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Combination of ADMSCs and chondrocytes reduces hypertrophy and improves the functional properties of osteoarthritic cartilage

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SUMMARY

Objective: To evaluate the therapeutic efficacy of Adipose derived MSCs (ADMSCs) in combination with chondrocytes in counteracting oxidative stress in chondrocytes *in vitro* and in rat model of osteoarthritis (OA).

Method: Cultured chondrocytes were exposed to oxidative stress with 200 μM Hydrogen peroxide (H_2O_2), followed by co-culture with ADMSCs or chondrocytes or combination of both cell types in a transwell culture system for 36 h. The cytoprotective effect was assessed by immunocytochemistry and gene expression analysis. *In vivo* study evaluated therapeutic effect of the above mentioned three treatments after transplantation in OA rats.

Results: The Combination of ADMSCs + Chondrocytes decreased the extent of oxidative stress-induced damage of chondrocytes. Enhanced expression level of Acan and Collagen type-II alpha (Col2a1) with a correspondingly decreased expression of Collagen type-I alpha (Col1a1) and Matrix metallopeptidase 13 (Mmp13) was maximally observed in this group. Moreover, reduced count of annexin-V positive cells, Caspase (Casp3) gene expression and Lactate dehydrogenase (LDH) release with concomitantly enhanced viability and expression of proliferating cell nuclear antigen (PCNA) gene was observed.

In vivo study showed that homing of cells and proteoglycan contents of knee joints were significantly better in ADMSCs + Chondrocytes transplanted rats. Increased expression of Acan and Col2a1 along with decreased expression of Col1a1 and Mmp13 indicated formation of hyaline cartilage in this group. These rats also demonstrated significantly reduced expression of Casp3 while increased expression of PCNA genes than the other cell transplanted groups.

Conclusions: Our results demonstrated that a combination of ADMSCs and chondrocytes may be a more effective therapeutic strategy against OA than the use of ADMSCs or chondrocytes separately.

Introduction

Articular cartilage is highly specialized avascular connective tissue within the diarthrodial joints. Cartilage provides smooth surface and low friction for load support, transfer and motion[1]. Osteoarthritis (OA) damages articular cartilage resulting in lesions and disturbed function of the joint. The repair potential of cartilage tissue is low due to its avascular, alymphatic and aneural nature[2]. Therefore development of successful treatment strategies represents a major challenge.

Autologous chondrocyte implantation (ACI) has proven effective against OA[3] and has been used in various clinical trials[4,5]. However, problems associated with maintenance of monolayer culture of chondrocytes, de-differentiation and their uneven distribution at the target site have raised practical difficulties[2,6,7]. Consequently, Adipose derived MSCs (ADMSCs) that can be isolated easily as compared to other sources of mesenchymal stem cells (MSCs) are gaining popularity for cartilage repair[8]. However, use of MSCs for cartilage repair has been reported to be associated with their hypertrophy in chondrogenic induction conditions and calcification after transplantation[9,10]. These issues merit the exploitation of alternative strategies with ability to address these limitations and treat cartilage injuries more effectively.

It has been reported previously that chondrocytes release paracrine factors that induce the chondrogenesis of MSCs[9,10], inhibit the terminal differentiation of chondrocytes[11,12] and also hypertrophy of MSCs in co-culture[13]. Hence, it may be hypothesized that the use of MSCs and chondrocytes in combination for the

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treatment of cartilage injuries may increase the therapeutic efficiency of both cell types by merging their beneficiary effects and by minimizing the problems encountered with transplantation of MSCs and chondrocytes separately.

Chondrocytes are susceptible to the attack of reactive oxygen species (ROS) during OA[14,15]. Hydrogen peroxide (H_2O_2) – a form of ROS causes apoptosis and senescence of chondrocytes leading to severe damage of cartilage[16–18]. Hence, H_2O_2 has also been used to induce *in vitro* oxidative stress injury for mimicking OA[19–21].

The present study is designed to investigate the efficacy of ADMSCs in combination with chondrocytes in alleviating H_2O_2-induced oxidative stress in chondrocytes *in vitro* as well as in improving OA in rats.

Methods

Animals

Male Sprague–Dawley (SD) rats aged 4–5 months were used. All animal handling and experimental procedures were in accordance with the guidelines of the Committee of Animal Care, National Center of Excellence in Molecular Biology, Lahore, Pakistan.

Culture of ADMSCs and chondrocytes

ADMSCs were isolated from adipose tissue of abdominal cavity as reported[22]. Chondrocytes were isolated from the heads of tibia and femur of SD rats as reported[23,24]. Cells at second passage were used for all subsequent experiments.

Oxidative stress induction in chondrocytes and co-culture experiment

Chondrocytes were plated in a 6-well plate (Becton Dickinson, USA) at a concentration of 1×10^4 cells/well and randomly divided into five groups. Injury was induced by exposing these chondrocytes to 200 μM H_2O_2 for 3 h (Merck)[25,26]. Equal numbers of freshly trypsinized ADMSCs, or chondrocytes, or ADMSCs + chondrocytes mixed in a ratio of 1:1 were placed on the membranes of inserts. The inserts containing these normal cells were placed in each well of 6-well plate having injured chondrocytes on completion of 3 h of H_2O_2 exposure. The normal cells above the inserts and injured chondrocytes underneath shared the same Dulbecco's modified eagle's medium (DMEM) but were not in direct contact with each other. The injured chondrocytes were then used for further analysis at 36 h of co-culturing.

Immunoflourescence for Acan, Collagen type-II alpha (Col2a1) and annexin-V

The treated injured chondrocytes were incubated with Anti-Aggrecan [6-B-4], Anti-Collagen II and Anti-Annexin-V (Abcam, MA, USA), followed by staining with respective secondary antibodies (Jackson Immuno Research Laboratories, USA). At least 30 images per group, captured from three separate experiments, were used to count positive cells for evaluation of expression of above mentioned markers in various study groups.

Western blot analysis

Anti beta-actin, anti Acan and anti Col2a1 in a ratio of 1:1000 (Abcam, USA) and horseradish-peroxidase (HRP) conjugated secondary antibodies (1:2000) were used for western blotting of protein extracted from treated chondrocytes, as reported[27].

In vitro gene expression analysis

RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA by using cDNA Synthesis Kit (Fermentas, MA, USA). All gene specific primers were designed online by using Primer3[28]. Semi-quantitative real-time Polymerase chain reaction (PCR) was performed for Acan1, Collagen type-I alpha (Col1a1), Col2a1, Matrix metallopeptidase 13 (Mmp13), Collagen type-10 alpha (Col10a1), proliferating cell nuclear antigen (PCNA), and Caspase (Casp3) genes (Table I) using SYBR® Green PCR Super Mix (BioRad Lab, CA, USA). Beta-actin (β-Actin) was used as an internal control.

Cell viability assay and Lactate dehydrogenase (LDH) cytotoxicity assay

Viability of chondrocytes was assessed by XTT assay while cytotoxicity was analyzed using *In vitro* Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma Aldrich, USA) as reported[27]. Three separate experiments, each in triplicate were performed.

Rat model of OA

Surgically-induced rat model of OA was developed by transection of anterior cruciate ligament (ACL) along with partial meniscectomy (anterior cruciate ligament transection (ACLT) + meniscectomy (MMx))[29]. Operated animals were kept in separate cages and were allowed to move freely with an access to water and food *ad libitum*.

Transplantation of ADMSCs, chondrocytes and ADMSCs + Chondrocytes

A total number of 30 SD rats ($n = 6$ per group) were used for *in vivo* analysis. Animals were randomly divided into five groups. PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma Aldrich, USA) was used to label ADMSCs, chondrocytes and ADMSCs + Chondrocytes prior to transplantation. All cell types were transplanted at day 30 after transaction of ACL and MMx by direct injection of cells into the knee joint space at a concentration of 1×10^6 cells per 50 μl phosphate buffer saline (PBS) per animal. In ADMSCs + Chondrocytes treated group, we used mixture of ADMSCs (1×10^3 cells) + Chondrocytes (1×10^3 cells) in 50 μl PBS per animal. All animals were euthanized at day 42 after transplantation.

Table I
Primer sequences of genes used in the study.

Gene	Sequence of primer (5′ → 3′)	Product size	Annealing temperature
β-Actin	F: GCTGTGTTGTCCCTGTATGC R: GAGCGCGTAACCCTCATAGA	106 bp	56°C
Agc1	F: ACTGAAGGACAGGTTCGAGTG R: CACACCGATAGATCCCAGAGT	133 bp	60°C
Col1a1	F: CAAGATGGTGGCCGTTACTAC R: TTAGTCCTTACCGCTCTTCCAG	198 bp	57°C
Col2a1	F: GACTTTCCTCCGTCTACTGTCC R: GTGTACGTGAACCTGCTGTTG	171 bp	60°C
Mmp13	F: GAGTTGGACTCACTGTTGGTC R: GCAAGAGTCACAGGATGGTAG	216 bp	58°C
Casp3	F: ACAGAGCTGGACTGCGGTAT R: TGCGGTAGAGTAAGCATACAGG	110 bp	57°C
PCNA	F: TGACCCTCACCGATACAACA R: CTGTACAGCACAGCCACGTT	110 bp	57°C
Col10	F: AGGCACTAAAGGTGAGACACGT R: CAACAAGACCTCGAAGACCAG	248 bp	59°C

Safranin-O/fast green staining and Mankin's scoring

Sections of knee joints were stained with 1.5% Safranin-O (ICN Biomedicals, Germany) and counter stained with 0.02% alcoholic Fast Green. The sections were mounted and observed under Olympus BX61 microscope (Olympus, USA). At least 30 images were randomly taken for each group, quantified by image J and used to perform modified Mankin's scoring[30].

Immunoflourescence for Acan and Col2a1

Immunoflourescence was done using primary antibodies against Acan and Col2a1. At least 30 images per group were evaluated by counting the positive cells per high power field for the respective marker.

In vivo gene expression analysis

Cartilage from the knee joint ($n = 6$ rats each group) was minced by a homogenizer and used for RNA extraction. cDNA was synthesized from 1 μg of total RNA by using cDNA Synthesis Kit (Fermentas, MA, USA). Semi-quantitative real-time PCR was performed for Acan, Col2a1, Col1a1, Col10a1, Mmp13, PCNA, and Casp3 genes by already mentioned protocol.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.00 for Windows, GraphPad Software, USA). Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple-comparison posttest for the comparison of group mean differences against the control group. Two-tailed unpaired Student's *t* test was used to compare the means of two groups. ANOVA and Student's *t* test used, fulfilled the assumptions that the tested observations are (1) independent (as mean value of the replicates for each animal was used), (2) have a Gaussian distribution (Shapiro–Wilk test was used to check the normality of the data)[31,32], and (3) have homogeneous variance (Levene's test was used to check equality of variances)[33,34]. Statistical significance was considered at $P \leq 0.05$. At least $n = 3$ observations were included in statistics analysis.

Results

Effect on composition of extracellular matrix

Immunoflourescence, gene expression and western blot analysis [Fig. 1] revealed that expression of both Acan and Col2a1 increased in injured chondrocytes *in vitro* after treatment with ADMSCs, or Chondrocytes or ADMSCs + Chondrocytes. Most prominent increase was observed in injured chondrocytes in ADMSCs + Chondrocytes group.

On the other hand, down-regulation in the expression Col1a1 and Mmp13 was observed only in chondrocytes in group Chondrocytes and group ADMSCs + Chondrocytes as compared to group H_2O_2 untreated injured chondrocytes [Fig. 2(G) and Fig. 2(I)]. Col10a1 gene was down-regulated only in group ADMSCs + Chondrocytes injured chondrocytes [Fig. 2(H)].

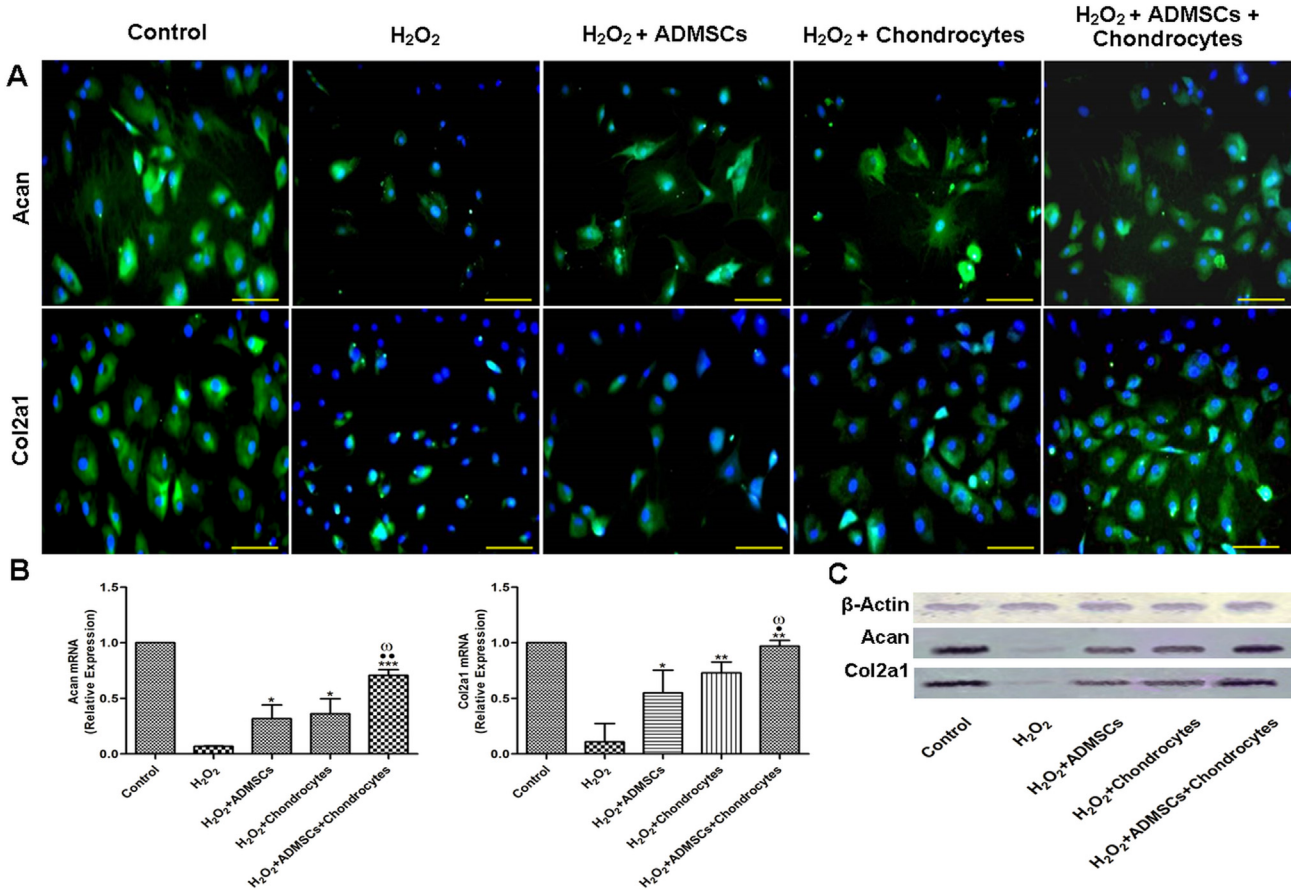

Fig. 1. Assessment of Acan and Col2a1 in various groups at 36 h of treatment. (A) Immunoflourescence analysis of Acan and Col2a1 (green). Blue nuclei indicate 4′,6 diamidino-2-phenylindole (DAPI). Bar ~100 μm. (B) Gene expression analysis of Acan and Col2a1. (C) Western blot analysis of Acan and Col2a1 protein expression.

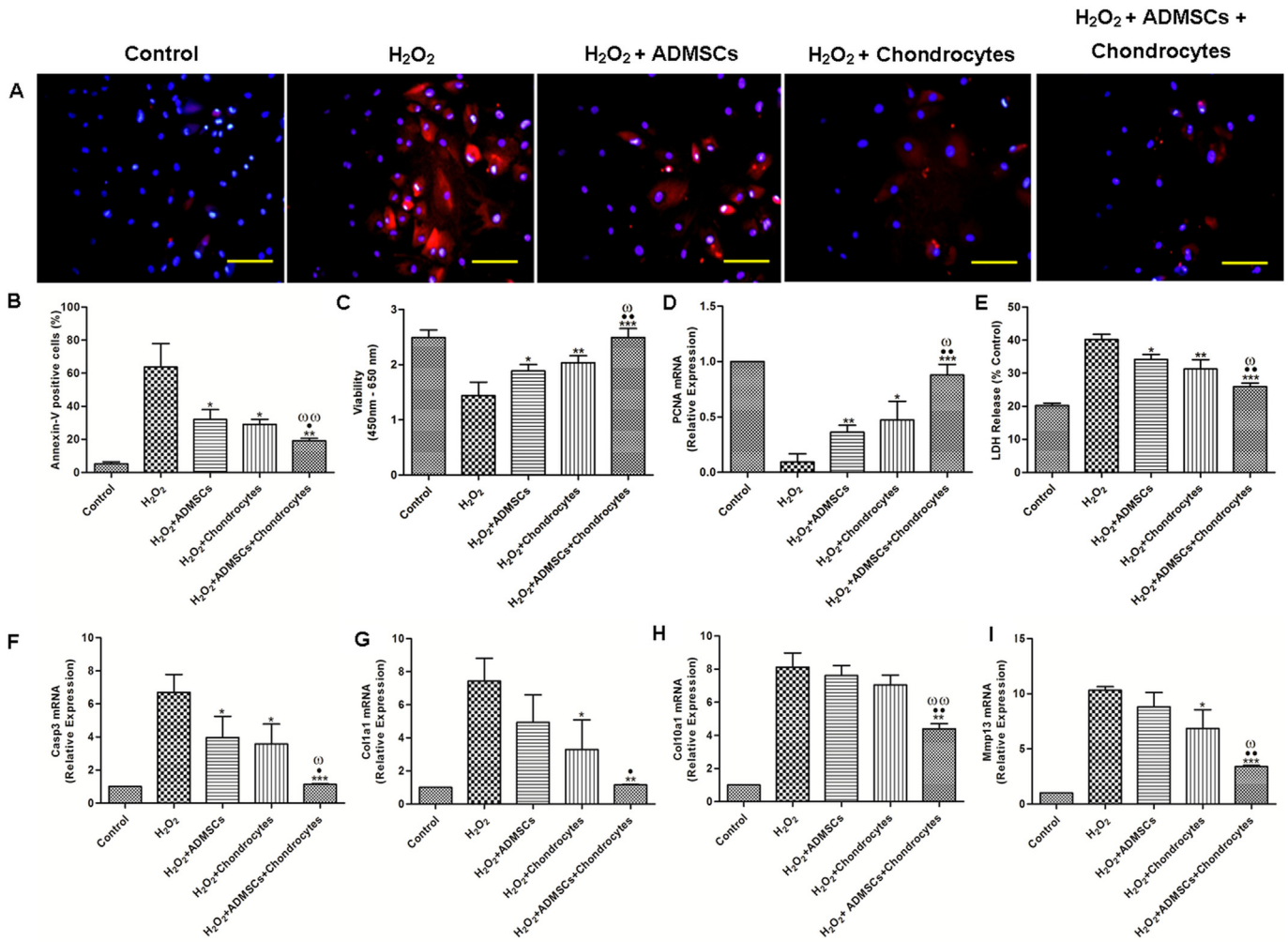

Fig. 2. Assessment of apoptosis, viability, cell damage and hypertrophy in various groups of oxidative stress induced chondrocytes at 36 h of treatment. (A) Annexin-V staining (red). Blue nuclei indicate DAPI. Bar ~100 μm. (B) Quantification of percentage of annexin-V positive cells. (C) Viability assay. (D) Analysis of relative expression of PCNA gene. (E) LDH release assay. Semi quantitative gene expression analysis of: (F) Casp3; (G) Col1a1; (H) Col10a1; and (I) Mmp13. Data is presented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs group treated with H_2O_2. $^{\bullet}P \leq 0.05$, $^{\bullet\bullet}P < 0.01$ vs group ADMSCs treated. $^{\omega}P \leq 0.05$ vs group treated with chondrocytes.

Analysis of apoptosis and cell damage in oxidative stress induced chondrocytes

The expression of annexin-V positive cells, LDH release and Casp3 gene in group ADMSCs + Chondrocytes treated chondrocytes was significantly less than both the group ADMSCs and group Chondrocytes treated chondrocytes [Fig. 2(A)–(B) and Fig. 2(E)–(F)].

Viability and proliferation of chondrocytes

The analysis of XTT assay [Fig. 2(C)] and expression of PCNA gene [Fig. 2(D)] revealed that viability of oxidative stressed chondrocytes increased after treatment with group ADMSCs + Chondrocytes was significantly more than the viability found in group ADMSCs and group Chondrocytes treated chondrocytes.

Homing of transplanted cells

Tissues from ADMSCs + Chondrocytes transplanted group demonstrated significantly higher number of PKH26 labeled cells (22 ± 2.04 cells/field) as compared to ADMSCs transplanted group (9 ± 3.13 cells/field) and Chondrocytes transplanted group (14 ± 4.01 cells/field) as shown in Fig. 3(A)–(B).

Assessment of proteoglycan contents of cartilage tissue and Mankin's scoring

Markedly decreased proteoglycan contents, with increased fibrillation was observed in osteoarthritic knee. Decrease in fibrillation was observed in knee in Chondrocytes transplanted group as well as ADMSCs + Chondrocytes transplanted group. However, uniformity of proteoglycan was more prominent in ADMSCs + Chondrocytes transplanted group only [Fig. 4(A)–(B)]. Mankin's scoring also revealed less severity of OA in ADMSCs + Chondrocytes transplanted group [Fig. 4(C)].

Immunoflourescence for Acan and Col2a1

Immunoflourescence analysis (Fig. 5) revealed that percentage of Acan and Col2a1 positive cells in ADMSCs + Chondrocytes transplanted group (85.5 ± 3.82% and 74.4 ± 5.17% respectively) was significantly higher than in ADMSCs transplanted group (39.4 ± 4.9% and 36.8 ± 5.9 respectively) as well as Chondrocytes transplanted group (46.7 ± 4.7% and 47.80 ± 5.8% respectively).

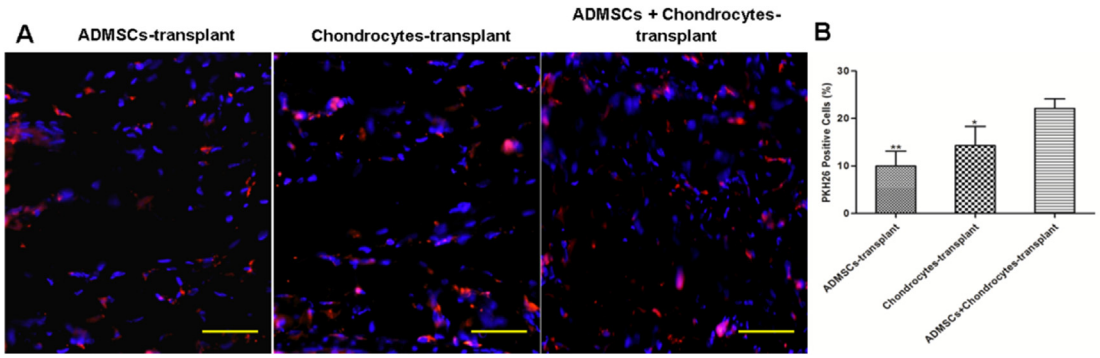

Fig. 3. Assessment of homing of transplanted cells in OA cartilage at 42 days of transplantation. (A) PKH26 positive cells (red) in various groups. Blue indicates DAPI labeled nuclei. Bar ~100 μm. (B) Percentage of PKH26 positive cells (determined by counting the positive cells from randomly selected 30 images per group). Data is presented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P < 0.01$ vs ADMSCs + Chondrocytes-treated group.

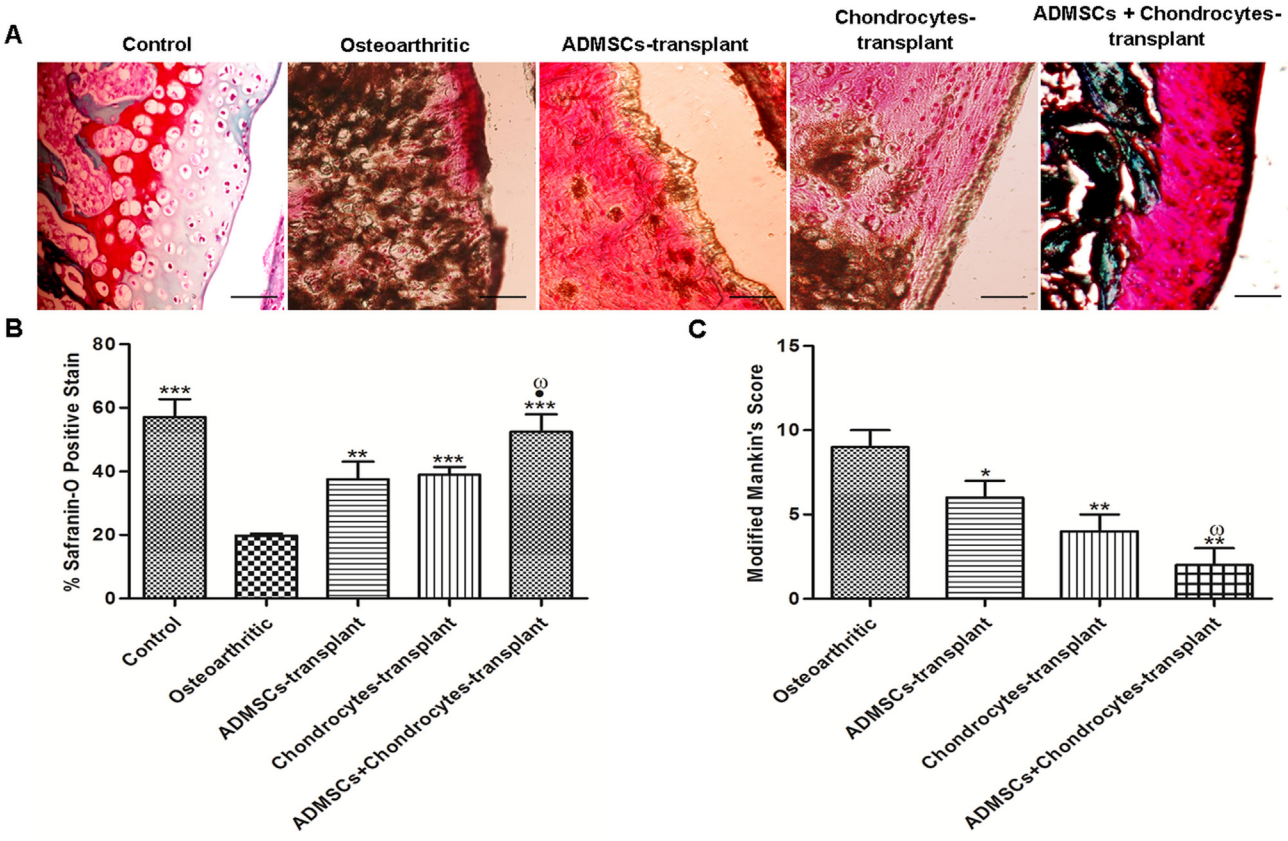

Fig. 4. Assessment of proteoglycan contents in various groups at 42 days of transplantation. (A) Safranin-O/Fast Green staining. Bar ~100 μm. (B) Quantification of percentage of safranin staining. (C) Quantification of OA severity with Mankin's score.

Gene expression analysis of cartilage tissue

Gene expression analysis of the cartilage tissue showed a significantly increased expression of Acan (0.96 ± 0.01-fold in ADMSCs + Chondrocytes transplanted group; 0.57 ± 0.19-fold in ADMSCs transplanted group; and 0.58 ± 0.20-fold in Chondrocytes transplanted group) and Col2a1 (0.82 ± 0.29-fold in ADMSCs + Chondrocytes transplanted group; 0.49 ± 0.07-fold in ADMSCs transplanted group; and 0.54 ± 0.07-fold in Chondrocytes transplanted group) in ADMSCs + Chondrocytes transplanted group as compared to other groups [Fig. 6(A)–(B)].

On the other hand expression of Col1a1 [Fig. 6(C)], Col10a1 [Fig. 6(D)] and Mmp13 genes [Fig. 6(E)] was significantly decreased only in ADMSCs + Chondrocytes transplanted group as compared to OA control group. No significant decrease in expression of these genes was observed in ADMSCs transplanted and Chondrocytes transplanted groups.

The expression of Casp3 gene was significantly decreased [Fig. 6(F)], whereas that of PCNA was significantly increased [Fig. 6(G)], in all cell transplanted groups.

Discussion

The idea of co-culture of MSCs and chondrocytes, to address the problems associated with ACI or transplantation of MSCs, has recently gained strength due to promising results observed with

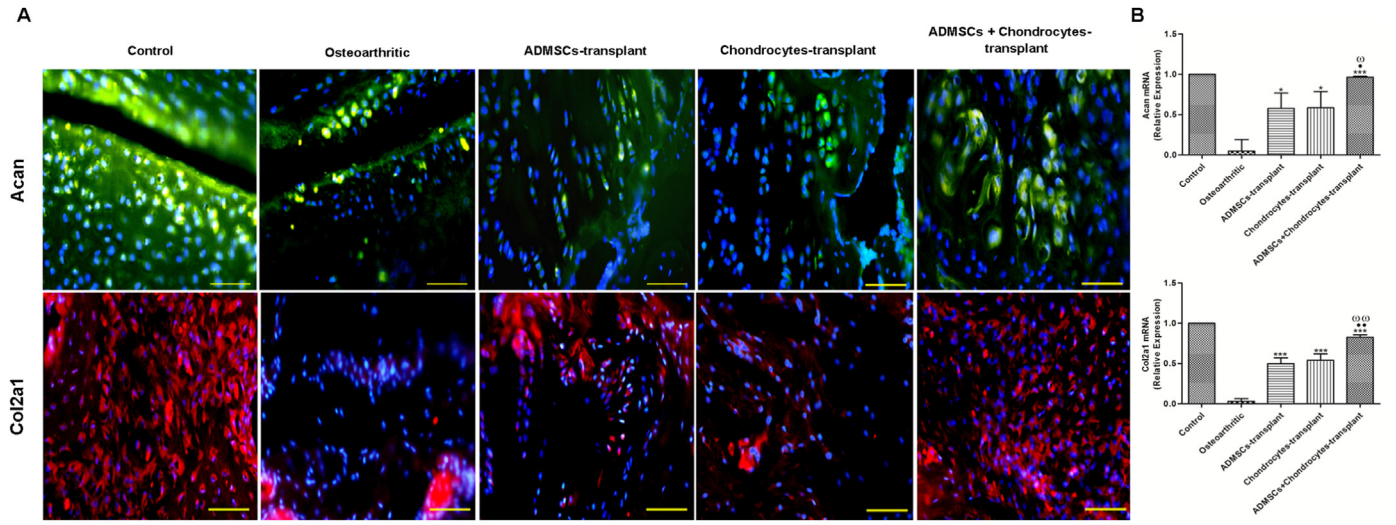

Fig. 5. Immunoflourescence expression of Acan and Col2a1 in various groups at 42 days of transplantation. (A) Acan and Col2a1 staining (green) in various groups. Bar ~100 μm. (B) Quantification of Acan and Col2a1 positive cells. Data is presented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs osteoarthritic group. $^{\bullet\bullet}P < 0.01$ vs group ADMSCs transplanted. $^{\omega}P \leq 0.05$, $^{\omega\omega}P < 0.01$ vs chondrocytes-transplanted group.

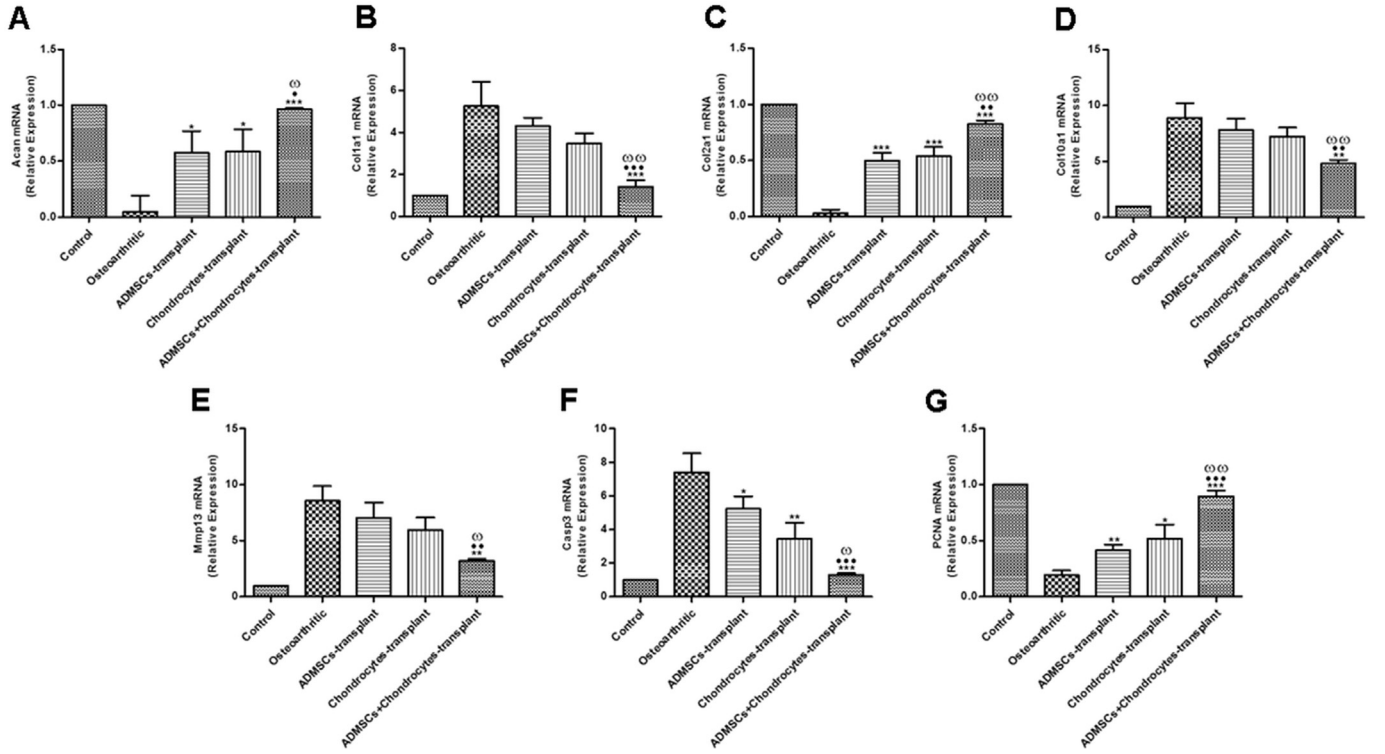

Fig. 6. Semi-quantitative gene expression analysis of cartilage tissue in different groups at 42 days of transplantation. (A) Acan. (B) Col2a1. (C) Col1a1. (D) Col10a1. (E) Mmp13. (F) Casp3. (G) PCNA. Data is presented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs group osteoarthritic group. $^{\bullet}P \leq 0.05$, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet\bullet}P < 0.001$ vs group ADMSCs transplanted. $^{\omega}P \leq 0.05$, $^{\omega\omega}P < 0.01$ vs chondrocytes-transplanted group.

their use in previously reported literature. The co-culture of human MSCs and chondrocytes in hydrogels[35], pellet cultures in the presence of chondrogenic induction medium containing factors like transforming growth factor beta (TGF-β), has been previously demonstrated to promote chondrogenesis of co-cultured cells *in vitro*[36]. These studies have mainly focused on the co-cultured cells itself or chondrogenic induction of MSCs by co-culturing them with normal uninjured chondrocytes[35–38]. These studies have also shown that induction of chondrogenesis by TGF-β can cause hypertrophy of MSCs[37,38]. Moreover, some studies performed in other degenerative organs like liver and heart[39,40] have demonstrated that use of simple mixture of different cell types without use of any prior co-culture or differentiation results in improved repair of these diseased organs.

Keeping in view these reports, a simple approach was adopted in the present study. A combination of ADMSCs and chondrocytes in 1:1 ratio without any prior co-culture or *in vitro* chondrogenic induction of ADMSCs was evaluated as potentially improved

treatment modality for OA in comparison to separate use of ADMSCs or chondrocytes. The chondrocytes used in the study were cultured in monolayer without the use of any special growth factors like TGF-β. This study at first evaluated the effect of combination of ADMSCs and chondrocytes on hydrogen peroxide oxidative stress induced chondrocytes *in vitro* and later evaluated their effect in repair of OA cartilage in rats. This study also provides an insight about the comparative therapeutic potential of ADMSCs and chondrocytes for the treatment of OA.

The major collagen and proteoglycan expressed by chondrocytes in articular cartilage are Acan and Col2a1. These are responsible for maintaining the integrity of the cartilage tissue. Oxidative stress can cause damage to chondrocytes due to accumulation of ROS, advanced glycation end products (AGEs) and mitochondrial dysfunction[41]. H_2O_2 induced oxidative stress *in vitro* as well as OA *in vivo* is known to decrease proteoglycan contents in chondrocytes and knee joint respectively[20,42]. We also observed reduced extra cellular matrix (ECM) in our cultured H_2O_2 stressed chondrocytes as well as in OA knee joints in rats. Treatment with ADMSCs + Chondrocytes reduced this damage most significantly, suggesting an increased formation of hyaline cartilage showing enhanced cartilage function in ADMSCs + Chondrocytes transplanted group [Figs. 1 and 5]. This data corresponds to the previous report showing improved proteoglycan contents in the chondrocytes and bone marrow derived MSCs co-cultured in pellet cultures[9]. This strengthens the probable role of paracrine factors released by two cell types on co-culturing[43].

A well-known phenomenon of cellular death is apoptosis that can be measured with annexin-V[20] and analysis of the expression of the Casp3 gene[44]. Our data demonstrated most significant reduction in apoptosis and cell damage with improvement in viability and proliferation of injured H_2O_2 treated chondrocytes on treatment with ADMSCs + Chondrocytes [Fig. 2(A)–(F)]. This data also correlated with our *in vivo* data as observed by maximum down regulation of Casp3 along with up-regulation of PCNA gene in ADMSCs + Chondrocytes transplanted OA rats [Fig. 6(F)–(G)].

The over-expression of Col1a1 gene is associated with the formation of fibrocartilage rather than hyaline cartilage[45]. Col10a1 over-expression induces hypertrophy of MSCs[35], and that of MMP13 is associated with progression of Col2a1 degradation[44]. In this study, expression of these genes was up-regulated in H_2O_2-treated chondrocytes *in vitro* and in rat model of OA. This is in line with previous reports[24,44]. On the other hand, combined treatment with ADMSCs + Chondrocytes resulted in most significant decrease in expression of these genes in comparison to separate ADMSCs and chondrocytes groups both *in vitro* and *in vivo* [Fig. 2(G–I)] and [Fig. 6(C)–(E)]. Amongst group ADMSCs and group Chondrocytes, only group Chondrocytes was able to show significant decrease in expression of Col1a1 and MMP13 genes as compared to H_2O_2 group *in vitro*. It is worth mentioning that *in vivo* experiments demonstrated the decrease in expression of Col1a1, Col10a1 and MMP13 only with the transplantation of ADMSCs + Chondrocytes in combination. This implicates that use of ADMSCs + Chondrocytes can help to minimize the problems associated with the use of chondrocytes or MSCs separately[2,7] for the repair of damaged cartilage.

Conclusion

This study showed that ADMSCs + Chondrocytes treatment increased the proteoglycan contents and viability of injured chondrocytes with a concomitant reduction in apoptosis and cell damage *in vitro*. Further ADMSCs + Chondrocytes transplanted group was the only group showing significant reduction in the expression of fibro cartilage associated gene Col1a1 and hypertrophy associated gene Col10a1. Hence this implicates the beneficial role of ADMSCs + Chondrocytes in protecting chondrocytes from H_2O_2-induced oxidative stress *in vitro* as well as in OA rats over separate the use of ADMSCs and chondrocytes.

Authors' contributions

MRA performed experiments, data analysis and drafted the manuscript. AM participated in the design of study, data analysis and draft of manuscript. FB helped in data analysis and draft of manuscript. SNK and SR contributed in study design and final approval of the manuscript. All authors read and approved the final manuscript.

Role of funding source

The authors also declare that the study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Competing interest statement

The authors declare that they have no competing interests.

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