Original Article
Comparative Regenerative Effects of Allogeneic Bone Marrow and Patellar Ligament Fat Pad Mesenchymal Stem Cells on Experimental Superficial Flexor Tendonitis in New Zealand Rabbits

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Abstract
Background: Cell therapy is applied in tendonitis to speed the healing process of tendon tissue and restore its functional properties. Almost all types of stem cells can differentiate from the recipient cells after transplantation.

Objectives: The main goal of this study is to compare the effects of two sources of mesenchymal stem cells on tendon regeneration.

Methods: This study randomly divided 32 New Zealand rabbits into 4 groups. The bacterial collagenase was induced at the superficial digital flexor tendon (SDFT) of all rabbits, and the treatment was performed 48 hours after collagenase induction. Group 1 was treated with allogeneic bone marrow mesenchymal stem cells (BMMSCs). Group 2 was treated with adipose-derived stem cells (ADSCs) from the patellar ligament fat pad. Group 3 (sham group) was treated with 0.9% normal saline, and group 4 (control group) was left with no treatment. All rabbits were euthanized 2 and 4 weeks after surgery, and tendon samples were harvested. The histopathology was assessed by hematoxylin-eosin, Masson's trichrome, and Vangieson's dye, and tendon structure, fiber arrangement, cell nuclei, tissue inflammation, vascularity (angiogenesis), and density were surveyed.

Results: The tendon healing process in the BMMSC and ADSC groups revealed better regeneration than the control and sham groups (P≤0.05). Significant changes (P≤0.05) in some microscopic parameters were seen by comparing the BMMSC and ADSC groups.

Conclusion: According to the present study, the injection of mesenchymal stem cells (BMMSCs or ADSCs) showed beneficial results in tendon tissue healing. Furthermore, ADSCs showed better regeneration of the injured tendon tissue than BMMSCs.

Keywords: Regeneration, Stem cell, Histopathology, Tendonitis, Rabbit
1. Introduction

 Nowadays, stem cell therapy is one of the best approaches for curing the injured tissues of humans and animals (Oryan & Sahvieh, 2017). Stem cells are potential cells that could be harvested from any organs such as bone marrow, peripheral blood, connective tissues, and so on (Oryan et al., 2020; Sahvieh et al., 2021). Exogenous stem cell therapy is performed by adding these cells to the injured area for treatment and stimulating the bio-modulatory actions and cell secretions (Oryan & Sahvieh, 2021). The stem cells cause the stimulation properties and activation effect (Oryan et al., 2021). These cells can be harvested from many sources, such as bone marrow mesenchymal stem cells (BMMSCs), adipose-derived stem cells (ADSCs), peripheral blood mononuclear cells (PBMCs), and Wharton’s jelly stem cells (WJ-SCs) (Oryan & Sahvieh, 2020; Oryan et al., 2022). Stem cells have immune modulation advantages and tissue tropism with the angiogenic and anti-apoptosis effect, guaranteeing better tissue regeneration (Mardpour et al., 2019; Abat et al., 2018). Stem cells are used for treating acute tendonitis and can differentiate into tenocytes (Liu et al., 2017). Most tissues that are replaced in clinical cell therapy are muscle, tendon, bone, and cartilage (Von Bahr et al., 2012). These multipotential cells modulate the inflammatory responses in the injured tissue, stimulate specific factor secretions, and differentiate into the host cells. Although the paracrine activity of mesenchymal stem cells (MSCs) participates in the healing process (Marcucio et al., 2015), their action mechanisms have not been recognized completely. These cells have an interesting therapeutic efficacy in clinical trials and have been used by allogenic and exogenous sources for regenerative therapy (Berebichez-Fridman et al., 2017; Lukomska et al., 2019). Clinical investigation showed that stem cells can improve histological and biomechanical properties in various animal models (rabbits, rats, sheep, and horses) after tendon damage caused by surgical manipulation or collagenase administration (Docheva et al., 2015; Martinello et al., 2013).

The present study evaluated the healing effects of bone marrow mesenchymal stem cells and adipose-derived mesenchymal stem cells harvested from the patellar fat pad on experimental tendonitis in New Zealand rabbits.

2. Materials and Methods

Bone marrow mesenchymal stem (stromal) cells from bone marrow sampling (BMMSCs)

A bone marrow sample was taken from a healthy young male rabbit. For this purpose, the tuber of ischia was prepared surgically, and bone marrow was harvested gently by fine needle aspiration (FNA). The whole bone marrow was referred to the Hematology Department of Kerman Medical University, Kerman City, Iran. The density gradient centrifugation was used to pre-enrich mononuclear cells and improve the recovery of rare stem cells. Also, acid citrate dextrose-treated blood was centrifuged at 3500 rpm for 20 min. The obtained buffy coat was diluted with phosphate-buffered saline (1:1; pH=7.4) (Gibco-BRL) and layered on the Ficoll-Paque solution (Biosera, France). After centrifugation at 1500 rpm for 15 min, the isolated mononuclear cells were plated out in Dulbecco’s Modified Eagle’s Medium (DMEM-F12), 15% FBS (fetal bovine serum), and 1% penicillin-streptomycin antibiotic (Gibco-BRL) at a seeding density of 10×10⁶ cells per cell-culture dish. The culture medium was changed every 2 days, and suspended cells were discarded after each medium exchange. Then, the supernatant was aspirated, and cells were harvested by centrifugation. The final pellet was transferred into one dish containing DMEM-F12, FBS (15%), and antibiotics. Three passages were performed using the trypsin enzyme (Gibco-BRL).

Adipose-derived stem cells from patellar ligament fat pad sampling (ADSCs)

Adipose tissue from the patellar ligament fat pad was isolated completely by surgical procedure. However, the patellar ligament fat pad could be harvested by FNA from the caudal part of the patellar ligament or the proximal region of tibia tuberosity. In this study, the tissue was fully exposed and isolated for cell culture to get more cells. The adipose tissue was immersed in PBS solution and transported on ice to the cell culture laboratory. Fat tissue was extracted and digested in a solution of 1 to 3 mg/mL collagenase/adipose for 20 minutes at 37°C and then centrifuged at 1200 rpm for 5 min. Cell suspensions were seeded in 60 mm culture dishes (FBS), 100 U/mL penicillin-G, 100 μg/mL streptomycin, and 1 μg/mL amphotrepsin B and incubated at 37°C in 5% CO₂. The medium was replaced every 3 days. The stem cells were passaged 3 times, 1:5 with 0.25% trypsin/1 mM EDTA (ethylenediaminetetraacetic acid) every 5 days (Salavati Pour et al., 2021).

Animal model and group selection

Thirty-two male New Zealand white rabbits, 8 months old, weighing 2.0±0.5 kg, were housed in separate cages and fed a standard diet. All surgical procedures were carried out in the teaching hospital of the Veterinary Faculty of the Shahid Bahonar University of Kerman. The stem
cells were prepared at the Afzilpour Clinical Research Unit at Kerman Medical University.

The animals were randomly divided into 4 equal groups and had different therapies 48 hours after collagenase-induced tendonitis (groups 1, 2, and 3) as follows:

- **Group 1**: Treatment by BMMSCs (bone marrow mesenchymal stem cells) (n=8),
- **Group 2**: Treatment by ADSCs (adipose-derived stem cells) (n=8),
- **Group 3** (control): Collagenase injected (n=8),
- **Group 4** (sham): Normal tendon treated only by 0.9% normal saline solutions (n=8).

**Stem cell therapy procedure and cell implantation**

All rabbits were anesthetized by intramuscular administration of 40 mg/kg ketamine hydrochloride and 5 mg/kg xylazine (Alfasan International, Woerden, the Netherlands). The Achilles complex of animals was prepared surgically. Type I collagenase (Sigma-Aldrich #C0130-5G, 0.29 U/mg) was filtered, sterilized, and distilled at a dose of 0.3 mg (0.1 IU/0.1 mL distilled water) and injected into the core of the superficial digital flexor tendon, at a distance of 12.5 mm far away from the calcaneal tuberosity of each rabbit on the left hind limbs. The injection depth was considered 2 mm and limited by a sterile silicone separator on top of the insulin needle.

Two days after the first injection, the specific factors, including pain, warming of the position, swelling, reluctance to walk, and screaming during touch, were surveyed. After 48-hour induction of tendonitis, the stem cells were injected to the same point under general anesthesia, and sampling was performed 2 and 4 weeks after stem cell therapy. The volume of injection was 0.4 mL consisting of 1×10^6 count of cells.

**Sampling and histopathology**

The tendon samples were harvested 2 and 4 weeks after the cell injection. The whole area of the Achilles complex was resected gently, and tendon tissue was successfully fixed in 10% buffered formalin. Then, the samples were embedded in paraffin, and 5-µm sections at the center of the injection site were longitudinally prepared and stained by Hematoxylin-Eosin, Masson's trichrome, and Vangieson's dye. Light microscopic evaluations were performed by Olympus BX51 (Olympus Optical Co., Tokyo, Japan) (Ning et al., 2022).

The current study investigated 6 histopathologic parameters of tendon quality: Fiber arrangement, fiber structure, inflammation, nuclear rounding, angiogenesis, and cell density. A histopathological scoring system for grading the tendon repair was used based on the Carvalho et al. (2013) study (Table 1).

**Statistical analysis**

All data were analyzed by SPSS software, version 17 (SPSS Inc, Chicago, USA) using non-parametric ANOVA at the significant level of P≤0.05. When the differences were significant, pair-wise group comparisons were performed by the Mann-Whitney U test. The histopathological data are shown in Table 1.

**3. Results**

**Histopathologic evaluation of BMMSCs and ADSCs cell implantation after 2 weeks**

The normal saline (sham) group showed no microscopic changes like structure, round nuclei, inflammation, vascularity, or cell density. However, there were some changes in the fiber arrangement.

The control group showed degeneration, disruption of the normal linear orientation of collagen bundles, and obvious fiber fragmentation at the injection site. The ruptured area of the tendon was filled with loose to semi-dense connective tissue in an irregular pattern and a small amount of extracellular matrix. An increased number of rounded cells and vascularity was observed. Infiltration of inflammatory mononuclear cells was seen in tissues around the tendon (peritendonitis) (Figure 1c). The median total score of the lesion in the control group was 16 (range: 16 to 16), showing a significant difference in comparison with both treatment groups (P<0.05) (Table 1).

In the BMMSC treatment group, the affected areas were filled with wavy connective tissue in some samples. The abundant proliferation of tenocytes with rounded nuclei was seen. Cellularity qualification was more than the sham, control, and ADSC treatment groups. Inflammatory cells were mostly lymphocytes in the tendon and the surrounding tissues. Angiogenesis was seen at the repairing site. The average fiber structure and arrangement histopathology scores differed significantly from the sham and control groups. The rounding of nuclei was similar to the control group and showed a significant difference (P<0.05) with the ADSC group (Figure 1). The total median score of the lesion in the BMMSC group was 13.5 (range: 13 to 15) (Table 2).
Regarding various histopathologic parameters, the ADSC treatment group was superior to the BMMSC, sham, and control groups. A significant difference in comparison with the control and BMMSC groups was observed in the frequency of rounding nuclei. Also, fiber structure and arrangement showed significant differences (P<0.05) compared with the sham and control groups. The total histopathologic score in this group was 11 (range: 10 to 15) (Table 2).

Statistical analysis at week 2

There was no significant difference (P>0.05) between the BMMSCs group and ADSCs according to the pathologic parameters, including structure, arrangement, inflammation, vascularity, and density. There is a significant difference (P≤0.05) in cell nuclei parameters between treatment groups. Total parameters were significantly different (P≤0.05) in both treatment groups. There was a significant difference (P≤0.05) in structure

<table>
<thead>
<tr>
<th>Variables</th>
<th>Score and Criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber structure</td>
<td>0=Absence of linear areas</td>
</tr>
<tr>
<td></td>
<td>1=20%-50% linear</td>
</tr>
<tr>
<td></td>
<td>2≥50% linear</td>
</tr>
<tr>
<td></td>
<td>3=Linear</td>
</tr>
<tr>
<td>Fiber arrangement</td>
<td>0=Complete disorder of fibers</td>
</tr>
<tr>
<td></td>
<td>1=20%-50% fibers linear</td>
</tr>
<tr>
<td></td>
<td>2≥50% of fiber is uniform</td>
</tr>
<tr>
<td></td>
<td>3=Uniform in all fibers</td>
</tr>
<tr>
<td>Rounding of nuclei</td>
<td>0=Spindle shape nuclei</td>
</tr>
<tr>
<td></td>
<td>1=Multiform nuclei</td>
</tr>
<tr>
<td></td>
<td>2=Semi-round nuclei</td>
</tr>
<tr>
<td></td>
<td>3=Round nuclei</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0=Normal</td>
</tr>
<tr>
<td></td>
<td>1=Slightly increased</td>
</tr>
<tr>
<td></td>
<td>2=Moderately increased</td>
</tr>
<tr>
<td></td>
<td>3=Severely increased</td>
</tr>
<tr>
<td>Vascularity</td>
<td>0=Non-vascular</td>
</tr>
<tr>
<td></td>
<td>1=Light vascularity</td>
</tr>
<tr>
<td></td>
<td>2=Moderate vascularity</td>
</tr>
<tr>
<td></td>
<td>3=Hyperfuscular</td>
</tr>
<tr>
<td>Cell density</td>
<td>0=Normal</td>
</tr>
<tr>
<td></td>
<td>1=Slightly oval</td>
</tr>
<tr>
<td></td>
<td>2=Moderately round</td>
</tr>
<tr>
<td></td>
<td>3=Predominantly round</td>
</tr>
</tbody>
</table>
Histopathologic evaluation of BMMSCs and ADSCs cell implantation after 4 weeks

All parameters in the sham group had no changes after 4 weeks of microscopic evaluation and had significant differences (P<0.05) compared with the other groups (Figure 2c).

The amount of disorganized collagen with moderate angiogenesis in the affected area was increased in the control group after 4 weeks of induction of tendonitis. There was no special pattern in the collagen fibers. The infiltration rate of lymphocytes, angiogenesis, and rounded nuclei was reduced compared to the second week after collagenase injection in this group. The median total histopathologic score was 13 (range: 11-15), which showed a significant difference (P<0.05) with the ADSC group (Table 2).

Statistical analysis at week 4

There was no significant difference (P>0.05) between the treatment groups (BMMSCs and ADSCs) and the other groups (sham and control) regarding pathologic parameters, including arrangement, nuclei, inflammation, vascularity, and density. The tendon structure parameter significantly differed (P≤0.05) between the BMMSCs and ADSCs groups. Also, total parameters had a significant difference (P≤0.05) in both treatment groups (BMMSCs and ADSCs). Compared to sham and control groups, both treatment groups had no significant difference (P>0.05) in nuclei, inflammation, vascularity, and density parameters. Still, there was a significant difference in tendon fiber arrangements (P≤0.05). For the tendon structure parameter, there was no significant difference (P>0.05) between the control group and the BMMSCs group, and there was a significant difference (P≤0.05) between the control group and the AD-

Table 2. Statistical analysis of tendon healing factors

<table>
<thead>
<tr>
<th>Group</th>
<th>Week</th>
<th>Factor</th>
<th>Structure</th>
<th>Arrangement</th>
<th>Nuclei</th>
<th>Inflammation</th>
<th>Vascularity</th>
<th>Density</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4</td>
<td></td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
<td>0(0-0)*</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td></td>
<td>2.5(2-3)</td>
<td>3(3-3)</td>
<td>2(2-2)</td>
<td>1.5(1-2)</td>
<td>2(1-3)</td>
<td>2(2-2)</td>
<td>13(11-15)</td>
</tr>
<tr>
<td>BMMSCs</td>
<td>4</td>
<td></td>
<td>2(2-2)b</td>
<td>2(2-2)a</td>
<td>2(1-3)</td>
<td>1(1-2)</td>
<td>2(2-2)</td>
<td>1.5(1-2)</td>
<td>10.5(10-12)a</td>
</tr>
<tr>
<td>ADSCs</td>
<td>4</td>
<td></td>
<td>(1-2)b</td>
<td>2(1-2)a</td>
<td>1.5(1-2)</td>
<td>0.5(0-2)</td>
<td>1.5(1-2)</td>
<td>1(1-2)a</td>
<td>8.5(8-10)ab</td>
</tr>
</tbody>
</table>

BMMSCs: Bone marrow mesenchymal stem cells; ADSCs: Adipose-derived stem cells.

*Significant difference between the sham and the other groups (P≤0.05), *Significant difference with the control group (P≤0.05), *Significant difference between the BMMSCs and ADSCs groups (P≤0.05).
SCs group. After 4 weeks, the BMMSCs group had no significant difference (P>0.05) from BMMSCs in two weeks, and there was a significant difference (P≤0.05) in the ADSCs group between four and two weeks (Table 2).

4. Discussion

Stem cell therapy is used to treat many diseases and extensive defect areas. Allogeneic stem cells should be isolated from the same species, with minimum tissue invasion and less damage to the donor site. In this study, mesenchymal stem cells were harvested from two origins. The bone marrow was aspirated from the iliac crest percutaneously with less tissue invasion. The infra-patellar ligament fat pad is a new and non-invasive source of adipose tissue for harvesting. This fat pad is known as Hoffa’s fat pad (Fontanella et al., 2017). The patellar ligament adipose tissue sampling method is non-invasive because the fat pad was harvested from the craniomedial or craniolateral region of the patellar ligament by FNA without skin incision. It has been stated that the best model for tendon injury and healing investigation belongs to the rabbits (Stoll et al., 2011). Also, Malard et al. studied the injection of adipose-derived and bone marrow mesenchymal stem cells into the injured superficial digital flexor tendon of athlete horses. The results showed significant changes after treatment, and mesenchymal stem cells differentiated into the reticular fibers 14 days post-injection confirmed the results of our study (Malard et al., 2020). In another study, the ADSCs are essential sources for tissue regeneration and can show tenogenic differentiation in vitro through induction of growth differentiation factor 5 (GDF-5). In addition, tendon repair potential in rabbit models depends on histological parameters, immunohistochemical, and biomechanical factors (Chen et al., 2009). Our investigation showed that the best tissue for tendon regeneration is the adipose tissue, which is easy to extract and has the proper amount of cells for this purpose. Also, there is no adverse side effect of ASCs in early clinical utilization (Zuk et al., 2010). In another study, the author mentioned that ADSCs cells are easy to isolate, and extracting a substantial amount of ADSC is easier than MSCs. The new source of ADSCs showed fewer invasions compared to abdominal fatty tissues isolations and subcutaneous sources (Kokubu et al., 2020). ADSCs can inhibit collagen fibers, increase cellularity in the acute

Figure 1. The H&E and Masson’s Trichrome, and Vangieson’s staining showing the repair area formed at the damaged tendon (star) after 2 weeks of induction

The fat stem cell therapy (ADSC) group showed better organization than the bone marrow stem cells (BMMSCs) group. In the BMMSCs group, tenocytes with spherical nuclei are more than in other groups. The upper row is hematoxylin-eosin staining, and the lower is blue mason trichrome.
phase, and induction of healing processes in acute tendonitis and acute phase of tendinopathies (Oshita et al., 2016). Also, the researchers used sutures and ADSCs in the injured site on the flexor tendon of rabbits, and the biomechanical properties improved in the early stages of healing (Oshita et al., 2016).

In another study, the injection and suturing of the tendon preferred the scaffolds and biogels. They declared that many factors, such as growth, angiogenesis, and antiapoptotic factors, affect tendon healing and rehabilitation (de Lima Santos et al., 2020). Using ADSc in combination with vitamin C exhibited normal tendon structure, collagen bundles, mature collagen fibers, and low cell density, whereas using ADSc alone showed partial healing (Kang et al., 2017). Further, the human ADSc transplantation into rat tendon improved tendon healing, caused tenogenic lineage, and contributed to the rats’ protein in the healing site for 4 weeks after surgery (Lee et al., 2017). The tenogenesis markers, such as collagen type I and III, tenomodulin, biglycan, and tenascin C, increase in the presence of growth differentiation factor 5 (GDF-5) and GDF-5 (Chen et al., 2021). Likewise, other GDFs (GDF-6 and -7) stimulate tendon healing (Eliasson et al., 2008). The allogeneic source of stem cells is already available, and in a clinical survey, it has shown the healing potential to treat tendonitis in rat models (Lee et al., 2015). The bone marrow mesenchymal stem cells are a suitable cell line for tendon regeneration by the effect of proliferation and migration of tendon stem/progenitor cells (Yu et al., 2020). Vigano et al. (2019) believed that, in tendon disorders, microfragmented adipose tissue on inflamed cells and growth factors could play a role as fibrosis inhibitor and reduce catabolic markers, is the more conservative and adjuvant therapy for treating tendon injuries and is similar to the author’s concept. Another study on matrix exosome proteins released under hypoxic conditions (<2% oxygen) from tenocytes has shown multiple signaling pathways of extracellular matrix for repair and regeneration of tendon that approve our study result that ADSCs in tendon repair has a greater role directly and indirectly compared with BMMSCs (Thankam et al., 2020). Also, BMMSCs could regenerate the tendon tissue into osteocytes and

![Image](https://example.com/image.jpg)

**Figure 2.** The H&E and Masson’s Trichrome, and Vangieson’s staining showing the repair area formed at the damaged tendon (star) after 4 weeks of induction.

The adipose-derived stem cell (ADSCs) group showed more regular collagen fibers than the bone marrow stem cell (BMMSCs) group. The arrow indicates a healthy tendon. The upper row is hematoxylin-eosin staining, and the lower is blue mason trichrome.
ectopic ossification, but ADSCs induce neovascularization and inhibit inflammation in the early phase of tendon repair (Ramires et al., 2022).

BMMSCs are the primary source of stem cell therapy, and ADSCs are the second source used in tissue damage and cell therapy. In this study, tendonitis and tendon injuries treated with ADSCs showed better regeneration, proven by pathologic factors (fiber structure, fiber arrangement, nuclei shape, inflammation, vascularity, and cell density). Also, according to the authors, the closest tissue sources of allogeneic or autologous stem cells could provide better support and tissue regeneration. Future studies could claim this overtakes and need more investigations in similar or other injured tissues. Further studies could focus on the communication of histology of patients, mechanical outcomes of tendons, biochemical assessments, tendon function, and repair.

Ethical Considerations

Compliance with ethical guidelines

All the procedures were conducted following the supervision of the Faculty of Veterinary Medicine Ethics Committee, Shahid Bahonar University of Kerman.

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Authors’ contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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References


مقاله پژوهشی

مطالعه مقایسه‌ای اثرات ترمیمی سلول‌های بینیادی آلوده به منشأ مغز استخوان و پد چربی لیگامنت کشک بر روی تاندونیت تجربی در تاندون خم کننده سطحی در خرگوش نیوزلندی

"می‌آزمی ابدا نیکزاد،\nعلي‌پرایا افسانه،\nشهزاد عزیزی\n
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نتیجه‌گیری نهایی: ترتیب ترمیمی دیف ۱ یا دیف ۲ بهترین نتیجه به دلیل اینکه دیف ۱ سلول‌های یخچالی مغز استخوان را تزریق کرده است و دیف ۲ سلول‌های بینیادی را تزریق کرده است. نتایج نشان داد که دیف ۱ پایین‌تر از دیف ۲ بوده و دیافراگم اورژانس این دو دیفراگم را به سمت جلو می‌رساند.

- دیف ۱ (سری پایدار) یا دیف ۲ (سری قندی) را در این مطالعه تریمی مشاهده نکردیم.

- نتایج نشان داد که دیف ۱ برای ترمیم بهتر است.

- دیف ۱ نتایج بهتری نسبت به دیف ۲ داشته است.

- دیف ۱ برای ترمیم بهتر است.

- دیف ۱ نتایج بهتری نسبت به دیف ۲ داشته است.

- دیف ۱ برای ترمیم بهتر است.

- دیف ۱ نتایج بهتری نسبت به دیف ۲ داشته است.

- دیف ۱ برای ترمیم بهتر است.