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Research Article

Pain and Inflammation Profile of a Degenerated Intervertebral Disc

Sobia Ekram, Shafiqa Naeem Rajput, Shumaila Khalid, Faiza Ramzan, Asmat Salim, and Irfan Khan*

Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
*Correspondence: irfankhan@iccs.edu

Abstract

Degenerative disc disease (DDD) is characterized by progressive intervertebral disc (IVD) inflammation that ultimately causes lower back pain (LBP). In the present investigation, the rat needle punctured IVD degeneration (IVDD) model was established under a fluoroscopically guided system, followed by human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) transplantation. Quantitative real-time polymerase chain reaction (qPCR) was performed to quantify the pain and inflammatory molecular markers, including *C-JUN*, *C-FOS*, serine /threonine protein kinase *(AKT)*, osteoprotegerin (*OPG)*, receptor activator of nuclear factor κ B *(RANK)*, toll-like receptor 4 *(TLR4)* and interleukin 1 beta (*IL1-β*). Radiographic imaging and histological analyses showed significant IVDD after two weeks of injury, and the hUC-MSC group exhibited the restored nucleus pulposus (NP) region and the significant presence of proteoglycans in contrast to the degenerated group. When compared to healthy IVDs, the molecular expression of pain and inflammatory genes was significantly increased on days 2 and 5, resulting in an immunomodulatory response and a marked IVDD. Conversely, the hUC-MSC group at 2 and 5 days showed significantly downregulated expression of *C-JUN* (***p ≤ 0.001 and **p ≤ 0.01 respectively), *C-FOS* (**p ≤ 0.01), *OPG* (***p ≤ 0.001 on day 2), *RANK* (**p ≤ 0.01), *TLR4* (***p ≤ 0.001 on day 5) and *IL1-β* (**p ≤ 0.01 on day 2), compared to the IVDD group. MSCs transplanted group also showed significant upregulation on days 2 and 5 in the anti-inflammatory marker *AKT* (***p ≤ 0.001 and **p ≤ 0.01, respectively) expression. Moreover, the hUC-MSCs-treated group indicated significant NP hydration. The preclinical transplantation of hUC-MSCs can potentially reduce the pain and inflammation caused by disc degeneration. Therefore, the therapy can ideally deal with all the manifestations that occur due to such debilitating DDD.

Keywords: Degenerative disc disease, pain, regeneration, inflammation, intervertebral disc

1. Introduction

Intervertebral disc degeneration (IVDD) affects 70 to 85% of the population worldwide, manifesting a major health risk resulting in low back pain (LBP) (Hou et al. 2014). Approximately 40% of LBP cases are associated with IVDD (Li et al. 2022). IVD is composed of the central gel-like nucleus pulposus NP, collagen-rich AF, and hyaline cartilage that sandwich the AF and NP (Oegema Jr 1993, Vo et al. 2016). The central gel-like NP region results in the compressive ability of the disc followed by the molecular attraction of water molecules from the glycosaminoglycan (GAG) chains on individual monomers of aggrecan and proteoglycan in the disc (Watanabe, Yamada, and Kimata 1998). Since IVD is an avascular tissue, the inflammatory response implies degenerative cascades that cause structural disruption. These cascades are initiated by declining cellular nutrition and the accumulation of cellular waste, which further compromises cell viability (Urban, Smith, and Fairbank 2004). Endogenous factors and extracellular matrix (ECM) breakdown products increase the expression of catabolic enzymes such as a disintegrin and metalloproteinase with thrombospondin motifs-

4/5 (ADAMTS-4/5), matrix metalloproteinases (*MMPs*), interleukin-1 *(IL-1),* and tumor necrosis factor-alpha (*TNFα)* which are thought to be important in the early stages of IVDD (Grant et al. 2016, Sampara et al. 2018). The etiology of inflammation and its association with LBP is unclear. Inflammatory cytokines increase the expression of neurotrophic factors such as nerve growth factor (*NGF)* and brain-derived neurotrophic factor *(BDNF)* in the disc resulting in excessive innervation in the NP region (Noorwali et al. 2018). Aggrecan degradation occurs, followed by increased inflammatory cytokines, resulting in the loss of hydration and becoming more fibrotic, ultimately affecting the integrity of the NP and AF. Several studies have been conducted to investigate the pathogenesis of DDD, including unhealthy habits, spine infection, endplate calcification, excessive mechanical load, and genetic predisposition (Moore 2006, Benneker et al. 2005, Adams and Roughley 2006, Battié et al. 2009, Cook et al. 2014, Cheung et al. 2009, Hristova et al. 2011, Koes, Van Tulder, and Peul 2007).

Predominantly, inflammation has been recognized as detrimental to degenerative cascades (Sun et al. 2013). Researchers are investigating the physiological role of inflammation in maintaining tissue homeostasis (Medzhitov 2008). Nevertheless, to restore and maintain IVD function-associated pain, it is also important to recover a balanced inflammatory response and the biological behavior of the resident cells (Santos et al. 2013). An imbalanced response may set off the inflammatory process. The reduction of *MMP*, *IL-1* inhibition, protease, and cytokine production caused by degradative fragments of fibronectin (Marom et al. 2007), fibromodulin (Sztrolovics et al. 1999), and hyaluronan (Arroyo and Iruela-Arispe 2010) promotes monocyte migration while modulating the infiltration of inflammatory cells *in vitro.* Endogenous inflammatory cells have been observed in human IVD with an increased transcriptional expression of inflammatory and catabolic genes, including *IL-6, IL-8,* cyclooxygenase *(COX)-2,* and metalloprotease-*1-13* (Quero et al. 2013). Nociceptive nerve fiber also contributes to angiogenesis resulting in pain associated with LBP (Freemont et al. 2002).

In recent years, cell-based therapeutic approaches enhancing IVD regeneration have emerged. These therapeutic approaches utilized embryonic stem cells (Sheikh et al. 2009), immortalized NP-cell lines (Iwashina et al. 2006), hematopoietic stem cells (HSC) (Haufe and Mork 2006), autologous IVD chondrocytes (Hohaus et al. 2008), fetal spine cells (induced pluripotent SC (Chen et al. 2013), olfactory SC (Murrell et al. 2009), bone marrow-derived MSCs (Sakai et al. 2005), and human umbilical cord blood stem cells. The MSCs derived from human umbilical cord tissue are one of the most promising candidates for IVD regeneration. As reported in the canine model, MSCs increased the expression of collagen type II while lowering cell apoptosis in IVD (Serigano et al. 2010). In the rodent model, MSC engraftment has been observed for up to 24 weeks which potentially resulted in the restoration of degenerated IVD (Miyamoto et al. 2010). Besides, MSCs' proficiency in multilineage differentiation and immunomodulation effects has been suggested to improve the degeneration cascade. The role of MSCs as cytokine-releasing factories has been explored; these factories interact directly with injured cells (Prockop and Oh 2012). MSCs' influence on IVD inflammatory response still has not been fully understood due to their multifactorial complexity (Bertolo et al. 2011). However, proinflammatory cytokines, including *TNF-α, IL-3, IL-6, IL-11, IL-15,* and *MMPs* were reportedly reduced when cocultured with rat NP cells (Miyamoto et al. 2010). MSCs were either injected into NP immediately (Orozco et al. 2011) or implanted into the IVD using sponge fibers (Yoshikawa et al. 2010). It has been reported that directly injecting MSCs into the IVDD model promoted an analgesic effect resulting in IVD height improvement (Orozco et al. 2011). Recently,

IVD injection of umbilical cord-derived MSCs has been a promising therapy to mitigate chronic LBP (Pang, Yang, and Peng 2014, Pettine et al. 2015, da Silva Meirelles et al. 2009). Therefore, the demand for MSCs for various culture conditions and preclinical models is increasing to translate into clinical settings. Standardizing protocols and their associated results related to IVD required physiologically accurate alternative *ex vivo* models. This approach for degenerating IVD could bring more advancement to exploring the inflammatory mechanism in the field of stem cell research. For the successful regeneration of degenerated discs, it is crucial to address the inflammatory cascade. Therefore, in the present study, the needle-punctured model of the rat was established. The pain and the inflammatory profile of the injured disc and hUC-MSCs transplanted disc were studied. The strategy could be able to balance the inflammatory response leading to a closer step toward IVD regeneration and bringing relief to sufferers of LBP.

Table 1: Experimental Groups

Group I	Normal Control
Group II	IVD Degenerated on Day 2
Group III	IVD Degenerated on Day 5
Group IV	IVDD transplanted with MSCs on Day 2
Group V	IVDD transplanted with MSCs on Day 5

2. Materials and Methods

2.1. Ethical Approval and Sample Collection

The study conducted on the human umbilical cord tissue was approved by the local independent ethics committee for the human subjects of the Dr. Panjwani Center of Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi (UoK), under an approved protocol (IEC-009-UCB-2015). Human cord samples were collected from healthy donors with informed consent undergoing a c-section procedure at Zainab Panjwani Memorial Hospital (ZPMH).

2.2. Isolation of Mesenchymal Stem Cells (MSCs) from Human Umbilical Cord (hUC) Tissue

The sample was collected in a sterile bottle containing phosphate buffer saline (PBS) supplemented with 0.5 % ethylenediaminetetraacetic acid (EDTA) and stored at 4 °C until it was promptly shifted to the laboratory followed by processing within two hours, as described by Beeravolu *et al*., 2017. The processing protocol was performed aseptically in a biosafety cabinet (BSC) Class II, type A2 (LA24A1, ESCO, Singapore). First, the sample was washed with chilled PBS to remove the blood clots and then dissected into small pieces or explants of 1–3 mm size to expose Wharton jelly. The explants were plated in a vented T-75 tissue culture flask (708003, Nest Biotechnology, China) containing 12 mL high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 units/mL penicillin, 4 mM L-glutamine and 1 mM sodium pyruvate. The flasks were placed in an incubator (NU5500E, NuAire, USA) maintained at 37 °C and 5 % CO_2. The DMEM was replaced every third day.

2.2.1. Sub-Culturing and Expansion of Adherent MSCs

Cells from the explant were released and adhered to the plastic surface of the culture flask. After 10-15 days, when the flask was 80 % confluent, the explants were re-cultured to another T-75 flask. At

Table 2: Primer sequences for Pain and Inflammatory Markers

Gene	Forward (5′-3′)	Reverse (5′-3′)	Ta (°C)
rGAPDH	5′- CTAAAGAGACAATCCATTTCCA -3′	5′-TTTAGGCCAGCCCTTACTTATA-3′	57
rC-JUN	5′-CCGGAGAAGAAGCTCACAAG -3′	5′-CTCTGGGTCAGGAAAGTTGC -3′	60
rC-FOS	5′-CCTGCCTCTTCTCAATGACC -3′	5′-GATGCCGGAAACAAGAAGTC -3′	60
rAKT	5′-ACTCATTCCAGACCCACGAC -3'	5′-ACCACGTTCTTCTCGGAGTG -3'	60
rOPG	5′-TGGGAATGAAGATCCTCCAG -3′	5′-CCTCTTTCTCAGGGTGCTTG -3′	57
rRANK	5′-AACTCCAACTCAACGGATGG -3′	5′-TGGGAAGGCCTATGCTGTAG -3′	60
rTLR4	5′-TGCTCAGACATGGCAGTTTC -3′	5′-TCAAGGCTTTTCCATCCAC -3'	60
rIL1-β	5′-AGGCTTCCTTGTGCAAGTGT -3′	5′-TGAGTGACACTGCCTTCCTG -3'	60

this stage, the cells were labeled as passage zero (Po). Once the cells achieved 70-80% confluency, the medium was removed, and the cells were washed with 1X PBS. Cells were detached using 2 mL of 0.25 % (1X) trypsin-EDTA solution (25200-056, Gibco, UK) and centrifuged at 1000 rpm for eight minutes (Centrifuge 5804R, Eppendorf, Germany) for pellet formation. Finally, the pellet was resuspended in DMEM and re-cultured into a T-75 flask at a cell density of 2.98×10^4 cells/cm^2 containing DMEM, followed by incubation at 37 ºC in 5 % CO_2 for further expansion. MSCs in passages 2 to 4 were used in all subsequent experiments.

2.2.2. *Morphological Characterization of MSCs*

The cells exhibited fibroblast-like morphology. The images were acquired with a charge-coupled device (CCD) camera and processed with a NIS element (Nikon, Japan).

2.3. *Experiment Animals*

Male Wistar rats of 2–6 months and weight between 250-300 g were taken in this study. The *in-vivo* study was performed at the Animal Resource Facility of the PCMD, ICCBS, University of Karachi. The Institutional Animal Care and Use Committee of the ICCBS approved the study under protocol number# 2017-0051, following international guidelines for the care and use of laboratory animals. Animals were supplied with purified water and food ad libitum in a room maintained at 21 ± 1 °C temperature with a relative humidity of 55 ± 5 % at 12 h standard light: 12 h nocturnal cycles. Thirty rats were used for the qPCR analysis, with three animals used at each of the following time points: on day 2 and day 5, normal IVDs control, IVDD, and MSC transplanted IVDD groups. At two weeks after injury and cell transplantation, twelve mice were used for histological analysis.

2.4. *IVDD Model Development*

All the surgical procedures were performed in an aseptic environment in the animal resource facility of PCMD, ICCBS, and UoK. Rats were anesthetized with an intraperitoneal injection of a combination of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (7 mg/kg). To ensure complete anesthesia, the animal's reflexes were checked by the "Tail Pinch Test". The surgical procedure was performed as previously reported (Ekram et al. 2021). Under a fluoroscopic guided system (Glenbrook Technologies Bench-Top Lab Scope Instrument), the degeneration was induced with a 21 G x 1 1/2 needle in coccygeal Co6/7. The Co5/6 was included as positive control or normal IVD. The coccygeal Co6/7 were located and

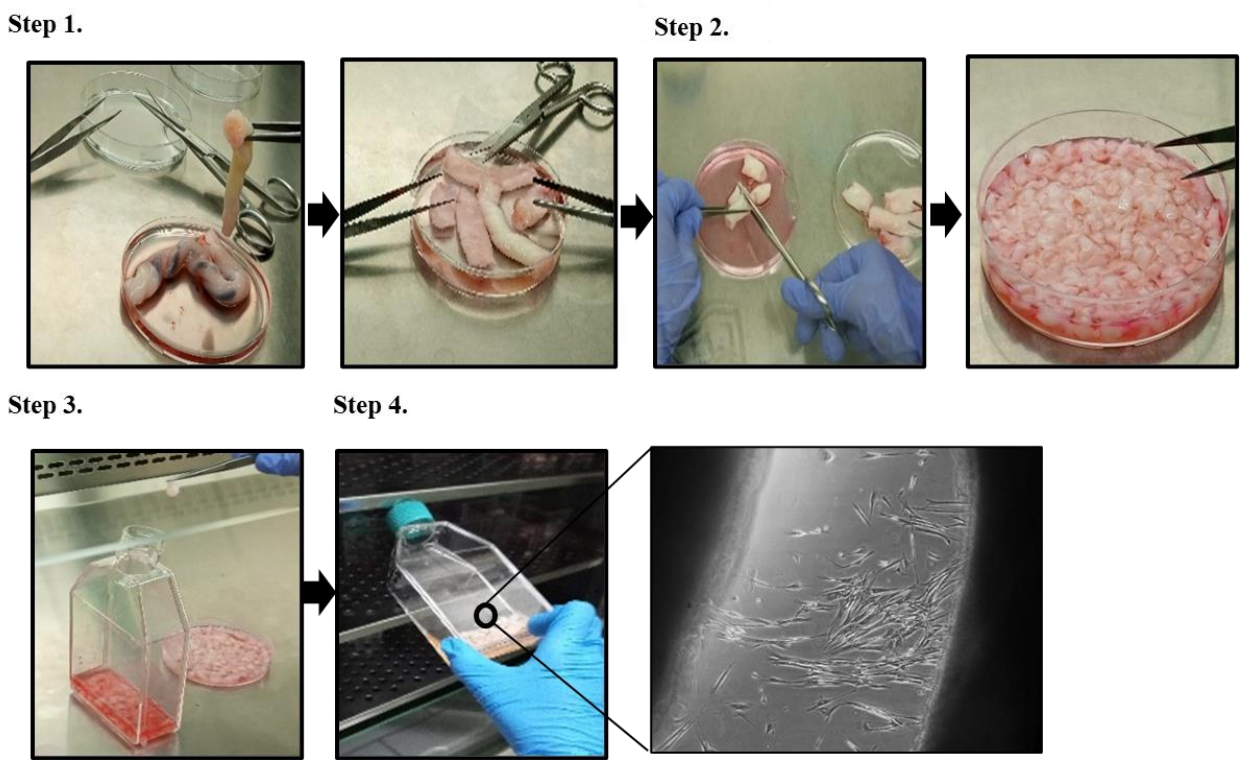

Figure 1A. Schematic Representation of Explant Culture: I. Cord sample washed and cleaned from blood cells with PBS. II. The cord was cut into 2-5 mm pieces with dissecting scissors. III. The explant tissue pieces were cultured in T-75 flasks with 13 mL complete DMEM. IV. Placed in CO_2 incubator at 37 °C with 5 % CO_2. Images showing morphology and population of MSCs, isolated from umbilical cord explant at day 7 of isolation.

confirmed by fluoroscopy. The needle was penetrated perpendicularly to the tail crossing the NP up to the contralateral AF. After penetration, the needle was rotated at 360° twice and held for 30 seconds. Following the procedure, the rats were placed back into cages and regularly monitored with standard feed prepared at the ARF facility. The animals (N = 18) were randomly divided in the qPCR study, as mentioned in **Table 1**. In this study, the animals were randomly categorized into five groups for days 2 and 5 qPCR temporal gene expression studies: one normal control group (n=6), two IVD degenerated groups on days 2 and 5 (n=6), two IVDD transplanted with the hUC-MSCs group on day 2 and 5 (n=6). Moreover, one normal, punctured, and IVDD transplanted with hUC-MSCs group (n=4) for histological and water content analysis after two weeks of post-injury and transplantation. The animals placed in the non-punctured group served as normal control IVDs.

2.5. Cell Labeling and Transplantation

Briefly, hUC-MSCs were detached, washed with PBS, and centrifuged at 1000 rpm for 10 minutes. After pellet formation, the cells were incubated with light-sensitive lipophilic cationic indocarbocyanine (DiI) membrane labeling dye (V-22885, Vybrant® DiI cell-labeling solution, Invitrogen, USA) for 5 minutes at 37 °C. The unbounded dye was inactivated by adding 6 mL complete DMEM, followed by washing twice with PBS and resuspending in 100 µL of resuspension buffer. Labeled MSCs (1×10^6 cells) were transplanted immediately after inducing degeneration in the coccygeal position Co 6/7 of the rat spine by using a sterile 100 U syringe (30 gauge × 5/16″; 0.3 mm × 8 mm). After 14 days of transplantation, animals were sacrificed, and discs were precisely harvested and demineralized in 11

% formic acid for 1 hour. Tissues were preserved in optimal cutting temperature (OCT) medium (Surgipath, FSC22, Leica Microsystems, USA) at -20 °C.

2.6. Characterization of IVD Degeneration

The IVD degeneration was evaluated based on histology, qPCR, and water content.

2.6.1. Histological Analysis

After two weeks of post-transplantation, the animals were euthanized with an intraperitoneal injection of 120 mg/kg sodium pentobarbital. The discs were harvested precisely using a scalpel blade and demineralized in 11% formic acid for 2 hours. Finally, the tissue was transferred in OCT small molds containing optimal cutting temperature (OCT) medium (Surgipath, FSC22, Leica Microsystems, USA) and kept at − 20 °C to turn into a frozen block. For cryosections, 10 µm thick sections were cut by cryostat microtome (Shandon, Thermo Electron Corporation UK). The section staining was performed using H & E and alcian blue stain to observe the ECM's tissue morphology and proteoglycan deposits.

2.6.2. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total mRNA was extracted from the tissue samples by Trizol method (Thermo Fisher Scientific), followed by homogenization as described in the literature (Lee JTY *et al*., 2015). Quantification and purity of isolated RNA were analyzed *via* spectrophotometry 260/280 and 230/260 absorbance, respectively. For cDNA synthesis, 1 µg of RNA and RevertAid First-Strand cDNA Synthesis Kit (K1622, ThermoScientifc, USA) were utilized. qPCR was performed using cDNA Master master mix (A600A, Promega, USA), and levels of *C-JUN, C-FOS, AKT, OPG, RANK, TLR4*, and *IL1-β* were evaluated using specific primers as mentioned in **Table 2**. Relative gene expression was evaluated as a ratio to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH).* The experiment was carried out in a triplicate manner.

2.6.3. Water Content Analysis

Total (n=9) IVDs were precisely harvested and kept in vials, instantly weighed, and then kept in the oven for 24 hours, evaluating the dry weight by balance with ± 0.04 mg repeatability (n=3). Water content was assessed as given below.

(%H_2O) = 100% × (wet weight-dry weight)/wet weight).

2.7. Statistical Analysis

Data were statistically analyzed using SPSS (version 19). Values are presented as means with their standard errors of means ± SEM, and the significance of differences between *in vivo* experimental groups was calculated by one-way ANOVA along with Bonferroni post hoc multiple comparisons, with values of $P \leq 0.05$ considered as statistically significant.

3. Results

3.1. Isolation and Propagation of Human Umbilical Cord-Derived MSCs

MSCs were isolated by the explant culturing method. The pictorial representation of the explant method is presented in Figure 1A. Isolated cells show a heterogeneous population of the adherent colony of the cells. On the third day of culturing, non-adherent oval-shaped blood cells were removed, and the exhausted medium was replaced with a completely fresh medium. The cells from the explants displayed tri-polar-shaped cells released from the umbilical cord tissue pieces. After 10 to 15 days, cells gradually propagated and formed a non-uniform monolayer. The explant was removed after reaching 80% confluence. These cells were considered Po. After the second week of subculture, they seemed to develop clonally and show predominantly plastic-adherent, typical spindle-shaped fibroblast-like morphology represented in Figure 1B.

3.2. Development of a Fluoroscopic IVDD Model

The rat model of IVDD was successfully established under fluoroscopic guidance through needle puncture, as a detailed illustration is shown

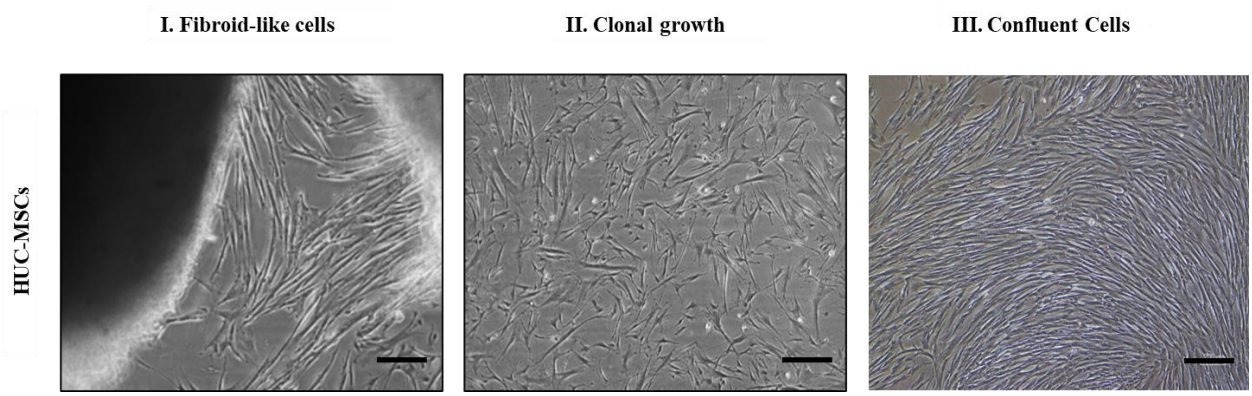

Figure 1B. Morphology of MSCs: I. Representative images of primary culture of hUC-MSCs outgrowth from the explants of hUC showing epithelioid-like morphology under phase contrast microscope. Migrating cells appear at the edge of explants. II. After 10-15 days, clonal growth of adherent cells at P_0. III. Confluent cells of hUC-MSCs at P_1. Scale bar 100 μm.

in Figure 2. MSCs were transplanted into the NP region of the Co6/7 coccyx IVDs. At 2 weeks post-transplantation, fluoroscopic images of the coccyx disc were captured with the rats under anesthesia. The mean disc height (DH) of the MSCs transplanted IVDD (Co6/7) group was significantly higher than the degenerated IVDs (Co6/7) group. The differences in DH were significant at two weeks of post-transplantation.

3.3. Histological Analysis

Histology was performed to observe the regeneration and repair of the disc by the cell therapy approach. Hematoxylin and eosin (H and E), and alcian blue staining were performed to analyze IVD tissue sections. The punctured discs after 2 weeks of degeneration are shown in Figure 3. H and E analysis of punctured IVDs showed decreased cellularity as a fissure appearance in the NP region. In addition, transplanted MSCs in the NP region exhibited that the cellularity and structure of the NP progressed better than IVDD. The transplanted MSCs integrated and dispersed in the injured areas of the NP and had higher glycoprotein content as compared to the IVDD, evident by alcian blue staining. The degeneration of the disc was greatly reduced in the cell therapy group.

3.4. Overall Gene Expression of Degenerated and MSCs Treated IVDD

To explore the changes at molecular levels in IVDs, RNA was isolated from 2, and 5 days' degenerated IVDs for real-time cDNA synthesis and real-time quantitative PCR (qPCR). Normal discs were used as control, and *GAPDH* was used as a housekeeping gene. Primers corresponding to rat-specific pain and inflammatory genes *C-JUN*, *C-FOS*, *AKT*, *OPG*, *RANK*, *TLR4*, and *IL1-β* were used to identify the degeneration of NP. The gene expression analysis indicated the successful development of the IVDD model. Their expression level showed that all pain and inflammatory genes were increased after the development of the IVDD model, which displayed various patterns at different degeneration intervals. In general, we observed a steady increase in the level of expression as the time of degeneration progressed. Furthermore, gene expression gradually dropped after day 5 and would very definitely return to normal levels. Therefore, we selected time points of days 2 and 5 for temporal expression studies of the acute model of IVDD in the coccygeal spine of rat tail. These include significant upregulation of inflammatory genes *TLR4* and *OPG* (***$p \leq 0.001$) at day 2 and (*$p \leq 0.05$ and **$p \leq 0.01$ respectively) at day 5, whereas on day 2, only *RANK* (***$p \leq$

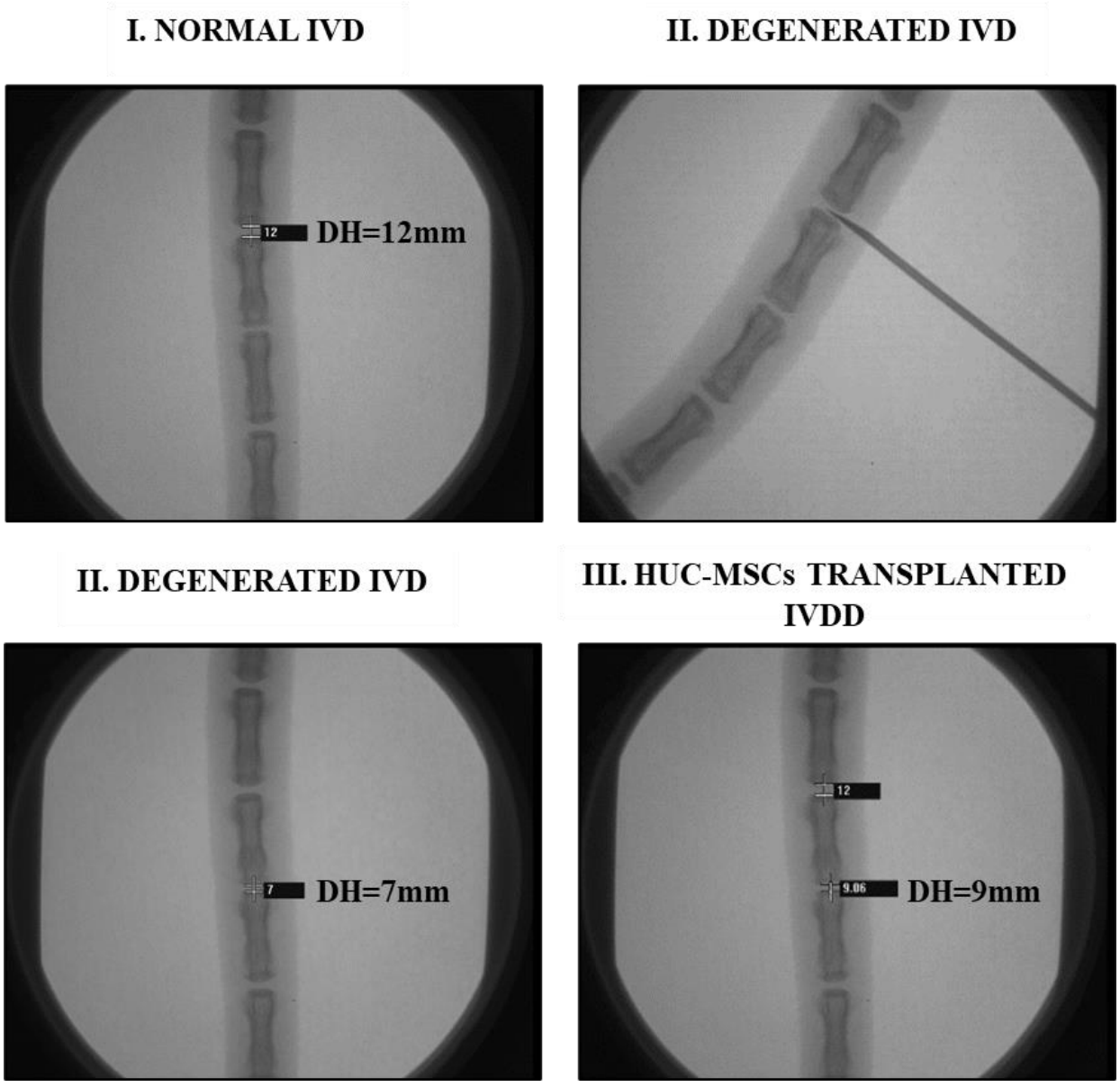

Figure 2. IVD X-Ray Fluoroscopic Visualization and Disc Height: I. Disc height of normal IVD [C0 5/6] is 12 mm. II. IVDD model by needle injection to puncture IVD [C0 6/7]. IVDD model [Co 6/7] showing the least disc height 7 mm. III. Degenerated IVD [Co 6/7] are injected with autologous MSCs for 2-week increased disc height by 9 mm as compared to the IVDD model 7 mm.

0.001) gene showed significantly higher expression in the punctured coccygeal disc of IVDD model as compared to control. Inflammatory genes *C-JUN*, *AKT*, and *IL1-β* showed a significant increase at day 2 (*C-JUN* with **p ≤ 0.01; and *AKT* and *IL1-β* with***p ≤ 0.01 respectively) and at day 5 (***p ≤ 0.001). However, on day 5, only *C-FOS* (**p ≤ 0.01) gene showed significantly higher expression in the IVDD group as compared to the control. The bar diagram of each inflammatory gene compares the expression levels of control and IVDD groups on days 2 and 5 for analyzing the IVDD model and then compares each IVDD group on days 2, and 5 with the MSCs transplanted IVDD for evaluating the regenerative ability of cell therapy**.** Bar graphs

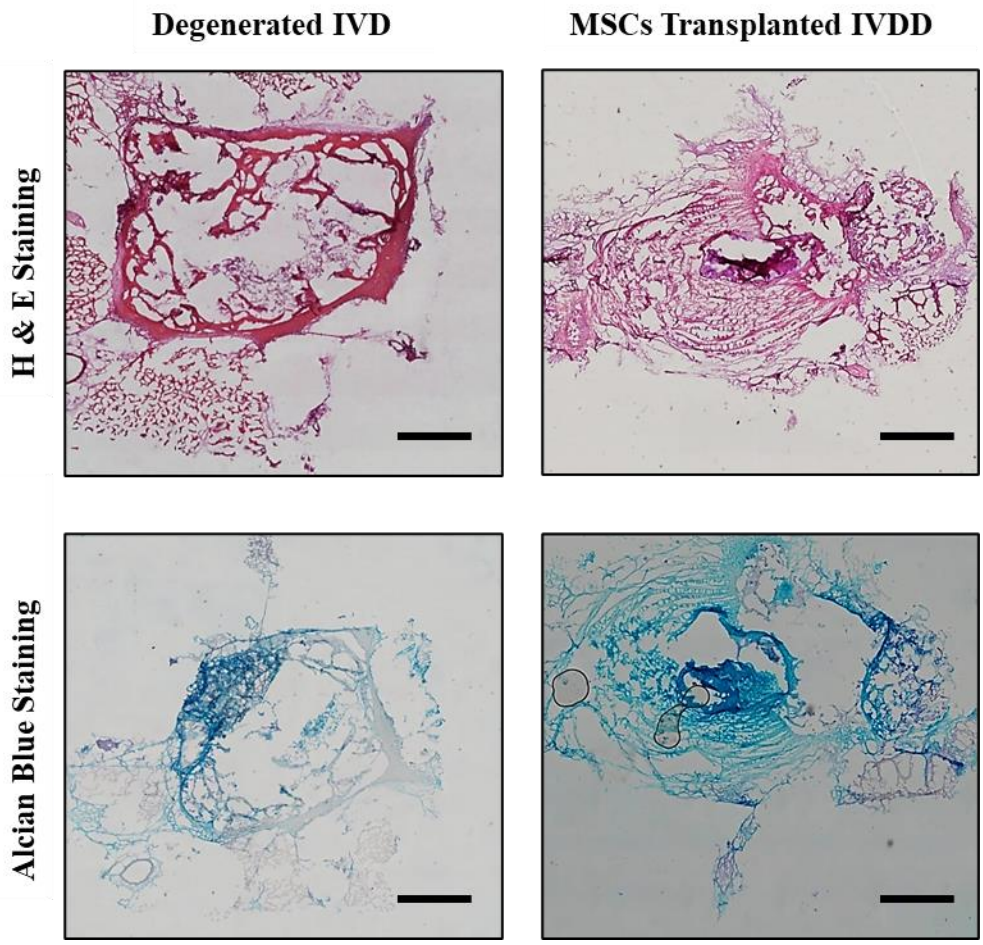

Figure 3. Histological Assessment using H and E and Alcian Blue Staining: Fissure appearance in the NP region show degenerated regions after the development of IVDD model. The MSCs transplanted group showed marked regeneration and proteoglycan production in contrast to the degenerated group. The images are captured under bright field microscope at 10X magnification, scale bar 100 μm.

representing quantitative 2-fold (2 -ΔΔCT) changes in inflammatory genes in normal, degenerated, and cell therapy groups. Graphs indicated that the expression of pain and inflammatory genes showed a positive upregulation in the IVDD model; however, downregulation in the IVDD group transplanted with hUC-MSCs compared to the IVDD group on 2 and 5 days of post-transplantation (***$p < 0.05$). MSCs were transplanted into the punctured IVDs for 2 and 5 days. No significant change was observed in the *TLR4* at day 2; and *OPG* and *IL1-β* at day 5 in the MSCs transplanted IVDD group as compared to IVDD at day 2 and day 5, respectively. Moreover, *C-JUN, OPG* with ***$p \leq 0.001$, *C-FOS*, and *IL1-β* with **$p \leq 0.01$ at day 2; and *C-JUN*, *C-FOS*, *RANK* with **$p \leq 0.01$, and *TLR4* with ***$p \leq 0.001$ at day 5 were significantly downregulated in MSCs transplanted groups as compared to degenerated IVDs. Some anti-inflammatory genes, such as *AKT* with ***$p \leq 0.001$ at day 2 and **$p \leq 0.01$ at day 5 in MSCs *in vivo,* showed significantly higher expression than degenerated IVDs. Data are represented as mean ± SEM of the triplicate measurements Figure 4 I-VII.

3.5. Water Content Analysis

The comparison of restoration of water content in normal, IVDD, and MSCs treated IVDD in percentages is presented in **Figure 5.** The water

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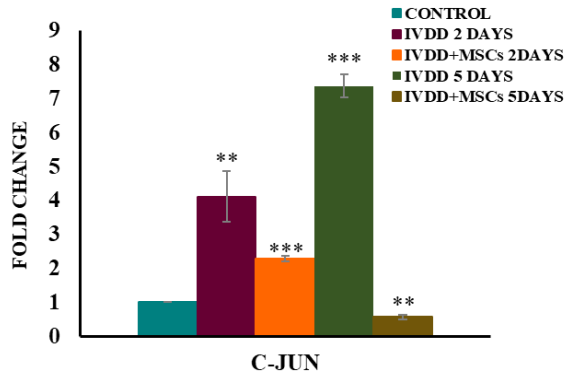

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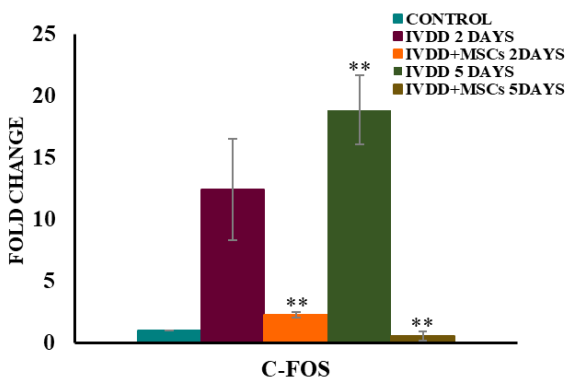

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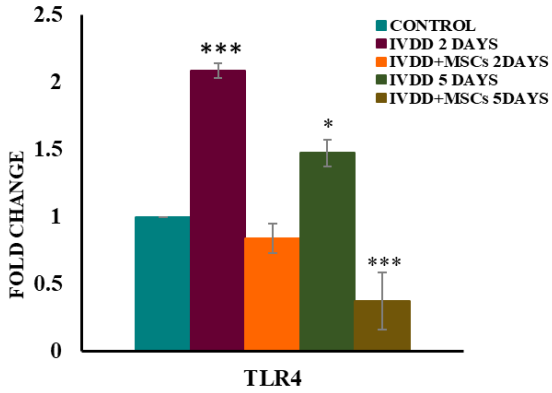

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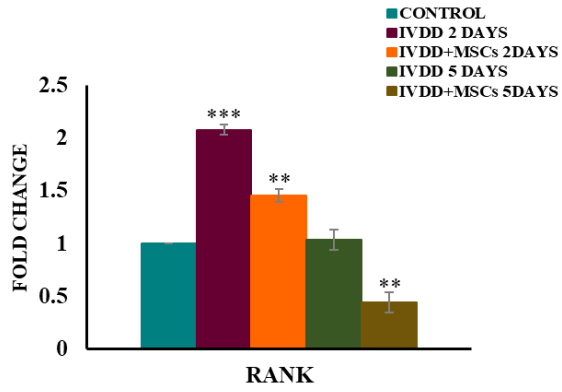

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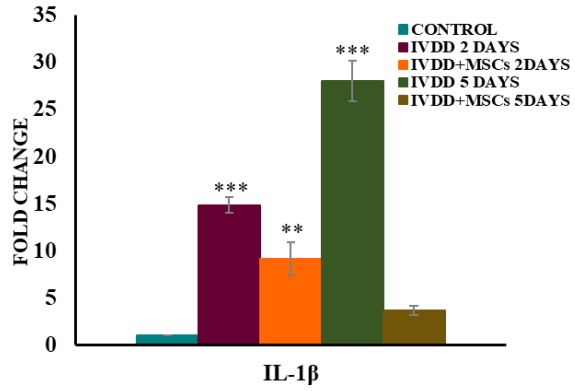

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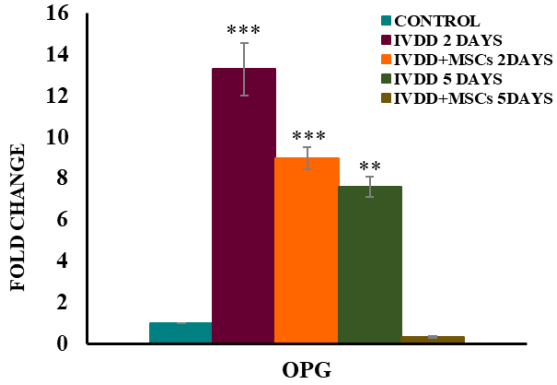

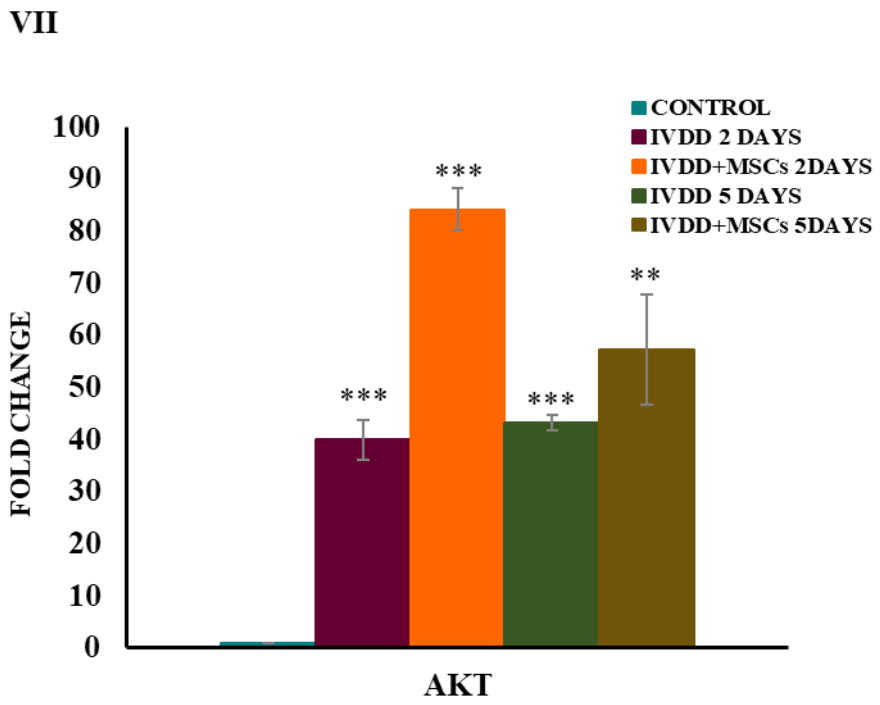

Figure 4. Gene Expression Analysis of Pain and Inflammatory Markers: Expression of inflammatory markers *C-JUN, C-FOS, AKT, OPG, RANK TLR4,* and *IL1-β* in normal, degenerated, and MSCs groups and was statistically subjected to ANOVA and Bonferroni post hoc comparison Bar graphs representing quantitative 2-fold (2 - ΔΔCT) changes. Discs were punctured using 21G needle. Significantly greater expression of *C-JUN* with **p ≤ 0.01, while *TLR4, RANK, IL1-β, OPG*, and *AKT* with ***p ≤ 0.001 at day 2 only were observed in IVDD. Inflammatory genes such as *C-JUN, IL1-β*, and *AKT* with ***p<0.001; *C-FOS*, and *OPG* with **p<0.01; and *TLR4* with *p<0.05; showed significant upregulation at day 5 in IVDD as compared to healthy IVDs. Comparing expression of MSCs transplanted group with degenerated IVD. I. MSCs transplanted group showed significant downregulation at day 2 and day 5 (*p ≤ 0.001 and **p ≤ 0.01 respectively) in the *C-JUN* expression. II. MSCs transplanted group also showed significant downregulation at day 2 and day 5 with **p ≤ 0.01 in the *C-FOS* expression. III. Significantly decreased expression was observed at day 5 in MSCs transplanted group in the *TLR4* gene IV) MSCs transplanted group also showed significant downregulation at day 2 and day 5 with **p ≤ 0.01 in the *RANK* expression. V. MSCs transplanted group showed significant downregulation at day 2 with **p ≤ 0.01 and no change at day 5 in the *IL1-β* expression. VI. MSCs transplanted group showed significant downregulation at day 2 with ***p ≤ 0.001 and no change at day 5 in the *OPG*. VII. MSCs transplanted group also showed significant upregulation at day 2 and day 5 (***p ≤ 0.001 and **p ≤ 0.01, respectively) in the *AKT* expression. The data represent the mean ± SEM of the triplicate measurements.**

concentration was significantly decreased by weight in mg in IVDD as compared to the normal IVDs water content. Results show successful and significant replenishment of water content in compressed IVDD upon treatment with MSCs.

4. Discussion

IVD is a complex fibrocartilaginous tissue connecting adjacent vertebral bodies that enable spinal movement. Human degenerative illnesses, such as IVDD, cause a significant economic burden on the healthcare system, spending billions of dollars (Wuertz and Haglund 2013), IVDD is a complicated, multifaceted condition caused by hereditary and environmental factors affecting people worldwide. One of the primary causes of persistent LBP is IVDD. Disc degeneration critically progresses due to long-term proinflammatory conditions (Molinos et al. 2015). It has been reported that inflammatory

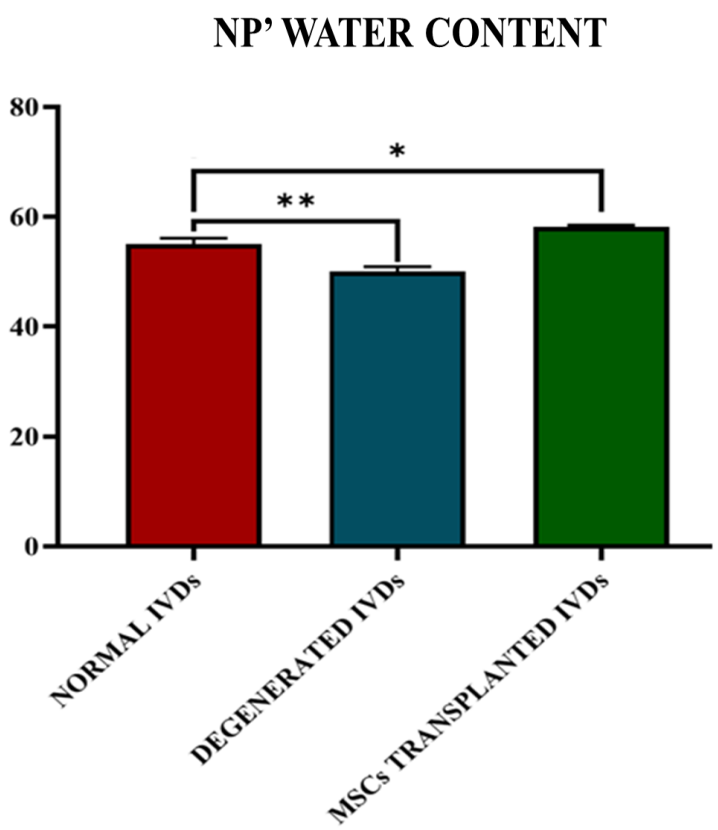

Figure 5. Graphical Representation of Water Content: The comparative analysis of normal, IVDD, and hUC-MSCs transplanted groups; data represents the mean ± SEM from three independent biological experiments in each group. Bar graphs representing changes in degenerated and transplanted IVDs as compared to healthy IVDs. Data were statistically subjected to ANOVA and Bonferroni post hoc comparison. The level of confidence is $p < 0.05$.

mediators and their degradative products are key factors for the progression and onset of disc degeneration (Navone et al. 2017). Increased recruitment of inflammatory cells worsens degeneration leading to a reduction in the NP cells (Cunha et al. 2018). Stem cell treatment is thought to be effective in treating chronic degenerative diseases. For efficacious cellular treatment, the survival of grafted cells into IVD tissue is essential. MSCs have been considered an appropriate therapeutic option due to immunosuppression and multilineage differentiation capabilities to restore IVD function compared to conventional medicine (Vadalà et al. 2016). Investigations have shown that MSCs obtained from perinatal sources showed a lesser risk in contrast to bone marrow-derived MSCs and developed a greater ability to regenerate the ECM, which is a primary component of NP (Li et al. 2016). As reported, the manual cord dissection approach is a robust and simple method that expands a homogenous population of MSCs (Beeravolu et al. 2017). In the present study, the MSCs derived from human umbilical cord explants showed fibroblast-like morphology and expressed CD90, CD105, CD44, CD29, and CD73 (Khan et al. 2019). These results agree with prior reports (Khan, Ali, and Salim 2019).

Research has been carried out to establish a fluoroscopically guided IVDD rat model to study disc height before the transplantation of hUC-MSCs. When we evaluated our outcomes two weeks after the injury, a progressive disc degradation was detected in disc height. This

progressive damage provides an accurate model of IVDD in rat spine [Co 6/7], and it was used to evaluate the efficacy of hUC-MSCs treatment after two weeks of transplantation. Furthermore, the substantial correlation between disc height values in mm suggests that disc height may be used as an effective injury indicator. Histological analysis showed that the structure, cellularity, and glycoprotein has decreased significantly in degenerated IVDs. The MSCs treated group was successfully regenerated compared to the degenerated group. After two weeks post-transplantation, results showed a marked decrease in the presence of proteoglycans and glycoproteins in the IVDD model, which conversely increased in MSCs treated group, restoring the ECM. These results were also analyzed findings that displayed enhancements in cellularity as well as glycan utilizing cell-based treatment in the disc of animal models. As in a previously reported study, the phenotype of the nucleus pulposus played a key role in IVD regeneration.

We evaluated the effect of pain and inflammation on the degeneration and regeneration of disc NP using the genes chosen to compare expression in the IVDD rat model and hUC-MSCs transplantation in the rat IVDD model *in vivo*. The inflammatory markers *C-FOS*, *C-JUN*, *AKT*, *OPG*, *TLR4*, *IL1-β*, and *RANK* were evaluated. It has been previously described that *IL1-β* expression is extensively elevated in IVD degenerated cells and tissues (Burke et al. 2002). *C-JUN* N-terminal kinase (*JNK*) signaling pathway performs crucial roles in cartilage endplate degeneration. Researchers have primarily focused on *IL1-β* and *TNFα*, which are the key inflammatory mediators. *IL1-β* is a crucial cytokine and exhibits robust proinflammatory activities by promoting the formation of various proinflammatory intermediaries, including cytokines, chemokines, and *MMPs* (Zayed et al. 2008). Thus, *C-FOS* regulation is important in regulating the pathological processes of IVD damage and homeostasis. *C-FOS* activation depends on mitogen-activated protein kinases (*MAPK)* pathways. Another study showed that *IL1-7A* increases *COX-2* expression and prostaglandin E2 synthesis through the *p38*/*C-FOS* and *JNK*/*C-JUN* signaling pathways in NP cells to facilitate IVD inflammation. *RANK* ligand (*RANKL*) is a member of the tumor necrosis factor ligand superfamily specifically recognized to regulate bone metabolism (Li et al. 2016). *RANK* and its ligand (*RANKL*) have been demonstrated to perform essential osteoclast activation and differentiation functions. *OPG* is a solvable decoy receptor for *RANKL*, which prevents its binding to *RANK*. *RANK*/*RANKL*/*OPG* expression systems have recently been observed in human articular cartilage (Kwan Tat et al. 2009). In a recent study, *RANKL (G*ruber et al. 2011) and *OPG* (Rutges et al. 2010) contributed to the advancement of degenerative human IVD. We investigated the mRNA transcriptome of inflammatory markers in the normal disc, degenerated, and treated IVDs *via* qPCR. The finding showed that the pattern of expression appeared to be reliant on the time of degeneration. Expression of *C-JUN, C-FOS, AKT, OPG, RANK, TLR4*, and *IL1-β* was significantly upregulated after inducing degeneration. In addition, a few genes at the end of day 5 began to decrease gradually and would almost certainly reach normal values. There was a marked reduction of *C-JUN, C-FOS,* and *IL1-β* expressions on day 2 followed by the higher expression on day 5 of degeneration. *TLR-4* significantly upregulated in the degenerated group on day 2 followed by a gradual decrease on day 5 of degeneration. *OPG* and *RANK* expression was upregulated on day 2 followed by a gradual decrease at day 5 of degeneration. *RANK* expression was normalized after day 5 of degeneration. Moreover, *AKT* was upregulated on day 2 of degeneration. Their levels had gradually increased at day 5, but they were still significantly elevated as compared to the control. It displays that the degenerated disc event is an inducer of specific genes.

In the hUC-MSCs transplanted group, *C-JUN* and *C-FOS* were significantly downregulated on 2 and 5 days. However, *TLR4* showed significantly lower expression in the MSCs group on day 5, while *IL1-β* showed significantly lower expression on day 2. Furthermore, *OPG* expression was also markedly decreased at day 2 after the MSCs regeneration period. Also, *RANK* showed lower expression levels in MSCs at the end of 2 and 5 days of the regeneration period. The expression of *AKT* was markedly increased in MSCs transplantation groups at 2 and 5 days. Nevertheless, the expression levels of these factors differ with the subsequent periods of degeneration. Based on these findings, we can conclude that the expression of pain and inflammatory markers on day 5 after transplantation may regulate the immune response and reduce inflammatory mediator secretion in acute deteriorated disc inflammation. The recent investigation of the various cellular therapeutic approaches now being utilized for degenerated disc disorders illustrates the deficient viability and absence of functional recovery of engrafted stem cells because of the harsh IVDs microenvironment (Sakai et al. 2005). To improve the treatment efficacy of hUC-MSCs, different inflammatory mediating responses with degenerative periods should be closely examined to make them more effective against damaged degeneration situations. The strongest evidence of NP restoration was a rise in water content in discs transplanted with MSCs. hUC-MSCs had a significantly stronger influence on water content than degenerated discs. Thus, evidence of IVDD regeneration is largely based on the combined results of histological, molecular, and biochemical studies. Additionally, it is vital to believe that MSCs transplantation initiates cytokine release into the damaged IVDs that might signal endogenous cells to migrate and stimulate, as well as secretion of other paracrine factors, promoting degenerated disc regeneration and repair (Molinos et al. 2015, Shamji et al. 2010). These results indicate that the establishment's accurate IVDD model, along with cellular and molecular evaluation, explored the role of pain and inflammation in studying the hidden etiology of pain.

5. Conclusions

In conclusion, the study demonstrated that coccygeal disc damage of rat tail using needle puncture method induced degeneration successfully under a fluoroscopic guided system. Moreover, the progressive augmentation of pain and inflammatory gene expression were successfully observed in the IVDD model. This further confirmed rat model of IVDD may be suitable for studying the pathogenesis of IVDD and inflammation. *In vivo* transplantation of hUC-MSCs has been proven to reverse the degeneration and restored the ECM. Furthermore, the decline in inflammatory marker expression by hUC-MSCs treatment mitigates inflammation, alleviating IVDD. Therefore, hUC-MSCs therapy for the degenerated disc provides an understanding of stem cell biology for treating LBP.

Conflict of Interest

The authors declare that they have no competing interests.

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Study Approval

The study was approved by the Institutional Review Board ASP# 2017-0051.

Consent Forms

Formal consent was obtained from umbilical cord donors.

Authors Contributions

SE performed the experiments and wrote the manuscript, SNR helped in experimentation, and writing, SK, helped in experimentation and data evaluation, FR helped in experimentation, AS evaluated the data and help in writing, IK designed the experiments, analyzed the data, secure the funding, and finalized the manuscript.

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