IRANIAN JOURNAL OF VETERINARY SURGERY (IJVS)
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# Use of Undifferentiated Cultured Bone Marrow-Derived Mesenchymal Stem Cells for DDF Tendon Injuries Repair in Rabbits: A Quantitative and Qualitative Histopathological Study

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## Abstract

**Objective-** To investigate the effect of intratendinous injection of bMSCs on the rate and extent of tendon healing after primary repair in a rabbit model.
**Design-** Experimental study.
**Animals-** Twenty seven skeletally mature New Zealand white rabbits weighing 1.8- 2.5 kg were used. Twenty rabbits were used as the experimental animals, and seven others were used as a source of bone marrow-derived mesenchymal stem cells.
**Procedures-** Under general anesthesia an experimental tenotomy was made through the midsubstance of the DDF tendon .The transected tendon was immediately repaired with use of a locking-loop suture. No treatment was given to control group (n = 10). Rabbits in treatment group (n = 10) were subjected to receive bone marrow-derived mesenchymal stem cells. Operated limbs were immobilized for two weeks post operatively. Samples from operated tendons were harvested at weeks of three and eight of operationfor histopathlogicalevaluation , which included evaluation of quantitative and qualitative assessment (twenty specimens).
**Results-** Histological findings revealed that there were significant improvements in structural characteristics of granulation tissue.Neovascularization and cellular proliferation also increased at the synovial layer of the epitenon (increased thickness)in the bone marrow-derived mesenchymal stem cells intreatment group compared to the control group at the week three ($P<0.05$). At the week eight there were no differences between the groups with regard to histologic characteristics ($P>0.05$).
**Conclusion and Clinical Relevance-** Intratendinous application of bone marrow-derived mesenchymal stem cells following primary tendon repair can significantly improve the histologicalparameters in the early stage of tendon healing. Early timeperiod during tendonhealing is crucial in the treatment of tendon injuries.

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**Key Words-** Bone Marrow-Derived Mesenchymal Stem Cell, DDF Tendon, Rabbit, Histological.

## Introduction

Tendons are soft connective tissues consisting of parallel collagen fibers embedded within an extracellular matrix. This organized structure allows tendon to withstand and transmit large forces between muscle and bone.[1,2,3]

Injuries to tendons are among the most common injuries to body. They range from the complete tendon ruptures to the incomplete injuries such as tendonitis. These injuries are not only responsible for large health care cost, but also result in lost work time and individual morbidity.[4] The successful resolution of tendon injuries can be limited by a number of factors: tendon is inherently slow to heal, healed tendon lacks the strength and elasticity of normal tendon and there is high incidence of injury recurrence.[5] After injury, the healing process in tendons results in the formation of fibrotic scar. The structural, organizational, and mechanical properties of this healed tissue are inferior to that of normal tendon[1, 2, 3].Although these properties improve over time, they do not return to normal levels even after long periods.[1,6] Despite improved surgical techniques and advances in postoperative therapy regimens, rupture of the repair site or the formation of adhesions still occurs.[7,8] Healing of damagedtendon tissue may require immobilization foreight weeks before adequate strength isregained.[9] Unfortunately, prolonged periodsof immobilization of a limb or joint may becomplicated by the atrophy of muscles and articularcartilage, osteoarthritis, skin necrosis, tendocutaneous adhesions andthrombophlebitis.[10,11] New methods are required to accelerate the healing of the tendon. Advances in the field of tissue engineering now allows for new approaches to treat these injuries.[4]

Tissue engineered cell therapies offer many new treatment options for repair of diseased and damaged tissue. Tissue engineers add cells to various delivery vehicles and introduce mechanical and chemical stimuli in culture to try and create safe and functional repair tissues.[12] Delivery of high concentrations of undifferentiated mesenchymal stem cells(MSCs) to connective tissue defect has shown promising results in animal studies for bone, cartilage and tendon repair.[13] Currently bone marrow aspirate is considered to be the most accessible and enriched source of MSCs[14].

It was hypothesized that mesenchymal stem cells (MSCs) in nucleated cells from bone marrow would differentiate into fibroblast-like cells once transplanted into healing connective tissue.[14,15,16] The objective of this study was to investigate the effect of cell based therapy on the rate and extent of tendonhealing after primary repair in a rabbit model.

## Materials and Methods

*Bone marrow-derived mesenchymal stem cell isolation and expansion*

MSCswere derived from bone-marrow aspirates from the iliac crest.[17,18] After the rabbits were anesthetized, bone marrow was aspirated from the iliac crest and collected into polypropylene tubes containing 1000 unit/ml preservative-free heparin.The bone marrow and heparin were mixed. Nucleated cells were isolated by density gradient centrifugation over Ficoll/pague (Pharmacia).The nucleated cell layers were carefully removed and resuspended in a culture medium containingof Dulbecco's modified eagle's medium (DMEM; Sigma Co. USA), 15% fetal bovine serum (Gibco), 100 unit/ml penicillin (Nasr, Fariman Iran) and 100 µ/ml stereptomycine (Nasr, Fariman, Iran).The nucleated cells were plated at the density of 5 million nucleated cells in T-75 flasks and grown at 37 °C, 5% $Co_2$ in humidified tissue-culture

incubator. After five days, the content of the flask were removed washed with medium, nonadherent cells were discarded and adherent cells were cultured. The medium was changed every 3 days.After about 14 days when the cells become 75% to 80% confluent,they were trypsinized and serially subcultured. The second-passage cells were injected in the repair site of tendon. Cells isolated with use of this technique have a fibroblast-like appearance (Fig. 1) and have been shown to be capable of multipotent differentiation.[13,14,19]

*Surgical Technique*

Twenty sevenskeletally mature New Zealand white rabbits weighing 1.9-2.5 kg were used. The experimental procedure and animal care were approved by the Ethics Committee of Urmia University. A group of seven rabbits were used as the source of bone marrow-derived mesenchymal stem cells. A second group of 27rabbits were used for experimental proper, which involved 20 animals assigned forhistological analysis at 3 weeks (10 rabbits) and 8 weeks (10 rabbits).

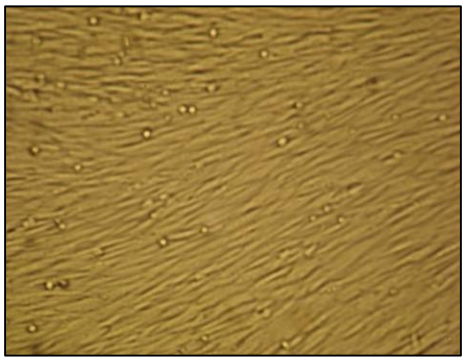

**Figure 1.** Rabbit bone marrow-derived mesenchymal stem cells are spindle shaped fibroblast-like cells. Original magnification x100

All animals were anesthetized with 35 mg/kg,IM,ketamine (Ketamine 5%, , Alphasan, Woerden, Holland), 5mg/kg,IM,xylazine (Xylazine 2%, , Alphasan, Woerden, Holland) and 1 mg/kg,IM,acepromazine (Acepromazine 2%, Hoogostraten, Belgium).[20] A longitudinal skin incision was aseptically made directly over the deep digital flexor (DDF) tendon. A complete transverse incision was made with a surgical blade through the midsubstance of the DDF tendon. This incision was immediately repaired with a locking-loop pattern with use of nylon 3-0 suture. In the treatment group, the bone marrow-derived mesenchymal stem cells (2 -3million) in 0.1 ml normal saline injected intartendinously at the repair site as well as externally around the repair site. The same procedure was repeatedin the control group, except that the bone marrow-derived mesenchymal stem cells were omitted from the normal saline. Skin closure was performed in a routine manner with use of nylon suture. Operated limbs were immobilized for 2 weeks.

*Histological Analysis*

At weeks 3 and 8 after the surgery all animals were euthanized and tissue samples of DDF tendon were harvested for routine histological study. Paraformaldyde-fixed longitudinal tissue section for histology were dehydrated, cleared with xylene, embedded in paraffin, sectioned, mounted on microscope slides, and stained with hematoxylin and eosin (H & E). The samples were studied for histological changes under light microscopy. We used qualitative and quantitative histological factors to assess the contribution ofbMSCs to tendon healing. The qualitativehistological changes such as the proliferation of fibroblast (cellularity),the formation of collagenband andthe density, regularity and crimp pattern of these bands and the quantitative histological changes such as the percentage of total area consisting of granulation tissue with longitudinal order of collagen fiber (in 2800 $\mu m^2$), average number of vessels transections observed in each field at x 100 (neovascularization), average number of vessels transections observed in epitenon at x 100, increase in capillaries and cellular proliferation at the synovial layer of the epitenon (increased thickness) and percentage of remodeling new collagen fiber next to severed edge of tendon, were all evaluated separately. The quantitative histological parameters were evaluated by means of graded lens.

*Statistical Analysis*

Statistical analyses were carried out using PASW Statistics (Release 18, SPSS Inc., Chicago, Illinois).The residuals were tested for normality by Shapiro-Wilk's test and normality plots (histograms and quantile-quantile plots) andfor homogeneity of variation by Levene's test and examining residual plot. Normality and/or homogeneity of variance assumptions for other variables were not satisfied and prior to statistical analysis these variables were logarithmically transformed to fulfill model assumptions. Statistical analysis of data was assessed using one-way analysis of variance (ANOVA). Post-hoc, Tukey test for pair wise comparisons was used, if required. Data are presented as mean ± standard deviation. The level of significance was set at $P< 0.05$.

## Results

The main histomorphometrics results are summarized in Table 1.

**Table 1.** Quantitive results of histomorphometrics studies (mean ± SD).

| | | | Parameters | | | | |
|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E |
| Groups | 3 weeks (n=10) | Control | 12.00 ±4.95 [a] | 39.60 ± 13.66 [b] | 18.60 ± 7.82 | 65.00 ± 12.74 [c] | 11.00 ± 3.16 |
| | | Treatment | 50.60 ±14.15 [a] | 93.20 ± 29.46[b] | 42.20 ± 8.40 | 142.00 ± 41.47[c] | 12.40 ±3.20 |
| | 8 weeks (n=10) | Control | 19.60 ± 5.50 | 19.20 ±10.10 | 50.20 ±9.03 | 48.00 ± 29.91 | 10.00 ± 2.91 |
| | | Treatment | 32.00 ± 9.90 | 19.00 ± 3.67 | 57.60 ± 21.20 | 73.00 ± 36.84 | 9.80 ± 2.86 |

A: Percentage of total area consisting of granulation tissue with longitudinal order of collagen fiber (in 2800 $\mu m^2$)

B: Average number of vessels transections observed in each field at x 100

C: Percentage of remodeling new collagen fiber next to severed edge of tendon

D: Epitenon thickness (µm)

E: Average number of vessels transections observed in epitenon at x 100

Histologically, at week 3, the mesenchymal stem cells treated samples exhibited more organized band of collagen with a better alignment of cells and collagen fibers and were more similar to the native tendon than untreated controls. Cell morphology was the reminiscent of tendon fibroblast compared with variable morphologies in the controls. The gradual increase in cellularity and more typical spindle shape fibroblast, the density and crimp pattern of these bands appeared to increase at 3 weeks in treatment group (Fig. 2).

All tendons showed marked increase in total area of granulation tissue with longitudinal order of collagen fiber (collagen fiber linearity), angiogenesis (neovascularization) inepitenon and healing lesion and epitenonthickness (cellular proliferation at the synovial layer of the epitenon ) in treatment group compared to the control group at 3 weeks ($P< 0.05$). Bone marrow-derived mesenchymal stem cell treated tendons percentage of remodeling new collagen fiber next to severed edge of tendon at three weeks were greater than the controls,

this difference was not statistically significant (P> 0.05). There was no significant difference in quantitive results of histological properties between the treatment and control groups at 8 weeks (P>0.05)(Table 1).

## Discussion

Our results indicate that bone marrow-derived mesenchymal stem cells injected intratendinosly at tendon repair site contribute to early tendonhealing –at 3 weeks– following primary repair, however no significant changes were identified at 8 weeks.

This study expands on the previously reported positive influences of bMSCs on tendon healing in vitro by examining the hypothesis that intratendinous injection of bMSCsat the repair site would enhance tendon healing in a clinical model of tendon rupture in rabbits.[13,18,19] We chose to use allogenic bone marrow-derived mesenchymal stem cells for a number reason. In fact, mesenchymal stem cells avoid allogenic rejection in human and in animal models. These findings are supported by in vitro co-culture studies.Three broad mechanisms contribute to this effect. Firstly, mesenchymal stem cells are hypoimmunogenic, often lacking MHC-II and co stimulatory molecule expression. Secondly, these stem cells prevent T cell responses indirectly through modulation of dendritic cells and disrupting NK as well as CD8+ and CD4+ T cell function. Thirdly, mesenchymal stem cells induce a suppressive local prostaglandins and interleukin-10 as well as by the expression of indoleamine 2,3-dioxygenase, which depletes the local milieu of tryphtophan.[15,21,22,23] Allogenic cells have been previously been used successfully in tissue engineering.[13,17,18,19]

In our experiment, we found that bMSCsincreased tendon healing rate and epitenonneovascularization, cellularity, collagen formation (specially in its linear form), fibroblast proliferation, percentage of remodeling new collagen fiber next to severed edge of tendon and epitenon thickness at 3 weeks treatment group.

Mature tendons are poorly vascularized and such intrinsic characteristics of tendons, may explain their slow rate of healing.However,blood supply increase in tendon during healing. During tendon injury, as with damage to any tissue, there is a requirement for cell infiltration from the blood system to provide the necessary reparative factors for tissue healing.[24,25] Gelberman et al., showed an initial vascular response in tendon injury of the flexor digitorumprofundus (FDP) in dog that was profuse and haphazard, and revealed that the early stages of repair were characterized by a marked increase in vascularity.[26] Kakaret al., noted neovascularization in the tendon sheath and epitenon of the FDP in the New Zealand white rabbit model after partial plantar laceration.[27] In this regard, our study showed that injection of bMSCs at tendon repair site can improve angiogenesis among the granulation tissue formed after tendon rupture. Neovascularization features among the important biological changes initiated at tendon healing time.[25] Increased neovascularization in the treatment groups (93.20 ± 29.46in treated groupversus 39.60 ± 13.66vessels transections in each field at x100 in control group) at 3 weeks is due to increasing metabolism and demand for blood supply in the repair site, which can facilitate tendon healing. Fibroblast proliferation and increased cellularity and formation of collagen, especially in its linear form, are crucial in tendon healing processas found histologically in our experiment.[5,29,30] Accordingto Potenza, proliferation of fibroblasts in the synovial layerof epitenonand at the injured ends of tendons accelerates the healing of the tendon. Increase in capillaries and cellular proliferation at the synovial layer of the epitenongive rise toincrease epitenonthickness, which we found in our study (142.00 ± 41.47micrometer in treated groupversus 65.00 ± 12.74micrometer in control group).

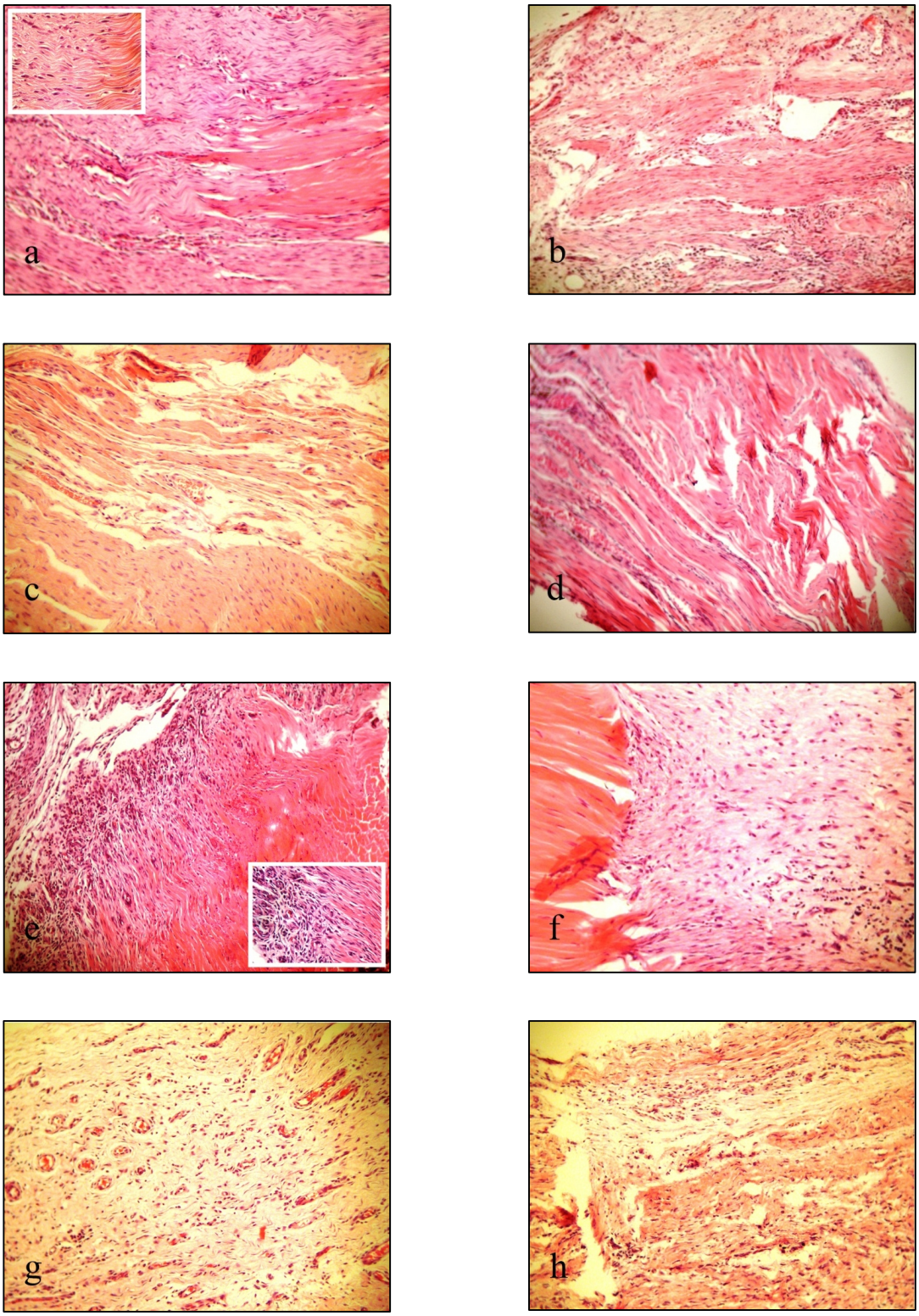


**Figure 2.**

**Figure 2.**
a: Treatment group (week 3): Photomicrograph of a longitudinal section of a rabbit tendon in the treatment group with bMSCs. There is relatively regular organization of the collagen tissue with the high volume density and crimp pattern of these bands and moderate increase in fibroblasts (H & E, x 100). Inset photomicrograph demonstrates x 400magnification(H & E).
b: Control group(week 3): Photomicrograph of a longitudinal section of a rabbit tendon of the control group without bMSCs. There is apparent disorganization of the collagen tissue with less crimp pattern with a mild increase in the number of fibroblasts (H & E, x 100).
c & d: Treatment and control (week 8): Photomicrograph of a longitudinal section of a rabbit tendon of the bMSCs treated group (c) and control group (d). There is no marked difference in histological parameters between two groups (H & E, x100).
e: Treatment (week 3): Photomicrograph of a longitudinal section of tendon in the bMSCs group. There is marked regular organization of the collagen tissue and an apparent increase in the capillaries (H & E, x100). Inset photomicrograph demonstrates capillaries sections at x 400magnification(H & E).
f: Control group (week 3): Photomicrograph of a longitudinal section of tendon in the control group without bMSCs, there is less organized band of collagen and a slight increase in the capillaries (H & E, x 100).
g & h:Treatment and control (week 3): Photomicrograph of a longitudinal section of epitenon .There is marked increase in capillaries and cellular proliferation at the synovial layer of the epitenon (increased thickness) in treated group (g)than those of control group(h)(H & E, x 100).

The cellular activity of fibroblasts located at the injured ends and epitenonalso play a major role in the healing process. A number of studies have demonstrated an increase in the number of fibroblasts in tendon healing.[9,31] Normal tendon is a relatively acellular tissue in which the degradation and production of extracellular matrix molecules is held in equilibrium by tendon fibroblasts. With injury, this equilibrium is disrupted and regulation of individual cell metabolism can be inadequate to repair the damage. One of the bMSCs functions may be to serve as a responsibility of differentiation potentials a storehouse of cells waiting to differentiate into needed lineage depending upon environmental needs and cues.[16] BMSCs have been shown in previous in vitro and in vivo studies to differentiate into fibroblasts [14,15] that is one mechanism which improved the tendon intrinsic healing capacity. These cells also have potential to be used as a molecular vehicle for therapeutic use to enhance healing of connective tissues.[14,15,18]
The better histological parameters at 3 weeks in the bMSCs treated group in our study suggest that bone marrow-derived mesenchymal stem cells improve rate of tendon healing and maturation. The findings parallel data from other studies that have shown that intratendinous cell therapy with bone marrow-derived mesenchymal stem cells following primary tendon repair can improve histological parameters in the early stages of tendon-healing.[17,18,19]

## Acknowledgments

This work was supported by a grant from the Education and Research vice chancellor of Urmia University, Urmia, Iran.

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**چکیده**

# استفاده از سلولهای بنیادی مزانشیمی مشتق از مغز استخوان در ترمیم جراحات زردپی خم کننده عمقی بند انگشتان در خرگوش: مطالعه هیستوپاتولوژیکی کمی و کیفی

**دکتر سارا جوانمردی [1]، دکتر فرشید صرّاف‌زاده رضایی [1]، دکتر نوروز دلیرژ [2]، دکتر رحیم حب نقی [2]، دکتر بهرام دلیر نقده [1]**

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**هدف-** بررسی اثر تزریق داخل زردپی سلولهای بنیادی مزانشیمی مشتق از مغز استخوان بر سرعت و وسعت ترمیم زردپی بعد از التیام اولیه در خرگوش.

**طرح مطالعه-** مطالعه تجربی.

**حیوانات-** بیست وهفت قطعه خرگوش سفید نیوزلندی بالغ با وزن 1/8 تا 2/5 کیلوگرم مورد استفاده قرار گرفتند.بیست قطعه از خرگوشها به عنوان حیوانات آزمایشی و هفت قطعه باقیمانده به عنوان منبع تهیه سلولهای بنیادی مزانشیمی مشتق از مغز استخوان مورد استفاده قرار گرفتند.

**روش کار-** تحت بیهوشی عمومی،عمل تنوتومی تجربی در قسمت میانی زردپی خم کننده عمقی بند انگشتان انجام گرفت. زردپی قطع شده بلافاصله با استفاده از الگوی حلقه بسته شده بخیه شد.در گروه کنترل هیچ گونه درمانی صورت نگرفت (10 قطعه). در صورتیکه خرگوشهای گروه آزمایش (10 قطعه) با سلولهای بنیادی مزانشیمی مشتق از مغز استخوان مورد درمان قرار گرفتند.اندامهای حرکتی جراحی شده به مدت دو هفته بعد از عمل گچ گرفته شدند.در هفته سوم و هشتم بعد از جراحی نمونه ها برای ارزیابی هیستولوژیکی اخذ گردیدند (بیست قطعه).

**نتایج-** یافته های هیستوپاتولوژی نشان داد که ویژگیهای ساختاری بافت جوانه‌ای و میزان رگزایی آن و همینطور میزان رگزایی و پرولیفراسیون سلولی(افزایش ضخامت) در لایه سینویال اپی‌تنوندر گروه درمان شده با سلولهای بنیادی مزانشیمی مشتق از مغز استخوان در هفته سوم بهبود چشمگیری نسبت به گروه کنترل داشت ($P< 0.05$). در صورتیکه در هفته هشتم از نظر آماری هیچ گونه اختلاف معنی داری در بین گروههای کنترل و آزمایش از نظر مشخصه‌های هیستولوژیکی وجود نداشت($P> 0.05$).

**نتیجه گیری و کاربرد بالینی-** کاربرد داخل زردپی سلولهای بنیادی مزانشیمی مشتق از مغز استخوان باعث بهبود معنی دار مشخصات هیستولوژیکی در مراحل اولیه التیام زردپی شد.دوره اولیه التیام زردپی جز مراحل حیاتی در ترمیم زردپی میباشد.

**کلید واژگان-** سلولهای بنیادی مزانشیمی مشتق از مغز استخوان، زردپی خم کننده عمقی بند انگشتان، خرگوش، هیستولوژیک.