

# Evaluation of the Hydroxyproline Content in the Frozen Allograft Tendon Impregnated With the Mesenchymal Cells & PRP in Lamb

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## Abstract:

**BACKGROUND:** Tendon grafting is mostly required to repair an injury to flexor tendon and due to the importance of this tendon; it needs methods to speed up the re-vitality of the tendon allograft.

**OBJECTIVES:** The aim was to investigate the efficacy of the mesenchymal cells and platelet rich plasma on the hydroxyproline content of frozen allograft tendons after grafting in lamb.

**METHODS:** In this experimental study, mid portion (five cm) of SDFT of fifteen lambs, from both forelimbs were removed and replaced with frozen allografts tendon. Animals were divided into 3 sub-groups of 5 lambs each: control, PRP and MSCs. The hydroxyproline concentration was measured by after 60 days. Data was analyzed using student t test at  $P < 0.05\%$ .

**RESULTS:** The normal mean values of hydroxyproline content was  $137.171 \pm 5.291$  mg/g dry matter which  $87.694 \pm 6.502$  in control group but  $99.694 \pm 1.839$  in PRP group and in group treated with MSC was recorded  $134.322 \pm 2.123$  mg/g dry matter ( $P < 0.05\%$ ) it was quite significantly different between control tendons with that of normal one of the same animal. There was marked increase in hydroxyproline content of MSC group when compare with that of control and PRP groups.

**CONCLUSIONS:** The results of this study showed that MSCs could enhance HP content in the frozen grafted tendon in lamb.

## Keywords:

Frozen tendon, Hydroxyproline, Lamb, Mesenchymal cell, PRP

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## Introduction

In working animal, injuries to tendons are among the most common injuries to the body. They include complete and incomplete tendon ruptures that occur from a single overload event as well as sometimes in the less dramatic form of tendinitis. These injuries are responsible for the high cost of health care besides leading to long period of rest, and even individual morbidity (Postachini & De Martino 1980). Although there are medical treatments for most of these conditions, continuing efforts need to be made to improve the effectiveness of the treatments and accelerate recovery. In the past, most of these efforts have been directed at improving surgical, pharmacological and rehabilitative techniques. Despite many improvements in these techniques, there remain significant limitations in our management of these conditions. The correct anatomic structure must be modeled and the desired experimental condition must be simulated. So far, many complete and incomplete injuries have been modeled differently and are being reported (Ehrlich et al., 2005). Another important factor is to determine the effectiveness of the technique being tested during ambulation and manipulation of the grafted area. Since the primary function of tendons is to transfer loads, injuries to these structures result in the interruption of load transfer and loss of function (Sasaki et al., 2012). There are several pathologic conditions that occur to tendons in the form of partial or complete tissue injuries. Clinically these injuries often do not heal properly leading to morbidity and often require reconstructive surgery. These conditions have been simulated through partial lesion, partial tissue laceration, tendinitis,

tendinosis, transections, incisional wound, dropping of limb, total transection, longitudinal lesion and hemi section being reported (Postachini; De Martino 1980 and Lin 2004). Tendon repair involves a complex orchestrated series of physiological events that include protein synthesis, cell migration, and regeneration of the extracellular matrix, particularly collagen (Bergman & Loxley 1969). An appropriate animal model should be available to study the tendon healing process. In this experiment using allograft tendon impregnated with Plasma rich platelet (PRP) and Mesenchymal cells (MSCs) in lambs shows its effectiveness by quantifying the amount of hydroxyproline formation as a sign of tendon regeneration (Sharifi et al., 2007a, Sharifi et al. 2011b, Sharifi 2007c)

## Materials and Methods

This research project was approved by the Experts Research Committee in the Faculty of Veterinary Medicine University of Tehran. Protocol 2345/95/2016. All lambs received full care in compliance with the experimental protocols of the Ethical Principles in Animal Experiments adopted by the Faculty of Veterinary Medicine Research committee (FVMRC). For in vivo study, fifteen lambs weighing 15 to 20 kg/bw, four months age were housed five per box and were given food and purified tap water ad libitum, all lambs were acclimatized to their new environment for 2 weeks. They were subsequently divided into 3 groups of five lambs each: Control, PRP and MSc group. Before operation lambs were taken off-feed for 12 hours and water was withheld for 6 hours. The ketamine hydrochloride was given intramuscularly 11mg/kg (ket-

amine HCL, Alfasan, Woerden, Holland) and 0.22 mg/kg Rompun (xylazine HCL, Alfasan, Woerden, and Holland) for sedation. Lambs were placed in a sterile field on a heated surgery table and after proper positioning endotracheal intubation was done and they were connected to anesthetic machine with isoflurane 1-2 % for maintenance. The distal portion of both forelegs shaved and subjected to disinfection with betadine 7.5 % for 3 minutes and last touch with mixture of betadine 10% and alcohol and then covered with a sterile surgical drape. The skin was cut longitudinally, on the caudo – lateral surface of metacarpal region and 5cm from the mid area of SDF tendon was removed from each lamb and was grafted with allograft tendon obtained from slaughter house and frozen at -20 °C for 45 days using 0-3 nylon. The samples were removed from deep freezer and were thawed overnight at 4 °C, then kept in ringer lactate solution 6 hours before grafting. 1 ml PRP solution was injected using 22-gauge needle into the anastomosis site. But for easy infiltration of MSCs solution needle 19 was used. After skin suturing a half limb cast was applied for two weeks.

**PRP Preparation:** In order to prepare autologous PRP, five lambs were selected and 10 ml venous blood was collected from each lamb and immediately transferred into tubes containing anticoagulant (ACD). Blood samples were centrifuged (Smart-PRP2, Centrifuge, Harvest Plymouth .MA) for 5 min at 2000 RPM so that three separate layers were formed based on density of blood components. The buffy coat layer was closely collected by a sampler and transferred into a separate tube which was centrifuged for a second time for 5 min at 2000 RMP so that PRP portion was taken

from the surface, 1.5 to 2.5 ml of PRP product was obtained. A volume of 1 ml of PRP containing  $882 \pm 199 \times 10^3$  platelet/ $\mu$ l was used for each lamb. (Peter 2006; Chaudhury 2013)

**Bone-Marrow-Derived MSCs:** In order to collect bone-marrow derived mesenchymal nonhemopoietic cells, one month before actual experiment, five lambs were selected and anesthetized, 10 ml bone marrow was collected from right iliac crest under strict aseptic condition using Jamshidi bone marrow needle, and immediately transferred into heparinized test tube. The bone marrow was diluted at a 1:1 ratio with phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS) and then layered on top of the Ficoll-Paque TM PLUS density gradient medium (STEMCELL Technologies, Vancouver Canada) in a 50 ml conical tube and centrifuged for 30 min at  $400 \times g$ . The mononuclear cells were washed once with PSB containing 2% FBS before being plated in tissue culture flasks at a density of 4000 cells per  $cm^2$ . The isolated MSCs were cultured in MesenCult® MSC Basal Medium supplemented with MesenCult® Mesenchymal Stem Cell Stimulatory Supplements (STEMCELL Technologies) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. The adherent MSCs were passaged at 60-80% confluence. At passage 2,  $1 \times 10^6$  MSCs were resuspended in 50  $\mu$ l culture medium and then mixed with 50  $\mu$ l 2% alginate, from the alginate mixture 40  $\mu$ l (containing  $4 \times 10^5$  MSCs) was added onto the PGA scaffold and alginate polymerization was subsequently initiated with CaCl<sub>2</sub> (100mM) Finally, MSCs were embedded within alginate gel. ( Martinello 2013)

**Postoperative care:** After surgery, the lambs were kept warm until they recov-

**Table 1.** The amount of hydroxyproline recorded in control, PRP and MSc groups after grafting with frozen allograft. <sup>ab</sup>Different letters among groups indicates significant differences ( $P < 0.05$ ).

series	Hydroxyproline content in normal tendon (mg/g) dry matter	Hydroxyproline content in control group (mg/g) dry matter	Hydroxyproline content in PRP group (mg/g) dry matter	Hydroxyproline content in MSc group (mg/g) dry matter
1	129.457	75.010	95.540	128.420
2	127.140	84.668	96.120	133.210
3	135.676	73.095	97.080	141.390
4	157.098	101.618	101.880	135.740
5	136.484	104.080	104.960	132.850
Mean	Mean± SE	Mean± SE	Mean± SE	Mean± SE
SD	137.171±5.291 <sup>a</sup>	87.694±6.502 <sup>b</sup>	99.116±1.839 <sup>b</sup>	134.322±2.123 <sup>a</sup>

**Table 2.** Comparison of Hydroxyproline content in the samples of three groups. <sup>ab</sup>Different letters among groups indicates significant differences ( $P < 0.05$ ).

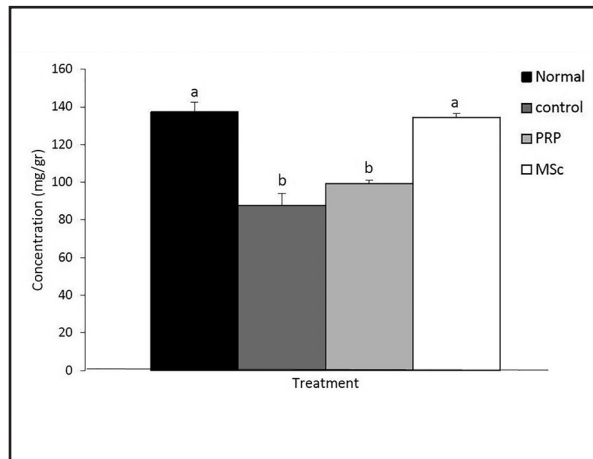
series	Differences between normal and control group (mg/g dry matter)	Differences between normal and PRP group (mg/g dry matter)	Differences between normal and MSCs group (mg/g dry matter)
1	54.447	33.917	1.037
2	42.472	31.02	6.07
3	62.581	38.596	5.714
4	55.48	55.218	21.358
5	32.404	31.524	3.634
	Mean± SE	Mean± SE	Mean± SE
	49.476±5.352 <sup>a</sup>	38.055±4.495 <sup>a</sup>	7.562±3.563 <sup>b</sup>

ered from anesthesia. Antibiotics such as procaine penicillin and dihydrostreptomycin sulphate (1 ml for each 25 kg) I.M., dexamethason 1ml I.M. and B Complex 1 ml I.M. were administered every 24 h for five days. During this time, they were kept housed, and skin sutures were removed on day 12 postoperative.

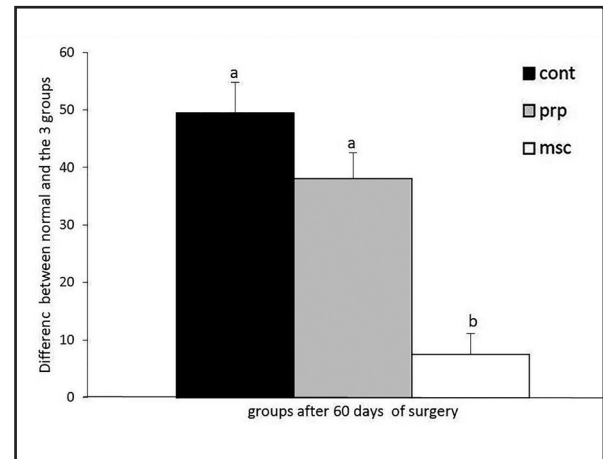
**Hydroxyproline analysis:** To find out if the anastomosis of frozen allograft tendon was effective, the hydroxyproline content was measured at sixty days of clinical observation at the end of study. The sample was collected exactly from attachment place and compared with the normal and experimental group. Specimens were intended for quantification of collagen. (Reddy1996; Reddy1998)

**Collagen quantification-4- hydroxyproline:** The collagen content was determined

by measuring the hydroxyproline content according to Reddy & Enwemeka. Dry weights of lyophilized samples (n=40 for each time point) were measured and transferred to tube culture, 13x100 mm (Corning, NY14831) containing 5 ml of 6N HCL. In separate tubes, 0 (blank) and 4, 6, 8, 10 and 12µg of the hydroxyproline standard (Sigma) were prepared to establish a standard curve for each experiment. Samples were hydrolyzed at 105 °C for 14-16 h and then oxidized by adding chloramine-T reagent and incubating at room temperature for 25 min. After oxidation, a chromophore was developed by adding Ehrlich’s reagents to each sample and incubating at 60 °C for 20 min. to remove interfering chromophores, hydroxyproline product in alkaline media was extracted into toluene and then into acid phase. The absorbance of acid phase



**Figure 1.** Hydroxyproline study of tendon for normal, control, PRP and MSCs groups, after 60 days of surgery. <sup>ab</sup>Different letters among groups indicates significant differences ( $P < 0.05$ )



**Figure 2.** Comparison of Hydroxyproline content in the samples of three groups. <sup>ab</sup>Different letters among groups indicates significant differences ( $P < 0.05$ )

was read at 543 nm and hydroxyproline content was calculated from calibration curve based on standard solutions run as in the same samples. In order to determine the percentage of dry matter (DM) in each tendon sample, 50-100 mg of each sample concurrent with sampling for hydroxyproline analysis was placed on a plate and dried at 100 °C in an oven for 3 h. Finally, hydroxyproline content of tendon samples were expressed in mg/g (Reddy 1996).

**Statistical analysis:** Data were statistically analyzed and values are expressed by mean normality of the variables assessed. Data were analyzed using the statistical package SPSS17.0 (Chicago, IL). Student t test was applied, significance was accepted at ( $P < 0.05$ )

## Results

The main results of the study are summarized in separated tables (1, 2) and figures (1, 2). The positive effect of MSc is observed in third group with 134.3±2.1 mg/g dry matter As compared to normal 137.1±5.2 mg/g dry matter and other two groups, the high amount of collagen for fiber orientation in

early tendon union was observed, which was very low in the control group (87.6±6.5 mg/g) and to some extent acceptable range in PRP group (99.1±1.8 mg/g dry matter) (Fig. 1, 2).

## Discussion

The healing of severed tendon usually due to limitation of vascular network takes a long time. The tendon tissue is not densely populated by cells and the tenocytes are low in their metabolism, which together lead to poor regenerative potential. Thirdly, the healing is accompanied by the development of scar tissue and adhesions to the surrounding area restricting the gliding capacity and thus the range of motion. This scar leads to poor mechanical properties, lower strength and elastic modulus respectively (Lin 2004; Gigante1996; Heidia 2002). To improve tendon healing as well as to increase tendon strength, PRP and MSCs infiltration at the site of anastomosis remarkably reduced adhesion formation by supporting scar-less healing mechanisms and to prevent re-ruptures during weight bearing too (Enwemeka 1989; Elliot 2013; Galatz et al.,

2015). Clinical observation was performed for weight bearing and evaluation of integration between frozen allograft and fresh host tendon (Sharma & Maffulli 2006; Tomopoulos et al., 2015; Wu & Tang 2013b). 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 the beginning of the third week but gradually improved in the treated group at the end of the third week, which demonstrated normal weight bearing after month. The swelling at the site of operation was less severe in the treated group by end of the 3rd week. 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 achieved local pain relief via indulging growth factors and accelerating tenocytes accumulation at the attachment sites (Sharifi 2007; Sharifi 2011). It seems to be due to increased 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 at the beginning of the 5th week. The clinical findings in this study provide indirect evidence that PRP and MSCs therapy local infiltration promote healing at the site of attachment even using allograft tendon by increasing fibroblast and tenoblast activity. Cumulative increase in hydroxyproline content usually has direct correlation in early maturation of fibroblasts and early parallel arrangement of collagen fibers and bundle formation (Sharifi et al., 2007). The normal mean value of hydroxyproline content

was  $137.1 \pm 5.2$  mg/g dry matter,  $87.6 \pm 6.5$  in control group and  $99.6 \pm 1.8$  in PRP group, but in group treated with MSc it was recorded  $134.3 \pm 2.1$  mg/g dry matter ( $P < 0.05\%$ ). This was quite a significant difference between control and PRP tendons compared with that of normal one of the same animal in that this difference was much less when treated tendon was compared with that of normal limb. There was marked increase in hydroxyproline content in third group using mesenchymal cells. Despite deep freezing of the allograft tendon, there was no drastic histomorphological changes in those tissues and preserving the biological graft material even  $-200$  C for 45 days withhold infrastructure of tendon similar to freshly obtained graft which is idealistic for replacing damaged tendon. At the site of attachment in the MSCs group there was an increase in mitogenic and anabolic and morphogenic responses of host tendon tissue that brought about the clinical success of using allograft.

Early studies (Lin et al., 2004; Gigante et al., 1996) showed a correlation exists among number of collagen fibrils, size, their organization and mechanical strength. The amount of regenerating tendons considered was a good indication of the relationship between mechanical strength and the absolute amount of collagen determined as hydroxyproline content and the healing tissue was reshaped. There was a corresponding decrease in cellularity, collagen and glycosaminoglycan synthesis (Burgisser, et al., 2016; Colgrave et al., 2008; Stoll et al., 2011)

**Conclusion:** This study indicates that direct local infiltration of mesenchymal cells at the attachment site of graft promotes and enhances hydroxyproline content in the treated tendon which is considered to be a

direct marker of the effect of Mesenchymal cells therapy on collagen content in injured tendon.

## Acknowledgments

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## Conflicts of interest

The author declared no conflict of interest.

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## ارزیابی محتویات هیدروکسی پرولین در بافت تاندون آلوگرافت منجمد پوشش داده شده با سلول‌های بنیادی مزانشیمی و پلاسمای غنی از پلاکت در بره

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### چکیده

زمینه مطالعه: در اکثر موارد، پیوند تاندون به منظور ترمیم آسیب به تاندون خم‌کننده و به علت اهمیت این تاندون مورد نیاز است. در نتیجه، همواره روش‌هایی برای افزایش سرعت در بازیابی خاصیت حیاتی بافت پیوندی آلوگرافت تاندون نیاز است.

هدف: هدف از مطالعه حاضر، ارزیابی تأثیر سلول‌های مزانشیمی و پلاسمای غنی از پلاکت بر روی محتویات هیدروکسی پرولین در بافت تاندون آلوگرافت منجمد بعد از پیوند در بره می‌باشد.

روش کار: در این مطالعه تجربی، قسمت میانی (پنج سانتی‌متر) تاندون خم‌کننده انگشتی سطحی در پانزده بره از هر دو اندام قدامی برداشته شده و با تاندون آلوگرافت منجمد جایگزین شد. حیوانات مورد آزمایش به سه گروه پنج‌تایی شامل: گروه کنترل، گروه پلاسمای غنی از پلاکت و گروه سلول‌های مزانشیمی تقسیم شدند. غلظت هیدروکسی پرولین بعد از گذشت ۶۰ روز اندازه‌گیری شد. داده‌ها با استفاده از student t test ارزیابی شد ( $P < 0.05$ ).

نتایج: مقدار طبیعی محتویات هیدروکسی پرولین  $137/171 \pm 5/291$  میلی گرم در هر گرم ماده خشک بود. این مقدار در گروه کنترل  $87/694 \pm 6/502$ ، در گروه پلاسمای غنی از پلاکت  $99/694 \pm 1/839$  و در گروه سلول‌های بنیادی مزانشیمی  $134/322 \pm 2/123$  میلی گرم در هر گرم ماده خشک بود ( $P < 0.05$ ). نتایج مابین گروه کنترل و تاندون‌های نرمال همان حیوان به طور کاملاً معنی‌دار متفاوت بود. همچنین محتویات هیدروکسی پرولین گروه سلول‌های بنیادی مزانشیمی در مقایسه با گروه کنترل و گروه پلاسمای غنی از پلاکت افزایش مشخص داشت.

نتیجه‌گیری نهایی: نتایج این مطالعه نشان می‌دهد که سلول‌های بنیادی مزانشیمی می‌توانند باعث افزایش محتویات هیدروکسی پرولین در تاندون آلوگرافت منجمد پیوند شده در بره شوند.

واژه‌های کلیدی:

تاندون منجمد، هیدروکسی پرولین، بره، سلول‌های مزانشیمی، پلاسمای غنی از پلاکت