Micronized Amniotic Membrane & Platelets Rich Plasma in Combination Improves the Knee Function

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Abstract:

Background: Osteoarthritis is the most common degenerative joint disease, causing pain and leading to functional disability. It greatly affects the quality of life and increases the economic burden on the health care system**. Aim:** To observe the therapeutic effects of PRP and Amniotic Membrane (AM) separately and in combination in the osteoarthritic rat models. **Material and Methods:** In this study, animals received intra-articular injection of Platelet Rich Plasma and micronized Amniotic Membrane, both separately and combined, the effect of which was assessed by RT-PCR, Safranin O staining, immunofluorescence assay, histological scoring, and gene expression analysis. **Results:** Treatment with PRP + micronized AM for knee osteoarthritis had more significant results than the exclusive treatment of PRP and micronized AM. There was an upregulation of proteoglycan, collagen type II alpha, Acan, TGF-α and β, as well as downregulation of collagen types 1 alpha and casp3 with the combined treatment of PRP and micronized AM. A remarkable increase in Col2a1, Aggrecan, and a decrease in caspases after 35 days of intra-articular injection were also observed. These all factors indicate the considerable improvement in the osteoarthritis knee. **Conclusion:** The combination of PRP and micronized AM is more potent in treating osteoarthritis than their individual therapies.

Keywords: platelet-rich plasma, micronized, amniotic membrane, osteoarthritis

Introduction

Osteoarthritis (OA) is the most prevalent form of chronically destructive articular joint disease. It is characterized by inflammation, pain, stiffness, progressive loss of the articular cartilage, remodeling of basic structural bones, and disability. In 2020, about 86.7 million people were affected by OA (Cui et al. 2020).Treatment of OA is strenuous due to the avascular nature of articular cartilage, which causes a decrease in regenerative capacities and limits the potential to heal the damaged joints (Cook & Smith 2018). Platelet Rich Plasma (PRP) and Micronized Amniotic Membrane for tissue regeneration may be new hope for treating degenerative diseases. Autologous PRP is a fast, easy, economical, and effective treatment used in regenerative medicine. When exogenous agents activate platelets, alpha granules secrete various growth factors. These platelet-derived growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and the vascular endothelial growth factor (VEGF), play a primary role in angiogenesis, cell migration, cell proliferation, anti-apoptotic function, and endorse the healing process of damaged tissues (Cook & Smith 2018). In-vitro animal studies reveal that PRP encourages the proliferation of chondrocytes, proteoglycan, collagen type II, and

a small amount of bone protein synthesis (Asjid et al. 2019). In addition to many growth factors and cytokines, PRP has shown a positive effect on cartilage repair (Filardo et al. 2015).

PRP's collaborated effect with Micronized Amniotic Membrane might be a breakthrough treatment of knee osteoarthritis. The Amniotic Membrane is a tissue of fetal origin derived from the human placenta that can be easily separated from the chorion. Micronized Amniotic Membrane is a minimally less invasive therapy with anti-inflammatory function and low immunogenic response (Mifune et al. 2013). This effect may be due to the downregulation of inflammatory markers (IL-1β and TGF-β), which are central synovial inflammation mediators in OA (Willett et al. 2014). It contains various growth factors, including collagen type IV, V, VII, proteoglycan, and fibronectin, which have the potential to attenuate inflammation and promote tissue repair (Mead & Mead 2020). Amnion might help in treating OA by depleting the pro-inflammatory mediators (Cazzell et al. 2018).

In this present study, we hypothesized that the synergetic effect of Platelet Rich Plasma + Amniotic Membrane could be a better treatment option for alleviating degenerative disorders. Using rat models, we have evaluated the effect of PRP and Micronized Amniotic Membrane in osteoarthritis.

Material and Methods

Animals

Thirty males, Sprague-Dawley (SD) rats weighing about 300-350g and aged 3-4 months were used. All the animals were kept in well-ventilated cages at the temperature of 20°C, under 12 hrs light and dark cycle with proper food and water supply. Animal care and experiments were followed under the Committee of Animal Care guidelines, Quiad-e-Azam University, Islamabad, Pakistan.

PRP preparation

PRP was prepared from autologous blood by centrifugation method. PRP was prepared from the whole blood drawn from a lateral tail vein, then processed through a centrifuge to separate cellular components of blood based on specific gravity. 1.5ml of venous blood was collected in the syringe pre-filled with ACD-A (Haemonetic USP) and centrifuged immediately at 300 g for 10 minutes (Kimmerling et al. 2020). The blood is separated into three layers from bottom to top; red blood cells, a buffy coat layer, and plasma. The buffy coat and plasma layer was shifted into another tube and then re-centrifuged to isolate PRP from platelet-poor plasma at 4000g for 8 mins.

Amniotic Membrane isolation and processing

Amniotic Membrane (AM) was taken from the human placenta of a healthy donor. The placenta was placed in sterilized normal saline containing antibiotics. Then the amnion was separated from the chorion mechanically under the biosafety cabinet. Separated amnion was then washed with PBS containing penicillin (100UL/ml) and streptomycin (100mg/ml) 2–3 times to eliminate all bacterial contaminants, blood clumps, and blood (Guner & Buyukbebeci 2013). Each rat

received an intra-articular infiltration in the right knee of the lyophilized human AM. Finally, 20 micrograms of micronized AM were transplanted in combination with PRP and separately to evaluate the effect of micronized AM.

Intra-articular injection of PRP + AM

The total number of 30 SD rats was divided into five groups, each having six rats. Surgically induced OA models were developed by transection of the anterior cruciate ligament (ACL) and medial meniscectomy. The first group was left normal as the control or naïve group. All groups were kept in separate cages and proper care and the environment was provided. The second group was left untreated, the third group received PRP and the fourth group received micronized amnion. A combination of PRP + Amnion was injected into each osteoarthritis rat model in the fifth group. In the PRP + Amnion treated group, we centrifuged amnion with PRP and injected 0.2 ml of the resultant mixture in each rat. On day 20th, after developing OA models, each group was given a single injection. After 35 days of treatment, all rat models were euthanized to obtain a knee section to evaluate the effectiveness of therapy.

Safranin-O/fast green staining

Knee sections were stained with 1.5% safranin-O (ICN Biomedicals, CA, USA), followed by a counterstain with 0.02% alcoholic fast green (Sigma Aldrich, MO, USA). The tissues were then fixed and examined under an Olympus BX61 microscope (Olympus, CA, USA) (Naseer et al. 2018). Twenty images from each group were randomly taken, computed by image J, and used for Mankin's scoring.

Immunofluorescence of Col2a1 and Aggrecan

Immunofluorescence was performed by adding primary antibodies against Col2a1 (collagen type II alpha 1), and Aggrecan (Santa-Cruz Biotechnology, USA). Twenty images of each group were collected and assessed the highly fluorescent cells for the particular markers.

Gene expression analysis

Knee joint sections of rats from each group were used to squeeze out RNA. cDNA synthetic kit (Fermentas, MA, USA) was used to transcribe complementary DNA from RNA. Then, qPCR was carried out for Aggrecan, Col2a1, caspases, and PCNA[xii].

Results:

Assessment of proteoglycan contents of knee joints:

Safranin-O/Fast Green staining was performed to determine the proteoglycan contents in knee joints and observed the percentage of positive stain cells in each section. Normal smooth cartilage was observed in the normal/control group. Proteoglycans content was found normal in percentage compared to the untreated group (72.4 ± 2.5 vs 36.2 ± 2.2 %; p: < 0.0001) and uniformly distributed in articular cartilage. Chondrocytes were normal in shape, uniformly distributed, and no loss was examined.

In the untreated control group, proteoglycan content was s significantly reduced compared to the normal healthy control group (36.2 ± 2.2 vs 72.4 ± 2.5%; p: < 0.0001). Treatment with PRP, Amniotic Membrane, and combination of PRP + AM resulted in significant improvement in proteoglycan content, (42.9 ± 3.0, 48.8 ± 4.6 and 60.0 ± 4.0%, respectively) as compared to untreated OA group (p-values; 0.035, 0.013 and 0.0008, respectively)(Fig. 1).

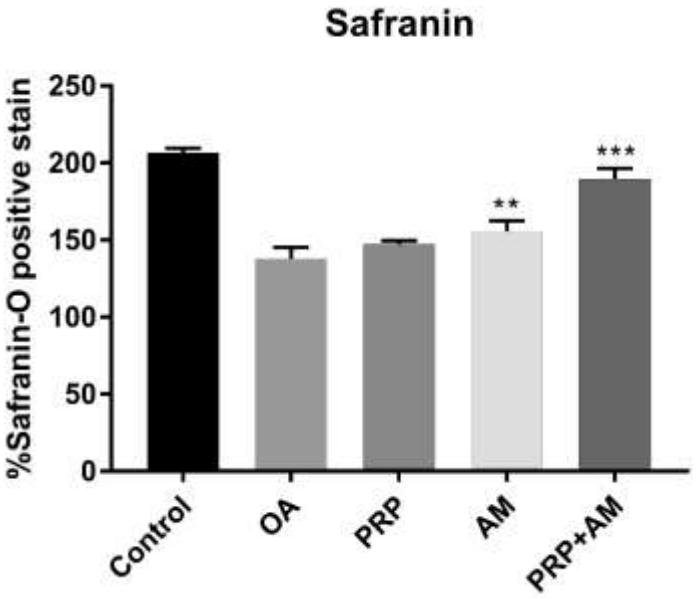

Figure. 1. Safranin-O/Fast Green staining determined the proteoglycan content of all the groups at 35 days after treatment. It shows the percentage of Safranin-O/Fast Green in the control or normal group, untreated OA group, PRP treated group, Micronized Amniotic Membrane group, and the combined PRP + AM treated group (p-value: 0.035, 0.013, and 0.0008, respectively).

values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

Loss of proteoglycans, osteophytes, and severe fibrillation was observed in osteoarthritis knee joints. However, the percentile of Safranin-O positive stain cells when examined after respective treatments was significantly higher than the osteoarthritic knee.

Platelet Rich Plasma and Micronized amniotic membrane transplanted knee joints showed that the effects of OA were reduced but with a serene loss of proteoglycans and minor fibrillation. In combined therapy of PRP+AM proteoglycans contents found significantly high as compared to their distinctive treatments (PRP + AM vs PRP [P=0.000 ± 00 vs 000] and PRP + AM vs AM ± 00%; [p: < 0.0001) respectively.

In combined therapy of PRP+AM treatment showed minor cartilage loss, no fibrillation, and chondrocytes distribution was very similar to the normal knee joint.

Histological score

Mankin score was higher in the osteoarthritic knee (13.1 ± 1.63), indicating cartilage damage. The higher score indicates worsening of the cartilage tissue.

The histological score of combined therapy (PRP+AM) was significantly low when compared with injured cartilage (6.2 ± 0.9 vs; 13.1 ± 1.63 p: < 0.0001), indicating improvement in damaged cartilage tissue. The histological score of PRP and AM transplanted groups (10.9 ± 1.3 and 9.7 ± 1.2) was also lower than the untreated group (13.1 ± 1.63) (p-values = 0.394 and 0.103, respectively), as shown in [Fig. (2)]. Moreover, the scoring is comparatively lower in combined therapy (PRP + AM) than PRP and AM treated group alone [p-value = 0.004 vs 0.394 and 0.103], respectively.

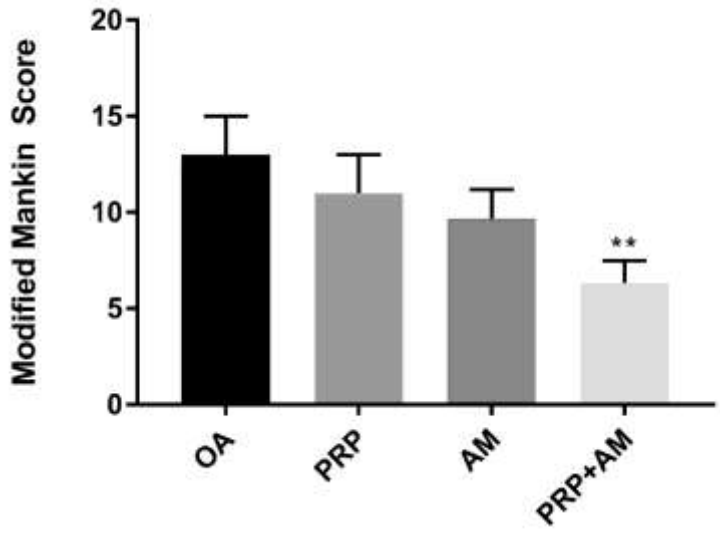

Figure. 2. Modified Mankin Scoring determined the severity of all the groups at 35 days after treatment. It shows the intensity of the untreated OA group, PRP treated group, Micronized Amniotic Membrane group, and the combined PRP + AM treated group (p-value: 0.394, 0.103, and 0.004), respectively.

values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

Immunofluorescence of knee joints for Acan and Col2a1

Immunofluorescence examination was done for Acan and Col2a1 [Fig. 3 and 4]. Expression of Acan was reduced in the osteoarthritic knee joint as compared with a normal knee joint (52.6 ± 2.05% vs 99.2 ± 1.3%; p < 0.001).

Immunofluorescence expression of Acan in PRP and AM separately transplanted groups ([57.1 ± 5.35%; p: 0.368] and [60 ± 1.63%; p = 0.069], respectively) was high as compared to the osteoarthritic knee joint. The combined therapy of PRP+AM showed higher expression [75.6 ± 1.24%; p < 0.001] as compared to the OA group [Fig.3].

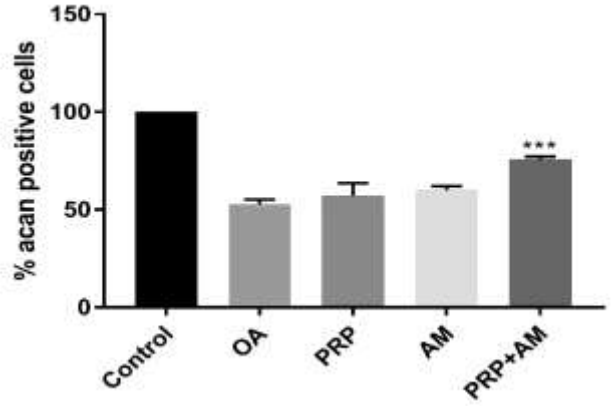

Figure. 3. Immunofluorescence determined the Acan positive cell of all the groups at 35 days after treatment. It shows the percentage of Acan positive cells in the control or normal group, untreated OA group, PRP treated group, Micronized Amniotic Membrane group, and the combined PRP + AM treated group (p-value: 0.368, 0.069, and 0.001, respectively).

values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

Similarly, in the untreated group, expression of Col2a1 was reduced in the osteoarthritic joint as compared with a normal joint (53.3 ± 0.94% vs 98.1 ± 2.1%; p < 0.001).

Immunofluorescence expression of Col2a1 in PRP and AM separately transplanted group ([60 ± 2.44%; p = 0.575] and [68.6 ± 8.2%; p = 0.049], respectively) was high as compared to the osteoarthritic joint. The combined therapy showed significant increase [80.3 ± 7.4%; p < 0.001] as compared to OA group [Fig.4].

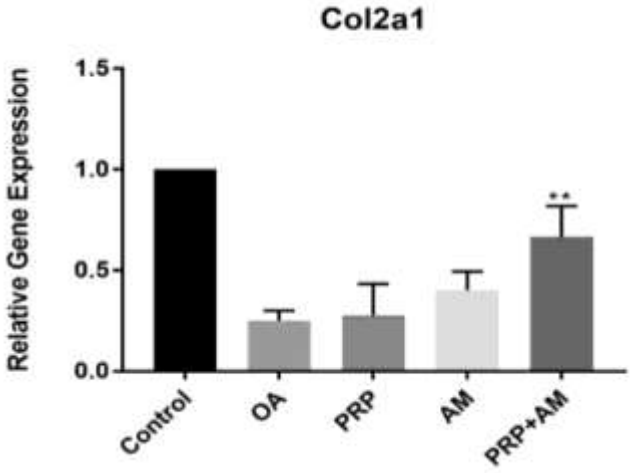

Figure. 4. Immunofluorescence determined the relative Gene expression of Col2a1 of all the groups at 35 days after treatment. It shows the percentage of Col2a1 positive cells in the control or normal group, untreated OA group, PRP treated group, Micronized Amniotic Membrane group, and the combined PRP + AM treated group (p-value: 0.575, 0.049, and 0.001, respectively).

values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

Gene expression analysis of cartilage explants

Semi-quantitative real-time PCR was performed for gene expression analysis of Casp3, PCNA, Acan, Col1a1, Col10a1, and Col2a1 genes.

The expression level of Casp3, Col1a1, and Col10a1 were observed to be upregulated in the osteoarthritic knee. At the same time, there was down-regulation in the expression levels of PCNA, Acan, Col2a1 genes when compared with normal knee joint.

In PRP and AM transplanted group, there was down-regulation in the expression of Casp3 (8.6 ± 0.47% and 8.3 ± .46% vs 10 ± .81%; p = 0.183 & p = 0.07), Col10a (11.6 ± 1.24% and 11.1 ± 1.59% vs 13.6 ± 0.43%; p = 0.271 & p = 0.096) and Col1a1 (6.5 ± 0.58% and 6.6 ± 0.51% vs 6.3 ± 0.49%; p = 0.105 & p = 0.096) genes compared with osteoarthritic knee and up-regulation in the expression level of PCNA (0.3 ± 0.08% and 0.35 ± 0.06% vs 0.15 ± 0.078%; p = 0.146 & p = 0.001), Acan (0.27 ± 0.12% and 0.40 ± 0.07% vs 0.2 ± 0.016%; p = 0.869 & p = 0.155) and Col2a1 (0.27 ± 0.11% and 0.44 ± 0.08% vs 0.25 ± 0.04%; p = 0.997 & p = 0.383) genes but not significant difference found.

On the other hand, in the combined therapy of PRP+AM, significant up-regulation of PCNA (0.8 ± 0.08% vs 0.15 ± 0.04%; p < 0.001), Acan (0.63 ± 0.12% vs 0.2 ± 0.016%; p = 0.002), Col2a1 (0.66 ± 0.12% vs 0.25 ± 0.04%; p = 0.003) and down-regulation of Casp3 (3.1 ± 0.81%vs 10 ± .81%; p < 0.001) , Col10a (6 ± 0.8% vs 13.6 ± 0.43%; p <0.001) and Col1a1(5.3 ± 0.4% vs 6.3 ± 0.49%; p = 0.007) was examined as compared to injured knee [Fig.5].

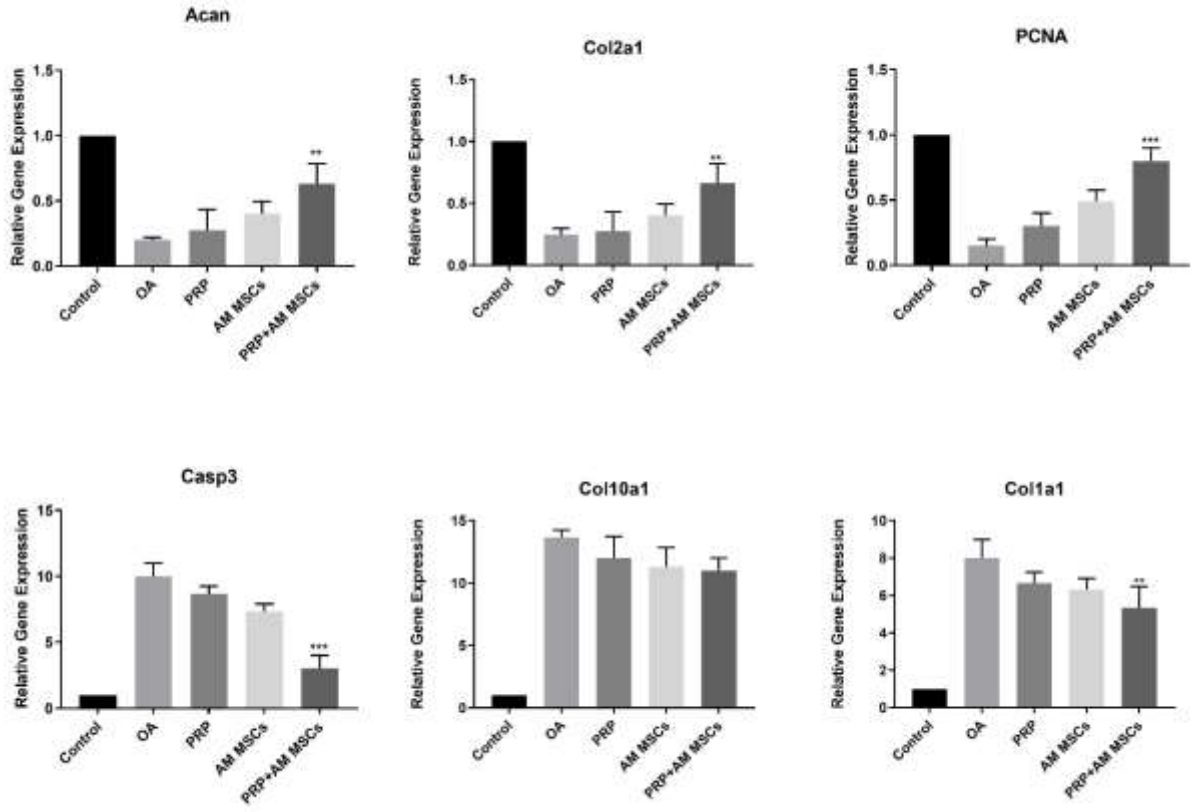

Figure. 5. Immunofluorescence determined the relative Gene expression of Acan, Col2a1, PCNA, Casp3, Col10a1, and Col1a1 of all the groups at 35 days after treatment. It shows the percentage of Acan, Col2a1, PCNA, Casp3, Col10a1, and Col1a1 positive cells in the control or normal group, untreated OA group, and PRP Micronized Amniotic Membrane group, and the combined PRP + AM treated group.

values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

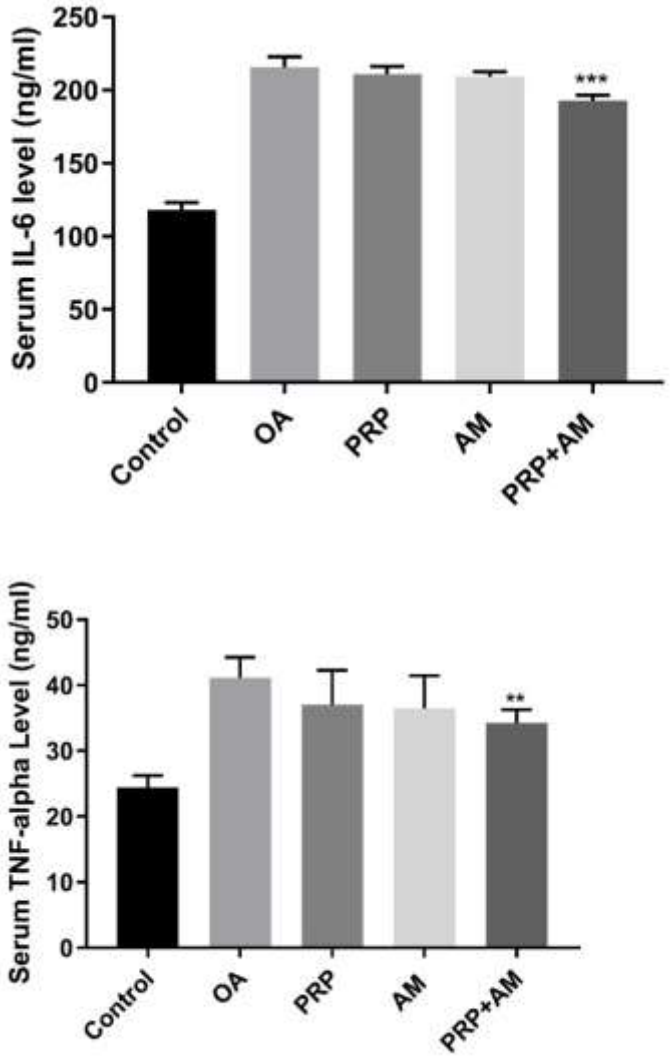

Figure. 6 Inflammatory Markers determined the relative TNF-α and IL-6 of all the groups at 35 days after treatment. It shows the IL-6 and TNF-α significantly decreased in PRP+AM as compared to untreated OA group, and PRP Micronized Amniotic Membrane group.
values are expressed as mean ± Standard Deviation. *p < 0.05; **p < 0.01; ***p < 0.001

Discussion

This study determined the regenerative potential of PRP and micronized amniotic membrane separately and in combination for induced osteoarthritis in rat models. Our study has proved that combined therapy is more effective than the individual treatment with PRP and AM. There was a substantial up-regulation of Col2a1, Aggrecan, PCNA, and a decrease in caspases after 35 days of injection compared to the untreated OA group.

Our study has demonstrated that the sole treatment with PRP and Amniotic membrane increased the expression of chondrogenic markers (Acan and collagen type 2 alpha1) as compared to the untreated group. However, the combined therapy of PRP+AM showed a significantly increased level of chondrogenesis when compared with the individual treatment. Willet et al., (2014) also noted that intra-articular injection of micronized dehydrated amnion/chorion membrane (μ-dHACM) controls osteoarthritis progression by elevating the level of TGF α, TGF β, fibroblast growth factors, PDGF, and epidermal growth factors in 21 days (Ahmed et al. 2014).

This study revealed that the combination of PRP and AM management induces an anti-inflammatory effect by reducing inflammatory markers (IL-6 and TNF-α) as depicted in fig.6. Treatment with PRP, AM, and the combined therapy of PRP and AM resulted in a remarkable decrease in IL-6 levels (211 ± 5.06, 209.71 ± 2.65 and 192.85 ± 3.57 %, respectively) as compared with the untreated OA group (215.57 ± 7.04 %). The combined treatment of PRP+AM resulted in a significant decrease in IL-6 levels, as compared with separate treatment (PRP + AM; $p > 0.001$, PRP; $P= 0.279$ and AM; $p = 0.066$). Similarly, the level of serum TNF-α was considerable decrease in PRP (37 ± 5.26 %), AM (36.42 ± 4.99 %) and the combination of PRP + AM groups (34.28 ± 1.98 %), as compared with untreated OA group (41.14 ± 3.13) (p-values 0.138, 0.08 and 0.006, respectively). However, in our study, the combined therapy of PRP+AM did not result in a better reduction in serum TNF-α levels than separate treatments (Willett et al. 2014).

Various other experimental studies have been executed to evaluate the effect of platelet-rich plasma and amniotic membrane in the management of osteoarthritis. Moussa *et al.* studied PRP's effect on the osteoarthritis cartilage and determined that chondrocyte propagation is directly proportional to the number of growth factors present in the PRP. Moreover, this study reported that chondrocyte apoptosis was significantly decreased with the administration of PRP therapy[1]. The expression of the Casp3 gene can measure apoptosis (Varley et al. 2005), and its increased expression has been reported with the progress of OA (Chen et al. 2012). Similar to Moussa et al., our results showed a significant reduction in apoptosis by down-regulating the gene expression of Casp 3.

Marino and his colleagues demonstrated that intra-articular injection of Amniotic Membrane improved the histological features of osteoarthritis cartilage and reduced the disease progression, which may be due to the AM growth factors that allow cell migration and re-epithelialization (Xu et al. 2012). Another study evaluated that AM has significantly increased the expression of collagen (COL2a1) and Aggrecan marker (ACAN) in the treated group and depicted a subsequent increase in the chondrogenesis and the extracellular matrix (Marino-Martínez et al. 2019). This present study showed improved histological features of OA cartilage by inducing

chondrogenesis and showed a significant increase in Col2a1 and ACAN. The immunostaining study revealed that the percentage of ACAN and Col2a1 is much higher in the combined treatment group (75.6 ± 1.24% and 80.3 ± 7.4%, respectively) when compared with the AM treated group (60 ± 1.63% and 68.6 ± 8.2% respectively). The PRP treated group (57.1 ± 5.35% and 60 ± 2.44%). Gene expression of Col2a1 and ACAN in cartilage reconfirmed that the combined PRP + AM transplanted group (0.66 ± 0.12and 0.63 ± 0.12, respectively), showed higher expression as compared with the AM treated group (0.44 ± 0.08% and 0.40 ± 0.07%, respectively) and the PRP treated group (0.27 ± 0.12% and 0.27 ± 0.11%, respectively). This might be due to the high content of transforming growth factors (TGFs), which upregulates the expression of chondrogenic markers (Naseer et al. 2018).

Conclusion

The present study results suggested that PRP in combination with the amniotic membrane is more significant for cartilage reformation. There was a considerable increase in proteoglycan, Col2a1, and Aggrecan expression after combined therapy of both PRP and amniotic membrane. Thus, it is concluded that combined usage of PRP and Amniotic membrane is therapeutically more significant than their exclusive therapy in the treatment of osteoarthritis. As this study was an animal trial, further research is needed to evaluate the long-term benefits of combined therapy for osteoarthritis's clinical treatment and make this treatment applicable in human beings.

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