



A STUDY ON THERAPEUTIC EFFECTS OF PLATELET RICH PLASMA ALONE AND IN CONJUNCTION WITH OZONE THERAPY FOR PARTIALLY RESECTED HAMSTRING TENDON REPAIR IN RABBITS

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ABSTRACT

In recent years, an interest in rehabilitation therapies has sufficiently increased, especially in the field of sports medicine. Hamstring tendon partial tears are very common in this regard and are mainly associated with trauma, chronic tendinopathies and degenerative conditions etc., which are very difficult to cure. Among their rehabilitation therapies along with standard surgical procedures include platelet rich plasma, medical ozone etc. PRP with higher platelet concentrations have abundant cytokines and growth factors that stimulate tissue healing. Medical ozone has special antioxidant, immunoregulatory and analgesic properties which may be highly useful to treat musculoskeletal injuries. In our studies, we selected a total of 18 rabbits that were allocated to 03 groups (n=06). Partial tendon resection was done, and surgical repair was managed with Krackow's suture pattern in all rabbits. Group A (sutures) served as control, while group B and C were administered with PRP and O₃-PRP, respectively @ 0.5 mL, four times a week. The study was carried out for 5 weeks and therapeutic regimens were monitored on day 0, 1, 7, 14, 21, 28 and 35 in terms of physiological vitals, hematology parameter, radiology, ultrasonography, histology and scanning electron microscopy. We noticed excellent tendon healing in group C (O₃-PRP) with abundant cytokines, growth factors, O₃ based oxidative stress and pathogen control. Group B showed good cure due to numerous thrombocyte mediators but it lagged behind group C, whereas group A revealed poor healing among all. Though the use of these regimens in vivo needs further trials, but in the complex scenario of hamstring injuries management we may suggest that 'medical ozone-PRP' had beneficial effects for the tendon partial rupture than PRP alone or the sutures.

Key Words: Hamstring tendon, medical ozone, ozonated saline, platelet rich plasma, tendinopathies, Achilles rupture

1. INTRODUCTION

Hamstring or Achilles is the thickest tendon of body mainly composed of gastrocnemius, superficial digital flexor and soleus muscles. Though it is amongst strongest tendons, but is quite prone to injuries

or ruptures, especially in animals with hopping gait (1, 2). Potential causes of damage may include forceful plantar flexion, traumas, tendinopathies, chronic steroid usage, overexertion etc. The most common rupture site is 2-4 cm above its calcaneal insertion (3).

Partial hamstring tear involves damage to varying number of tendon fibers. In very rare instances clinical signs may be asymptomatic but mostly an acute pain onset, audible pop and piercing sensations during load may be indicative of these ruptures. Physical examination usually reveals tendon site edema or muscular atrophy, depending on size and duration of injury (4).

Tendon healing occurs via Inflammatory, proliferative, and remodeling phases. Leukocytes, platelets and other mediators invade injured site during initial inflammatory phases resulting in necrotic tissue phagocytosis, growth factors release and hem-plug formation etc., that stimulate angiogenesis, fibroblasts proliferation and initiate collagen synthesis. During proliferative phase collagen-III formation reaches to maximum for about 6 weeks, while collagen-I peaks during remodeling phase resulting in tenocytes and collagen fibers alignment in stress direction (5)

For hamstring surgical repair, the ruptured tendon is accessed via longitudinal incision along tear. Full-thickness flaps are mirrored to reveal the torn part via paratenon. To appose tendon ends, two double-stranded Krackow sutures are used which are further strengthened with interrupted circumferential pattern (6).

As tendon problems are frequent, causing severe pain and impairments. Various treatment modalities may include splints, Icing, pressure bandages, electroshock therapy, antioxidants, and rehabilitation procedures etc. Regenerative therapies including platelet-rich plasma, stem cells, ozone therapy etc. have gained popularity in recent years to facilitate speedy regeneration of tendon and ligament injuries (7).

Platelet rich plasma (PRP) is among novel therapies to treat musculoskeletal injuries, especially in sports medicine. Numerous conditions like cartilage loss, epicondylitis, muscle or ligament strains, tendon ruptures etc. have manifested good rehabilitation when treated with PRP. It is bioactive blood component with higher platelet concentrations than normal plasma. Platelets are essential for the natural cycle of injury repair and intercellular communication regulation. Numerous cytokines and growth factors secreted by them serve as chemokines for inflammatory cells and mediators that promote healing by enhancing mitogenesis, neovascularization, collagen synthesis etc.(8).

Ozone is a gas with three oxygen atoms and a half-life of about 40mins. To produce ozone for medical purposes, an ozone generator is needed which uses 100% pure oxygen exposing it to an arc of electricity. Then two oxygen molecules are splitted and some being bound to three, forming ozone molecules. The resultant gas mixture is a combination of oxygen and ozone, with content ratio of 95-97% and 3-5% respectively, is mentioned as 'medical ozone'. Usually, ozone generator has capacity of producing ozone at concentrations between 1-80 g/mL (9).

Medical-ozone reveals various features of O₃ including analgesia, anti-inflammatory, antioxidant and immunoregulatory actions that may be helpful to treat numerous musculoskeletal problems including osteoarthritis, tendon affections, vertebrae issues etc. (10).

Usage of O₃ and PRP as combined therapy may be more efficient to treat ligament/tendon issues. Autologous PRP has abundant cytokines and growth factors while O₃ enhances tissue oxygenation, inflammatory mediators, shows analgesic effects etc. These integrated 'O₃-PRP' properties may enhance recovery pace for musculoskeletal injuries (11).

Partial and complete tears of hamstring tendon are quite common as sports injuries, which are difficult to cure. The aim of current study is to identify and compare the tendon rehabilitation capability of PRP alone and O₃-PRP combination, in terms of healing efficacy and to overcome associated complications including re-rupture, pains, delayed healing etc., that may be helpful for practitioners to tackle tendon problems with ease and speedy recoveries. To establish these therapies as routine clinical practice, further randomized controlled trials may be needed.

2. MATERIALS AND METHODS

2.1. Ethical approval

The study was carried out in accordance with guidelines approved by 'Ethical Review Committee' (NO: DR/32, Dated: 23-01-2023) University of Veterinary and Animal Sciences, Lahore, Pakistan.

2.2. Animal selection

A total of 18 adult rabbits of either sex or breed, weighing 1.5-2.5 kg with age 1-3 years were opted for studies. Ten days prior trials, all rabbits were shifted to experimental stations, Department of Surgery, UVAS-Lahore, to acclimatize them with surroundings. Ad-lib water and commercial feed were provided to all rabbits.

2.3. Experimental design and treatments

18 rabbits were allocated into three groups: A, B and C (n=06 per group) with numbers A1-A6, B1-B6 and C1-C6, respectively. For group A (control), partial tendon resection was repaired with polypropylene, 5/0 (Prolene[®], Ethicon) sutures only. In group B, it was managed with sutures and platelet rich plasma (0.5 mL, injected locally), while in group C repair was done with sutures along with medical ozone-platelet rich plasma combination (0.5 mL, injected locally). The injections were repeated four times a week in designated groups. The study was conducted for 5 weeks with treatment evaluations on days 0,1,7,14,21,28 and 35.

Before trials, pre-operative anomalies were ruled out by clinical examination, complete blood counts, renal/liver function tests. Ivermectin (Promectin[®], Livisto) @ 0.2mg/kg (SC) was injected for endo-/ecto-parasites. Prophylactic antibiotics, enrofloxacin (Encure[®], Nawan) @ 10mg/kg (IM), was administered to each animal.

2.4. Anesthesia and surgical procedures

Ketamine @ 20mg/kg (Ketasol[®], Indus Pharma) and Xylazine HCL @ 3mg/kg (Xylaz[®], Mylab) combination were administered intramuscularly, for induction. Isoflurane (Restane[®], Piramal pharma) @ 3% was used for anesthesia maintenance (12, 13). During operations, IV fluid-line was maintained with Lactated Ringer's Solution (Medisol[®], Medipak).

Surgical asepsis and related protocols were managed as per (14). For partial hamstring resection, a 3cm longitudinal skin incision was given on posterior aspect of the tendon. Afterwards, a transverse half thickness incision, 2cm above calcaneal tubercle was given on the Achilles to induce partial tear. Tendon repair was done with polypropylene, 5/0 (Prolene[®], Ethicon) by Krackow's suture technique. PRP and O₃-PRP were injected at site as per assigned groups. Skin incision was closed as per (15, 16).

2.5. Post-operative management

After surgery, rabbits were monitored for any post-operative complications. Antibiotics enrofloxacin (Encure[®], Nawan) was injected @ 10mg/kg, IM, for five days to avoid infections. Meloxicam (Melonac[®], ICI) was administered @ 0.2mg/kg, SC, to manage pains for 5 days.

2.6. Parameters evaluated

2.6.1. Clinical vitals

Clinical vitals including temperature, heart rate and respiration were recorded on designated days. Temperatures were monitored per rectum; heartbeat and respiration via heart/lung's auscultation.

2.6.2. Hematological indexes

1mL blood was collected from jugular vein in anticoagulant vacutainer (K₂-EDTA). Complete blood count was performed with 'Hematology Analyzer BC-2800 Vet (Mindray)' to rule out potential blood abnormalities including anemia, dehydration, infections etc.

2.6.3. Radiographic evaluation

Radiography was performed at day 0,7,14,21,28 and 35 to evaluate hamstring healing, suture dehiscence etc. at the Department of Small Animal Clinical Sciences, UVAS-Lahore with digital x-ray machine i.e. FDR smart-X (Fujifilm®).

2.6.4. Ultrasonography

With ultrasonography, tendon edges gap was monitored on stipulated days. All ultrasound procedures were performed with ultrasound machine (DUS60-EdanUSA®).

2.6.5. Histological examination

Histological evaluation was done to assess cellular changes and therapy effectiveness at tear sites. Animals were euthanized at trial's end and obtained tendon samples were stored in 10% buffered formalin and dispatched to 'histopathology lab' Department of Pathology, UVAS- Lahore (13). The slides were prepared by Hematoxylin-Eosin and Masson trichome stains, for tissue analysis and healing scores (17).

2.6.6. Electron microscopy

Tendon samples were stored in biopsy jars, containing 10% formalin solution. Onwards, the tissues were dried and mounted on slides for dispatch to "Laser Physics department", University of Engineering and Technology, Lahore, Pakistan for SEM analysis.

2.7. PRP Preparation

Whole blood was collected from jugular in vacutainer with sodium citrate (anticoagulant). To obtain PRP, first spin was performed @ 1600 rpm for 10mins that resulted in three distinctive layers. Upper layer containing platelets was collected in a new tube and second spin was done @ 2000 rpm for 10 mins, that resulted in layers of platelet poor plasma (upper 2/3rd) and platelet rich plasma (lower 1/3rd). The lower layer (PRP) was collected carefully and stored at 4°C for future use. (18, 19).

2.8. Preparation of medical ozone/ozonated saline

Ozone medical generator (APRUIO®) was used to prepare medical ozone. For this purpose, pre-chilled 30mL normal saline was utilized to make concentration of 40µg/mL. Generator's electric current was set at 0.54 volts and oxygen supply was maintained on ¼ liter (as per manufacturer's instructions). The process was allowed to take place for 20 minutes, till ozone bubbles were visible in saline, resulting in ozonated saline/medical ozone. Afterwards, it was stored at 4°C, for future use (20).

2.9. Statistical Analysis

Data was analyzed by statistical software GraphPad prism 6, by using 2-way ANOVA. Significance among groups was evaluated by Tuckey's test. Graphically presentation was done through GraphPad. Significant values were denoted by (P≤0.05).



A



B



C

Figure:

- [A]: Making of medical ozone
- [B]: Platelet rich plasma
- [C]: Ultrasonography procedures

3. RESULTS

3.1. Physiological vitals

Clinical vitals revealed an increase in body temperatures slightly above physiological limits (normal=101.5-104.2°F) in all groups, at day 1. On day 7, the values decreased to upper normal ranges. From day 14 to the trial’s end, temperatures were observed at median levels for all rabbits. A significant difference ($P \leq 0.05$) was observed on day 21 between group A and C (Figure-1). An increase in heart rate minutely above normal range (normal=180-350) was noted at day 1. From day 7 to 35, cardiac output values remained fluctuating within physiological limits, for all rabbits. Statistical significance ($P \leq 0.05$) was seen between groups A-B at day 7 and for A-B, A-C at day 21 (Figure-1). A mild surge in respiratory rate was observed at day 1, with all groups above normal limits (normal=35-45). Afterwards, values showed a downtrend, oscillating at mid ranges till the end of studies. Significance ($P \leq 0.05$) was noted at day 1 between groups A-C and B-C. It was ($P \leq 0.05$) also observed at day 7,14 and 28 for B-C (Figure-1).

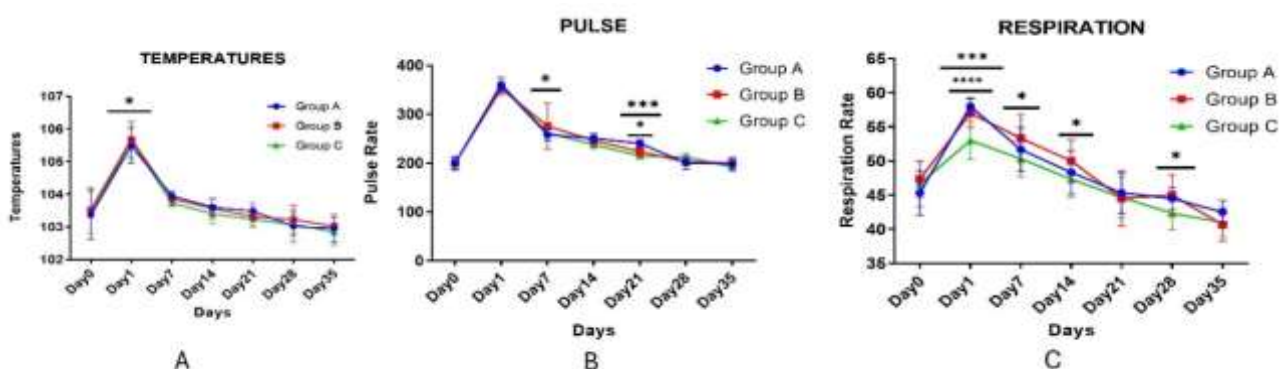


Figure-1. Time-line of changes in Clinical Vitals: (A) Temperatures, (B) Pulse, (C) Respirations. For temperatures, pulse and respiration: significant difference ($P \leq 0.05$) was noted in groups A,B,C at stipulated days.

3.2. Hematological indexes

Hematological evaluations manifested total leukocyte count at median physiological limits (normal= $6-12 \times 10^3/\mu\text{L}$) during studies, except at day 1 where the values were near upper normal range in all groups. Statistically, no significance ($P \geq 0.05$) for WBCs was seen among groups (Figure-2). A slight decline in platelet counts within normal limits (normal= $250-650 \times 10^3/\mu\text{L}$) was noted on day 1,

for all groups. Afterwards, thrombocyte levels rose a bit and fluctuated within normals, till the end of trials. Statistical significance ($P \leq 0.05$) among all groups was observed at day 1, 7, 14, 21, 28 and 35 except only non-significance ($P \geq 0.05$) for B-C at day 35 (Figure-2). A drop in RBCs and Hb at day 1, to lower physiological limits (normal=RBC: $4-7 \times 10^6/\mu\text{L}$; Hb:8-15g/dL) was noted in all groups. Onwards, an uptrend was seen for the parameters and values were observed at median normal, for the remaining time. A significant difference ($P \leq 0.05$) was noted at day 14 and 21 for groups A-B and A-C. At day 28 and 35, significance ($P \leq 0.05$) was seen for groups A-B and among all groups, respectively (Figure-2).

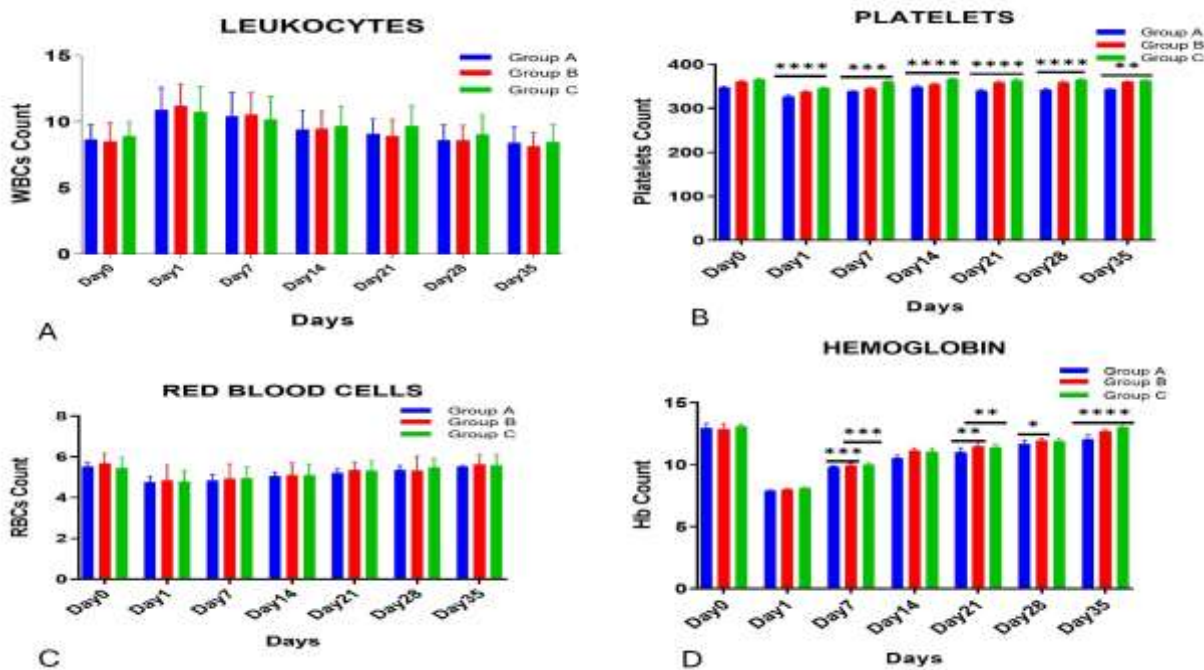


Figure-2. Time-line of in Hematology: (A) WBCs, (B) Platelets, (C) RBCs, (D) Hb. For Platelets and Hb: significant difference ($P \leq 0.05$) was noted in groups A,B,C at stipulated days.

3.3. Radiographic evaluation

Radiography revealed resected tendons healing on stipulated days. In group C, hamstring rehab was excellent and tendons of 03 rabbits regenerated fully on day 21, while complete healing in all animals was noted at day 28. In group B healing was good and 03 animals showed thorough healing on 28th day while at day 35, all rabbits manifested exhaustive cure. In group A, the healing process was slow and full tendon healing was non-evident till end of trials. At day 7, significant difference ($P \leq 0.05$) was seen between groups A-C. Significance ($P \leq 0.05$) was noted among all groups at day 14, 21 and 28 while at day 35, it was observed for groups A-B and A-C (Figure-3) (Figure-4).

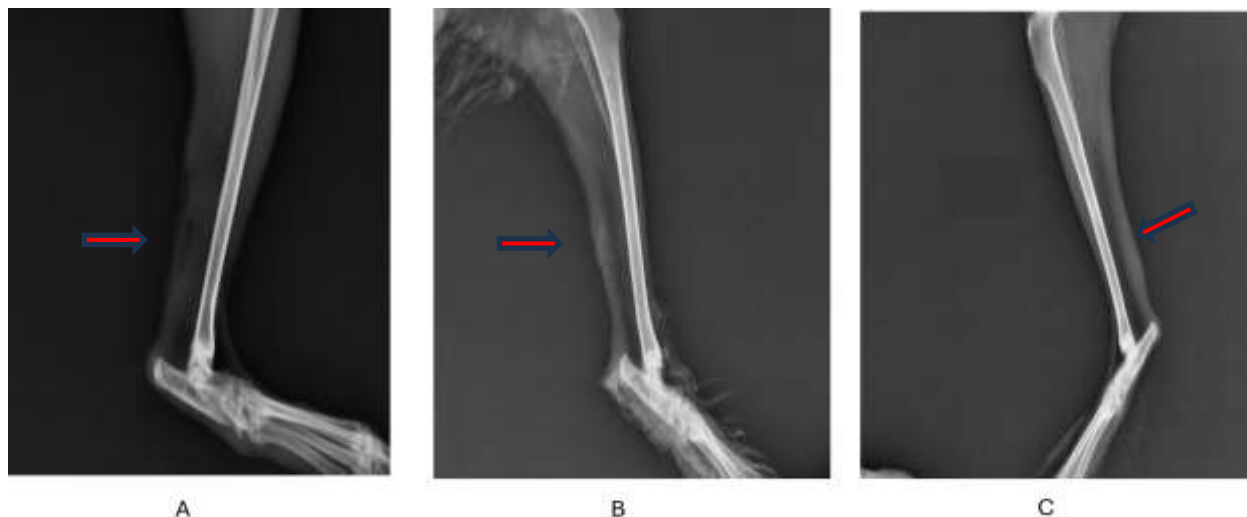


Figure-3. Radiology: Tendon healing comparison at trial end. Best to poor healing in group C, B and A respectively

3.4. Ultrasonography

Ultrasonography manifested reduction in tendon edges gap. In group C, a rapid decrease in gap was noted, where 50% animals showed complete recovery at day 21, while full reduction was observed on day 28, in all rabbits. In group B, the gap healing was good with 50% cure at day 28 while Achilles of all rabbits healed till day 35. Group A revealed a gap decrease linear curve than groups B and C (where it was steep) and was unable to show full recovery till trial's end. At day 7, statistical significance ($P \leq 0.05$) was observed between groups A-B and A-C. At day 14, 21 and 28 significance ($P \leq 0.05$) was noted among all groups, while at day 35 ($P \leq 0.05$) was seen for groups A-B and A-C (Figure-4) (Figure-5).

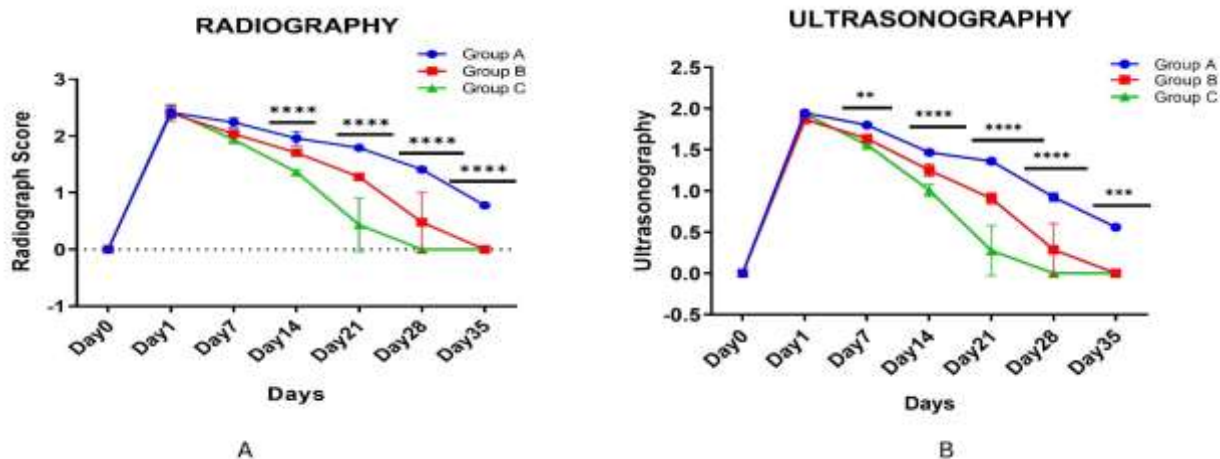


Figure-4. Time-line of changes in tendon healing: (A) Radiogr (B) Ultrasonography. For both parameters significant difference ($P \leq 0.05$) was noted in groups A,B,C at stipulated days.

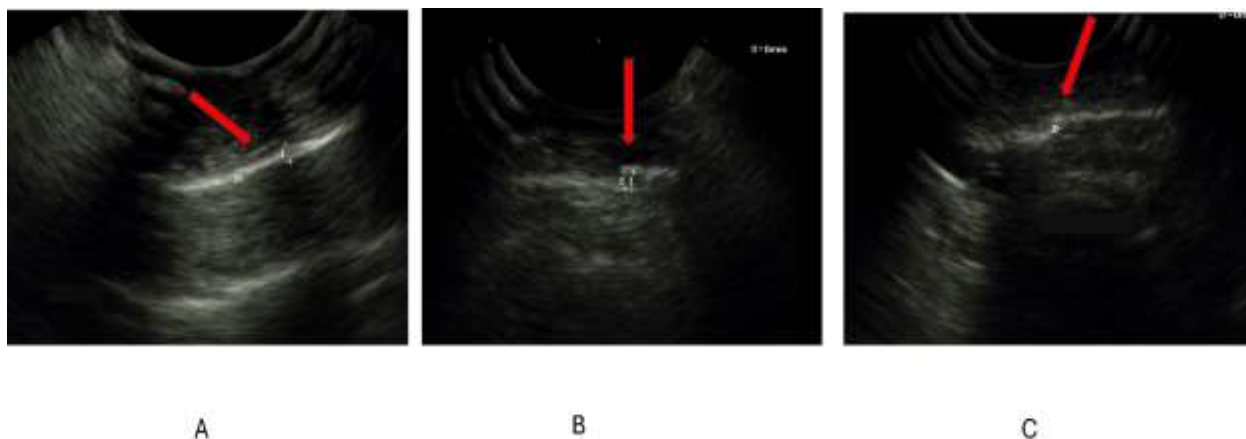


Figure-5. Ultrasonography: Tendon healing comparison at trial end. Best to poor healing in group C, B and A respectively

3.5. Histological evaluation

At end of trials, hamstring healing was histologically evaluated by Hematoxylin-Eosin and Masson-Trichome stains to identify tenocytes, ground substance, vascularity and collagen fibers array. Histological scoring was done as per ‘Bonar score system’ (21). Best scores relating cellularity, ground substance, angiogenesis and collagen were observed for group C (O₃-PRP), followed by group B (PRP) and group A (control), respectively (Figure-6)(Table-1)

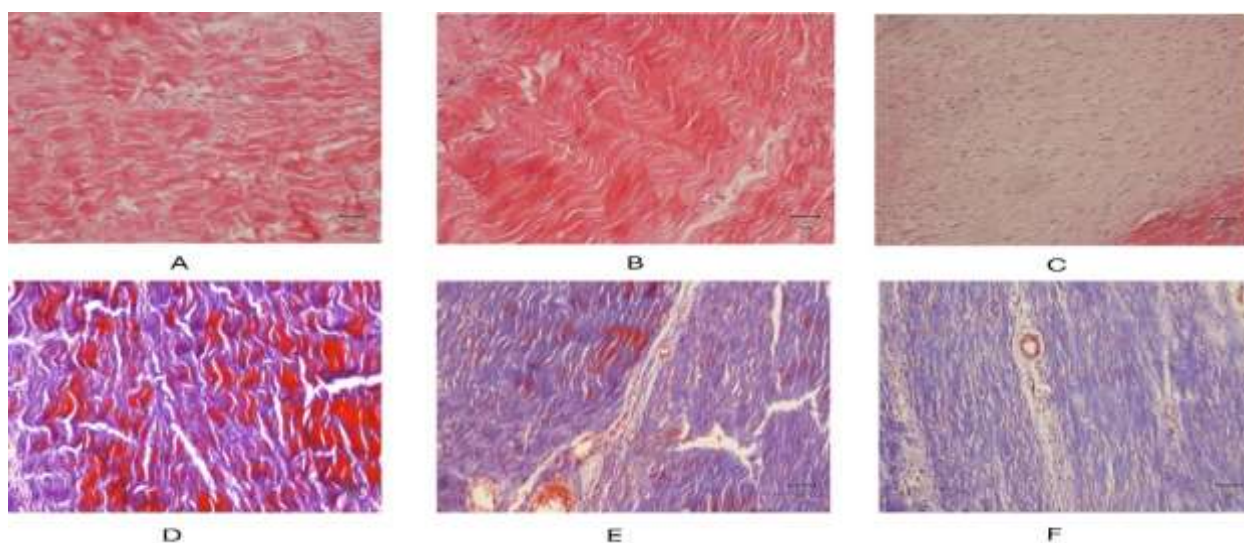


Figure-6. Histological findings-Tendon healing (A,B,C): H & E stain, (D,E,F): MT stain
H & E stain- Best to poor healing in group C, B and A respectively
MT stain Best to poor healing in group F (group C), E (group B) and D (group A) respectively

HISTOLOGICAL SCORING						
Rabbits	Hematoxylin & Eosin			Masson Trichome	Overall Individual Score	Group Score
	Cellularity	Ground Substance	Vascularity	Collagen Array		
A1	3	3	4	3	16	87
A2	4	3	3	2	14	
A3	3	3	4	2	15	
A4	3	3	4	2	14	
A5	4	2	2	2	13	
A6	4	3	3	3	15	
B1	1	2	3	2	10	
B2	0	1	4	3	10	

B3	1	0	2	4	10	59
B4	1	2	3	1	11	
B5	0		3	1	07	
B6	1	1	2	4	11	
C1	2	1	1	1	6	35
C2	1	0	2	2	6	
C3	2	0	4	1	7	
C4	1	0	3	1	4	
C5	2	1	1	1	7	
C6	2	0	0	2	5	

Table.1; Histological Scoring (Bonar Score System)

3.6. Electron microscopy

Scanning electron microscope (SEM) further endorsed histological findings by revealing various cells including tenocytes/fibroblasts that are vital for healing along with neo-vasculature and collagen deposition in tendon gaps. It was observed that group C (O₃-PRP) revealed excellent results in resected tendon gap reduction, group B (PRP) manifested reasonable healing while group A (control) was noted below par than both in this regard (Figure-7).

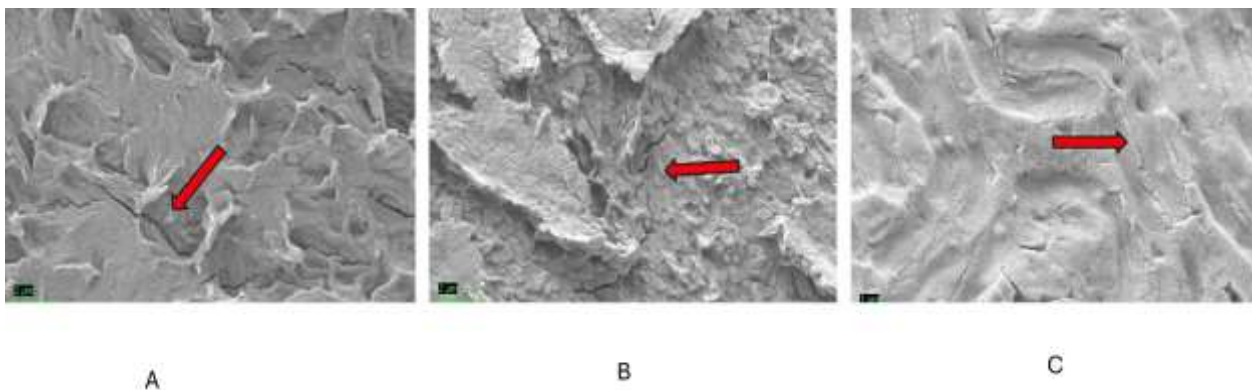


Figure-7. Scanning Electron Microscopy [A]: Poor tendon gap healing (control). [B]: Good tendon gap healing (group B). [C]: Excellent tendon gap healing (group C)

4. DISCUSSION

A mild hyperthermia was noted only at day 1, afterwards the temperatures were observed within normal range, for all groups. This initial rise may be associated with surgical stress, pain, inflammatory mediators etc. (22). Tachycardia and tachypnea at moderate levels were observed immediate post-surgery that may be related to hypoxia, hypercapnia, surgical tissue handling, acidosis and anesthesia recovery phases (23, 24).

Hematological indexes revealed, a mild leukocytosis near upper physiological limits at day 1. This TLC increase may be correlated to inflammatory responses, tissue healing cascade, tendon resection, operative stress etc. (25). Platelets slightly decreased on day 1 in all groups but overall, they remained within normal limits during studies. This initial mild thrombocytopenia may be due to tissue hemorrhages during manipulation and thrombocytes migration to site for hem-plug formation (26). A slight drop, within normal range, for RBCs and Hb was noted after surgical repair at day 1 and 7. Onwards, the values oscillated at mid-levels during trials. This initial decline could be result of peri-operative blood loss, reduced erythropoiesis due to inflammation and less GIT iron uptake after surgery (27, 28).

Radiography manifested resected hamstring tendon healing during studies. Group C showed excellent healing, scar formation and speedier recovery of the Achilles than group B and A. It was due to versatile properties of ozone i.e. ability to elicit oxidative stress that activated healing mediators, vasodilation, enhanced endogenous factors and as potent antiseptic (29). Moreover 'O₃-PRP' with its supraphysiological platelet levels resulted in thrombocyte factors, thus activating inflammatory

mediators and numerous growth factors (PDGF, TGFs, IL-1 etc.), triggering angiogenesis and speedy regeneration(30). Group B, showed good healing due to PRP's abundant cytokines and GFs but with deficient O₃, it lagged group C. Group A (control) was inferior to both groups, with noticeable delayed healing (31, 32).

Ultrasonography revealed tendon edges gap healing. The healing progress in group C was excellent and up to the mark, than groups B and A. This excellence may be due to medical ozone-PRP combo that manifested various characteristics i.e. neo-angiogenesis, fibroblast proliferation, collagen synthesis etc. (10). In group B, good gap reduction was seen that was due to PRP high platelet numbers and growth factors, but it was noted 2nd to group C (33). Healing quality and speed in group A (control) was poorer than both mentioned groups (34, 35).

Histological evaluation and scores, with Hematoxylin-Eosin/Masson Trichome stains showed abundant tenocytes and fibroblasts as hallmark of tendon healing, which produced sufficient ground substance that helped in greater tensile strength. The cells also assisted in increased vascularity and collagen development, for appropriate tissue healing. Distinctive O₃-PRP cumulative properties helped in greater chemotaxis of inflammatory cells/mediators, which in turn activated fibroblasts and manifested best healing than PRP alone or control group. For histology, excellent to poor results were manifested by group C, B and A, respectively (19).

Scanning electron microscope (SEM) helped to understand hamstring healing and tendon gap reduction in better precision. Tendon architecture remodeling was noted in terms of collagen fine fibrils, neo-angiogenesis, tenocytes, fiber array etc. For group C, the gap healing was excellent with complete recovery and morphology near to normal non-damaged tendon due to O₃-PRP duo qualities, as compared to group B (PRP) and A (control) which were noted at 2nd and 3rd tier, respectively (36, 37).

CONCLUSION:

Hamstring or Achilles is among the strongest tendons of the body, often revealing least or delayed healing intention in case of injury. The studies had revealed that ozonated-solutions and PRP can be employed as biological assistance, to quicken their repairs. This study may suggest that 'medical ozone-PRP' combination may be among the best choices for hamstring rupture healing than PRP or sutures alone, as it well enhanced healing quality with reduced cure time, due to unique properties. Moreover, these findings may be supported by further randomized control or clinical trials, due to some shortcomings associated with experimental station investigations.

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Conflict of interest

Authors declare no conflict of interests.

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