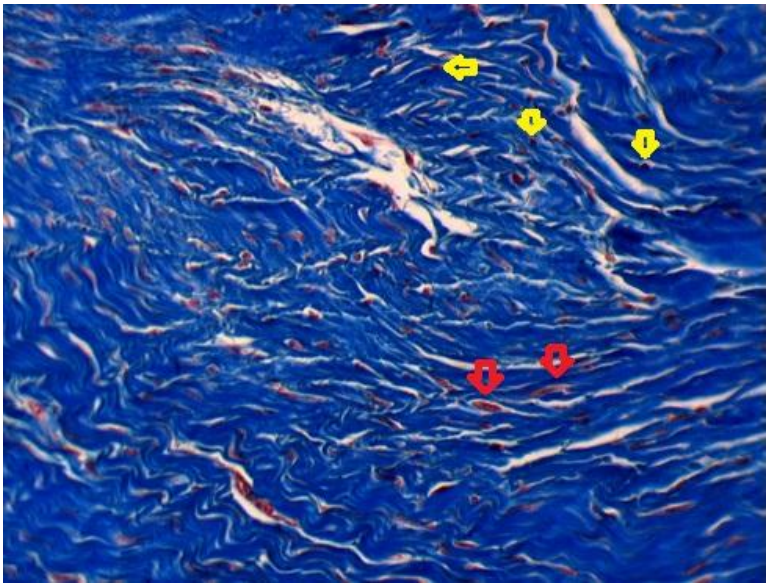


Figure 4-23: A histopathological section of the control group showed thin collagen fibers with small size fibroblast (yellow arrows) with formation of small blood vessels (red arrows). Masson Trichrome Stain 40X.

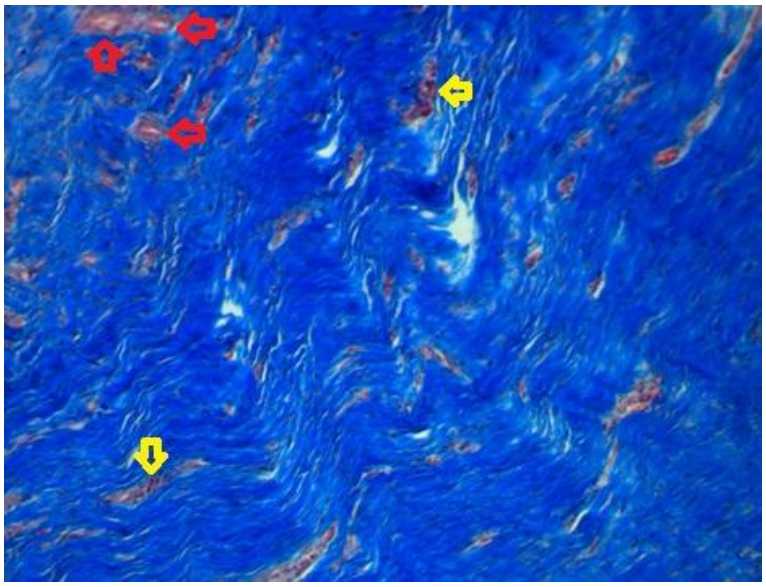


Figure 4-24: A histopathological section of MSCs group showed dark staining of nuclei of the tendon fibroblast are located in parallel rows flattened between collagen fibers (yellow arrow), blood vessels (red arrows). Masson trichrome stain 40X.

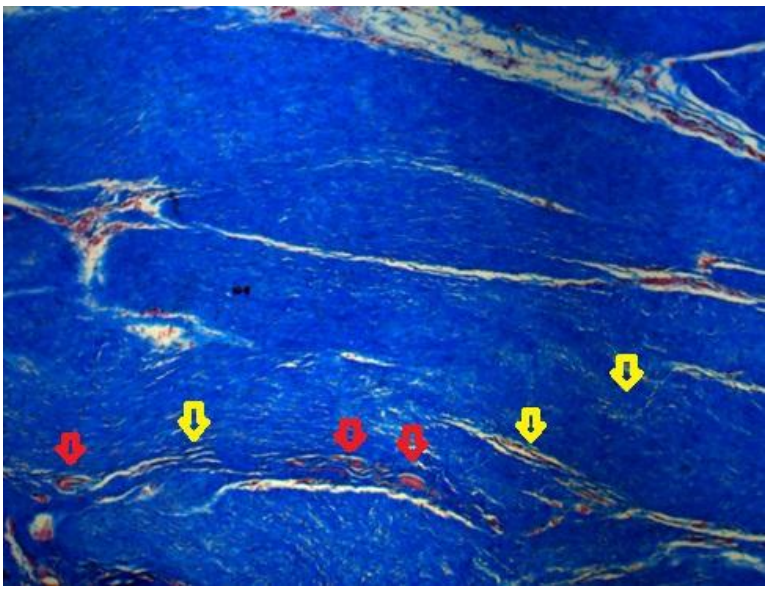


Figure 4-25: A histopathological section of PRP group showed longitudinal oriented collagen fibers, but there are some crimped fibers (yellow arrows) and there are some blood vessels (red arrows). Masson Trichrome Stain 40X.

Table 4-17: Histological scoring system

Pathologic characteristics / groups	ECM (extracellular matrix) organization of whole tendon	Cellularity/cell matrix- ratio	Cell alignment	Cell distribution	Cell nucleus morphology	Organization of repair tissue of the tendon callus	Vascularization in the area	Inflammation	Sum of points
Con1	1	1	0	1	1	1	0	1	6
Con2	2	1	1	1	1	1	1	1	9
Con3	1	1	0	1	1	1	0	1	6
Con4	1	0	0	1	1	1	0	2	6
Con5	1	0	0	1	1	0	1	1	5
PRP1	1	1	1	1	1	1	0	1	7
PRP2	1	1	1	1	1	1	1	1	8
PRP3	1	1	0	1	1	1	0	1	6
PRP4	1	1	1	1	1	1	0	1	7
PRP5	1	1	1	1	1	1	1	1	8
MSC1	1	1	1	2	2	1	1	1	10
MSC2	1	1	1	1	1	1	2	2	10
MSC3	1	2	2	2	2	1	0	1	11
MSC4	1	1	1	1	1	1	1	1	8
MSC5	1	1	1	1	1	1	1	1	8

According to Table 4-17 in the 60th day after surgery, the control group had less value than that in the other groups, but control group showed better status among the treatment groups in the characteristics of the organization of the extracellular matrix. However, in terms of the ratio of cell to matrix mesenchymal cell group, cellularity increased in compared to other groups. The matrix, Cell alignment, etc.; it was shown to be MSCs better than other groups, and the PRP group was better than control treated group. In addition, results showed little increase vascularization value in control and PRP treated group.

Chapter Five
Discussion & Conclusions

Discussion:

Tendon injuries are an important issue for orthopedic surgeons. There are obstacles on path to heal tendon injury. The blood supply to tendon is relatively poor giving their low metabolic rate, which results in slow healing after injury (Pastides and Khan, 2011). Healing does not lead to a normal surgical structure; healing occurs forming scar tissue whose quality is less than normal tendon. Therefore, a healed tendon does not have normal function and may suffer recurrent injury. (Sadegh et al., 2016)

Injuries of energy strong tendons (SDFT) are responsible for significant financial cost. It requires a better therapy to aid in the development of successful treatment regimens. A surgical models of a cute tendon injury was successfully developed and applied to sheep and horse energy strong tendons, this has potential for modeling tendon injury in horses and human (Tyelor, 2013)

It is more accurate and reliable experiment such as biomechanical testing is needed with a large animal model, such as sheep or dog, to obtain the results for judging the possibility of the use of MSC and PRP therapies to enhance the process of tendon reconstruction in human beings (Li et al., 2007).

There were a highly recorded lacerated tendons and acquired-congenital contracture of flexors tendons in the flock of sheep in the Veterinary Hospital in the Basra University. For studying basic mechanisms of frozen allograft tendon healing as well as therapeutic agents that could be translated to clinical care, the lamb model has been selected (Sharma and Maffulli 2006; Tomopoulos et al., 2015; Wu and Tang, 2013).

Tendons are dynamic tissues, and tendon pathologies significantly reduce quality of life. There are no effective pharmacological therapies

currently available to treat tendinopathies, and should tissue replacement be necessary (Youngstrom and Barrett, 2015). Tendon allografts play an important role in tendon reconstruction, particularly where there is a shortage of suitable available local tissue (Robertson et al., 2006).

Fresh allograft tissue is unsuitable for implantation because it is highly immunogenic and tissue typing is impractical. For rejection response, the process of fresh freezing allograft tissue significantly reduce the immunogenicity of the tissue (deep-freezing at –20"C, for 45) by destroying tenocyte cells minimize antigenicity within the tissue (Robertson et al., 2006).

The present study is for investigation ultrasonographical, hydroxyproline content, Biomechanical, and histological regeneration process of frozen allograft tendon in surgical procedure with replacement a 5 cm of superficial digital flexor tendon of lambs. The proposed treatments include MSC and PRP during surgery.

The majority of MSCs used for orthopedic applications are obtained from bone marrow tissue as these cells are relatively easy to access and provide relatively high numbers of MSCs compared to other sources. The iliac crest is the most common site for MSC harvesting, although a number of other sources have been identified (Chaudhury, 2012).

Before surgery, sheep were free of lameness, and ultrasonographic evaluation did not reveal any signs of present or previous tendon injuries.

After removal of the cast on day 15 after surgery, the grafted limbs in all the lambs clinically showed the same degree of lameness, which was almost identical due to similarity of the lesions. The lameness was mostly apparent during beginning of third weeks but gradually improved in the

treated group at the end of third week, which showed to have normal weight bearing after month. The swelling at the site of operation was less severe in the treated group by end of 3rd weeks. The result of the effect of PRP and mesenchymal cells at the site of attachment of allograft and the host tendon clinically reduced inflammatory process and lameness and also achieves local pain relief via indulging growth factors and accelerating tenocytes accumulation at the attachment sites (sharifi, 2007; sharifi, 2011; Bosch et al., 2010) .

It seems to be due to increase tenocyte proliferation and restoring tendon integrity gradually leading to restoration of biomechanical properties correlated to that of clinical signs of having full limbs weight bearing in MSc and PRP groups in being of 5th weeks. The clinical finding in this study provide the indirect evidences that PRP and MSCs therapy local infiltration promote healing at the site of attachment even using allograft tendon by increasing fibroblast tenoblast activity (Jackson et al., 1996).

Ultrasonography is one of the most accurate, well-tolerated and non-invasive tools to evaluate the tendon structure after an injury, follow-up the healing process, and establish prognosis (Mostafa et al., 2015; El-Shafaey et al., 2016).

Ultrasonography study showed a remarkable significant increase in Cross sectional area in MSCs treated group tendons. This is in agreement with the result reported by (Barreira et al., 2008) who affirmed that ultrasonographic difference in the mean values of the percentage of rupture collagen fibers in a cross sectional view occurred between the treatment and control groups after the administration of bone marrow derived mononuclear cells.

But, this is in contrast with the a study by (Fortier and Smith, 2007) who suggest that the implantation of Bone marrow derived MSCs did not

provoke worsening of the lesion or even tendon reaction, with no significant increase in tendon area in ultrasonographic examination.

In thickness, there is no significant difference between PRP and MSC groups but in fiber score there is significant decrease in PRP group. Collagen fiber proliferated more densely early after grafting and subsequent remodeling of the collagen fibers and approximation of normal tendinous tissue occurred earlier in PRP group than in other groups. Therefore, administration of PRP shortened the inflammatory phase and promote tendon healing during the proliferative phase (Takamura et al., 2017).

However, in Width and Echogenicity score, there is no significant difference between all groups this result in agreement with (de Mattos Carvalho et al., 2011 and Nixon et al., 2008) which affirmed no significant differences occurred in the ultrasonographic parameters between limbs that received that received cellular therapy and control limbs in horses.

In the same group after surgery and after 60 days of surgery, there was a significant improvement in Echogenicity and fiber score (Schnabel et al., 2009; Mostafa et al., 2015).

Improvement in echogenicity and fiber alignment occurred during tendon repair reflects the presence of immature granulation tissue with active fibrogenesis and collagen production associated with increased in the acoustic density (Mostafa et al., 2015).

About edema after 60 days of surgery, there was slight decrease in control group in comparison with PRP and MSCs treated groups but it is not significant. There is significant difference between times of surgery in the same groups, but in control group, there is not difference between the two periods (before surgery and after 60 days). These results came in

agreement with (Wu and Tang, 2013) who reported that the Edema in subcutaneous tissue and the tendon is an inevitable biologic process, Edema peaks a few days after surgery and persists as long as biologic healing processes are active.

During these ultrasonographic results showed that the frozen tendon allograft was improved in tendon replacement, but MSCs and PRP accelerate its regeneration.

The normal mean value of hydroxyproline content was 137.171±5.291 mg/g dry matter. While in control, it was 87.694±6.502, and 99.694±1.839 in PRP treated group. However, group treated with MSc was recorded 134.322±2.123 mg/g dry matter ($p<0.05\%$).

It was quite significantly different between control and PRP tendons with that of normal one of the same animals. This difference was quite less when treated tendon was compared with that of normal limb. However, there was marked increase in hydroxyproline content in third group using mesenchymal cells. These results indicate to a higher intensity in the MSCs treated group than control and PRP. The penefit repair using MSCs enhanced by using growth factors, MSC treatment in the tendons showed higher concentration of collagen and rapid matrix remodeling (Huo et al., 2009)

The MSCs from bone marrow would differentiated into certain cells in the healing allograft following adaption to the specific environmental conditions of the tendon, and it may be reasonable to expect that MSCs could differentiate as well as promote the regeneration and maturing of the graft. When the MSCs applied to the surface of tendon grafts may have served as a source of additional recruitable fibroblast-like cells for tendon

repopulation, or they may have been involved in activation and recruitment of local fibroblast precursors (Li et al., 2007).

PRP Hydroxyproline content had no significant increase compared to control; this result came in contrast with (Allahverdi et al., 2015) they suggest PRP treatment enhance hydroxyproline content in tenotomized tendon of rabbit.

In tensile strength evaluation, stress RMN and Absorbed energy, there significant increase in MSCs group, but in RMN/nm^2, PRP group also increased but not significant. In the rest of parameters, there were slight increase values in MSC group and lesser PRP group but it is not significant, This indicates that MCSs treatment gave the proper mechanical loading conditions, increase the proliferation of tendon stem cells as well as cellular production of collagen (Gulotta et al., 2012).

The increase in biomechanical properties for the MSC treated group tendons and relative magnitude of these results compared with those of control and PRP treated groups suggest that the MSC group had the potential for more rapid return to normal function, and indicate the improvements associated with introducing MSCs into the repair allograft tendon. The significant larger cross sectional area of the cells assisted repair and increase in the rate of structural properties could explain these results (young et al., 1998).

TGF-beta1 released from fibroblasts differentiated from cells MSCs can promote mechanical strength in healing allograft tendons by regulating collagen I and III synthesis, cross-link formation, and matrix remodeling (Hou et al., 2009). The histology of this study appears to support the biomechanical finding.

The PRP biomechanical result of this study comes in contrast with (Kraus et al., 2016) results, they suggest the biomechanical and immunehistological result did not show positive effects of the MSC groups on tendon remodeling in rat Achilles tendon defect. After 12 week, stem cells had not significant effect on biomechanical results.

In histopathological evaluation, Trichrome Masson staining was used for visualization of the SDFT tendon. Blue staining represents collagen in the tendon. The dark staining nuclei of the tendon fibroblasts are located in parallel rows flattened between collagen fibers.

The control group had less value than that in the other groups, but control group showed better status among the treatment groups in the characteristics of the organization of the extracellular matrix. Possible explanation for the slow healing of the tendon, the resulting formation of mechanically inferior extracellular matrix are probably because of the fact that tendon is a minimally vascularized tissue, present cells that exhibit diminished mitotic activity, and the presence few progenitor cells in the tissue (de Mattos Carvalho et al., 2011)

The orientation of collagen fibers in the MSC and PRP group were better organized in comparison with control group. The numbers of the vessels in the MSC were decreased compared to the control and PRP group suggesting that MSCs demonstrated accelerated remodeling (Gulotta et al., 2012).

PRP may have shortened the duration of the healing process (Lyras et al., 2009). Platelet derived growth factors increase cell proliferation and migration , and collagen production, and it shown to enhance regeneration of the tendon allograft (Bosch et al., 2010; Li et al., 2007).

However, in terms of the ratio of cell to matrix mesenchymal cell group, cellularity increased in compared to other groups. The matrix, Cell alignment, etc. it was shown to be MSC better than other groups, and the PRP group was better than control treated group. In addition, results showed increase in vascularization value in control and PRP treated group.

Bosch et al. in 2010 suggested that principle effect of PRP on the healing process of tendon injury is a (lasting) effect on cell proliferation and migration, and hence on overall metabolic activity, these finding supported by histological findings, with PRP treated tendons showing a higher regional cell density and more vascularization, indication a higher metabolic activity.

Despite the advantages of PRP therapy, there are concerns regarding the optimized use of growth factors. One of the major concerns is the short life span of these bioactive agents, which have limited their efficacy (Kiapour and Murray, 2014).

According to these result, PRP may induce migration, proliferation and neovascularization in the early stage of tendon healing. This result of PRP group came in agreement with (Takamura et al., 2017) who injected PRP in severed Achilles tendon of rabbit, they find out the number of fibroblast decreased significantly at the 6^{th} week compared to that at 2^{nd} week, also vessels diameter and number of vessels decreased in the PRP group. They suggest in inflammatory phase, growth factor released from PRP simulate fibroblast migration stimulate fibroblast migration and proliferation as well as neovascularization. The large number of migrating fibroblast leads to early initiation of the proliferative phase. Consequently, the remodeling phase is initiated earlier.

There were fewer vessels in the MSC group compared to control and PRP groups. This result suggested that MSC accelerate the healing process

because Angiogenesis is an essential step in the process of tendon healing and tendon graft remodeling, in which neovascularization prompts delivery of inflammatory cells, fibroblasts and growth factors to the wound site. Therefore, enhancing angiogenesis during stages of healing may be accelerating the remodeling of tendon graft (Li et al., 2007).

A higher regional cell density and more vascularization, indication a higher metabolic activity (Li et al., 2007; Pastides and Khan, 2011).

TGF-β plays a key role during the various tissue repairs that is secreted from fibroblasts differentiated from mesenchymal stem cells MSCs. TGF-β can enhance the production of vascular endothelial growth factor (VEGF). TGF-β regulates a various biological processes including cell proliferation, migration, differentiation, apoptosis and extracellular matrix deposition. In particular, TGF-β accelerates the proliferation and matrix synthesis of tendon and ligament fibroblasts. In addition, TGF-β modulates proteoglycan deposition and stimulates production of collagen by fibroblasts. VEGF is especially important during proliferative and remodeling phases (Kushida and Lida, 2014).

In inflammatory score, PRP treated group had decrease in comparison with control and MSCs treated group. PRP injection may be suppress tendon inflammation and hence reduce tendon pain, thus enhancing tendon function in the animals. Indeed, the Hepatocyte growth factor (HGF) in PRP was shown to have anti-inflammatory function (Zhang et al., 2013; Wang and Nirmala, 2016).

The results of PRP group in this study are in disagreement with Parafioriti et al 2011 they suggest that the use of a single injection of PRP appear not useful for Achilles rat tendon tear. It is not an adjuvant to complete recovery of functionality in this rat Achilles tendon tear model.

Histopathological evaluation revealed MSCs treated group in the first place and PRP treated group revealed improvement in tendon fiber organization diminish inflammatory infiltrate better than control group.

The cellular therapy model promotes increase perivascular inflammatory infiltrate, fibroblastic density and qualitative healing improvement of tendon extracellular matrix, in term fiber orientation and type I and III collagen ratio (de Mattos Carvalho et al., 2011).

Conclusion:

We have demonstrated that the application of MSCs and PRP for anastomosis and allograft tendon is effective in keeping intact certain structural properties during the early phase of tendon remodeling. MSCs treatment leads to enhance regeneration of tendon tissue and subsequent enhanced collagen deposition in the grafted area. Therefore, this technique may be a potentially useful tool for improving allograft tendon repair and regaining early tendon strength.

The application of PRP seems to be a promising method of tendon healing acceleration. In addition, the low cost of PRP in comparison to the use of MSCs and the theoretical benign nature of PRP may give priority to the use of PRP treatment in clinical studies.

Recommendations:

1. Additional study is required using gene expression to identify collagen type I after tendon allograft transplantation in large animal models.
2. Studying regeneration processes of tendon allograft using multiple injections PRP for long term of time.

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