International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2024.290.296

Research Article
Fasudil on the Chondrogenic Variation of Bone Mesenchymal Stem Cells

Kun Zhang, Tiezhu Yang, Ran An, Peifeng Shen and Wei Ren

Department of Emergency, The Second Affiliated Hospital of Inner Mongolia Medical University, Hohhot, 010010 Inner Mongolia, China

Abstract

Background and Objective: Osteoarthritis (OA) is a prolonged joint disease characterized by gradual degradation and expansion of the articular cartilage. So, a long-term remedy to this medical problem is required. The present study assesses the impact of fasudil (FAS) on the chondrogenic variation of bone mesenchymal stem cells (BMSCs) and its potential therapeutic application to osteoarthritis (OA). **Materials and Methods:** Primary BMSCs were isolated from each rat for *in vitro* study and BMSCs were treated without FAS. In the study, cell viability, chondrogenic variation and migration of BMSCs were assessed. *In vivo*, OA was induced in rats by a surgical procedure and treated with FAS (30 mg kg^{-1}, p.o.). In the cartilage tissue of OA rats, the occurrence of cytokines and the expression of JNK, ERK and p38 proteins have been measured. **Results:** The FAS 100 nM-treated BMSCs had significantly higher cell survival and migration than FAS 0 nM treated BMSCs. The mRNA expression of Sox9, Col2a1, aggrecan and Col10a1 showed substantial rises in FAS treated at 10 and 100 nM compared to 0 nM on days 7 and 14. The expression of JNK, p38 and ERK proteins was substantially reduced ($p<0.01$) than a control group of BMSCs. The FAS lowered cytokine and Transforming Growth Factor-β1 (TGF-β1) levels while increasing MMP-13 and Col II expression in OA cartilage tissue. **Conclusion:** Treatment with FAS protects against OA by encouraging the differentiation of BMSCs from the bone marrow into chondrogenic cells. Moreover, FAS reduces the level of cytokines and the activation of JNK/MAPK/ERK signaling pathways in OA rats' cartilage tissue.

Key words: Osteoarthritis, fasudil, JNK/MAPK/ERK signaling pathways, BMSCs, bone mesenchymal stem cells

Citation: Zhang, K., T. Yang, R. An, P. Shen and W. Ren, 2024. Fasudil on the chondrogenic variation of bone mesenchymal stem cells. Int. J. Pharmacol., 20: 290-296.

Corresponding Author: Wei Ren, Department of Emergency, The Second Affiliated Hospital of Inner Mongolia Medical University, Saihan, Hohhot 010010, China

Copyright: © 2024 Wei Ren *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoarthritis (OA) is a long-term joint condition characterized by the progressive deterioration and enlargement of the articular cartilage[1]. Various etiological variables, including improper loading, repeated injury, aging and obesity, contribute to the development of osteoarthritis (OA). The OA is a leading cause of disability worldwide, particularly among older individuals, because of restricted movement and discomfort[2]. Differentiation and condensation of MSC into chondrocytes contribute to the skeletal development of OA, commonly known as chondrogenesis. Adipocytes, osteoblasts and chondrocytes like the multi-differentiation ability of bone marrow MSCs in the field of orthopedic therapy and clinical research[3]. In OA, cartilage injury repair is stimulated by regulating the differentiation of chondrocytes from MSC and there are only a few pathways involved in its regulation[4]. According to the literature, various biological changes include the control of the MAPK/ERK pathway, including chondrogenic differentiation activation. Furthermore, Transforming Growth Factor-β1 (TGF-β1) increases chondrocyte growth and development by triggering the MAPK/ERK pathway. Thus, molecules triggering the MAPK/ERK pathway need to be explored in chondrogenic differentiation MSC for the treatment of OA. The Rho kinase (ROCK) inhibitors are clinically used for the treatment of subarachnoid hemorrhage and fasudil (FAS) is a ROCK inhibitor that shows very few side effects. The FAS normalizes the blood flow, protects the damaged nerve tissues and thereby prevents spinal cord injury[5]. Moreover, a study suggests that FAS enhances the differentiation of MSC by regulating the MAPK pathway for the management of spinal cord injury. This investigation intends to assess the impact of FAS on BMSC chondrogenic development and the therapy of OA.

MATERIALS AND METHODS

Study area: The current investigation was conducted at The Second Affiliated Hospital of Inner Mongolia Medical University from June to August, 2023.

Animal model of OA: In this study, 30 Sprague-Dawley rats (200-250 g) were procured from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd., in China. The animals were kept at 65±5% humidity and a temperature of 22±2°C. The animals were split into 3 groups (n = 10); normal, OA and OA+FAS (Asahi Kasei Pharma. Co. Ltd., Beijing, China) treated group (30 mg kg^{-1}; orally for 7 days). Each subject got an IV injection of xylazine (Sigma-Aldrich, Beijing, China) at a dosage of 7 mg kg^{-1} b.wt., as well as ketamine (Sigma-Aldrich, Beijing, China) at a dosage of 60 mg kg^{-1} b.wt. To induce OA, a surgical procedure was performed involving the incision of the medial side of the patellar tendon, dislocation of the patella and transection of the anterior cruciate ligament using a surgical blade. The repair of the medial retinaculum was performed, followed by the closure of the skin. After 7 days, rats underwent execution as a method of sacrificing, following which the articular cartilage was extracted from the medial tibial plateaus. The entire animal investigation process was authorized by the Institution (Reg No. 2022/OA/5383).

Chondrocyte isolation and culture: The isolation of primary BMSCs was conducted by the procedures described in a previous research study conducted by Zhou *et al.*[6]. The tibial plateaus and femora were obtained from the tested Sprague-Dawley rats and subjected to culture for 2 days in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C. The DMEM was enriched with TGF-β, sodium pyruvate, ascorbate and dexamethasone. The removal of non-adherent cells was accomplished by implementing medium shifts until the cells reached a confluence level of between 80 and 90%. The technique of flow cytometry was employed to assess the characteristics of BMSCs concerning their expression of commonly observed surface markers. Monoclonal antibodies targeting CD29, CD90, CD45 and CD105 were introduced to BMSCs by the instructions provided by the manufacturer.

CCK-8 assessment: The CCK-8 evaluation was performed according to the kit's manufacturer's directions to determine cell viability[7]. A total of 5×103 cells were injected into each well of a 12-well plate. Subsequently, the cells in each well were treated with varying doses of FAS (Asahi Kasei Pharma. Co. Ltd., Beijing. China) (0, 10 and 100 nM). Subsequently, the well plates were subjected to a 10 µL solution of CCK-8, which was then moved to an incubation chamber and maintained at a temperature of 37°C for 1 hr, while maintaining a CO_2 concentration of 5%. A microplate reader (BioTek Synergy H1 Multimode Reader, Agilent, Santa Clara, California, USA) was utilized to detect the intensity of absorption at 450 nm.

Transwell assessment: A transwell filter system was used to examine the cell migration as per the standard method of Ma *et al.*[8]. The BMSCs (200 µL) added in a serum-free medium were used to fill the top chamber and a complete medium (600 µL) was used to fill the lower chamber.

Chondrogenic induction of BMSCs: Induction of chondrogenic differentiation of BMSCs as per the reported method of Hu *et al.*[9]. The BMSCs were introduced at a concentration of 1×10^4 cells/well in each well on a 12-well culture plate and subsequently grown at 37°C until they reached 80% confluence. The chondrogenic-inductive medium was replaced in place of the culture medium in the presence and absence of FAS. The BMSCs were kept for chondrogenic differentiation for 14 days and later collected BMSCs were placed for further study.

Western blot assessment: Western blot assessment from BMSCs was followed by the standard method of Xin *et al.*[10]. The RIPA lysis buffer (Solarbio, China) was used to extract protein from BMSCs and the protein level was assessed by the bicinchoninic acid protein assessment kit (Sigma Aldrich, Shanghai, China). Then the proteins were separated by a 10% (w/v) solution of SDS-PAGE and then added to nitrocellulose membranes. Primary antibodies were added to the membranes after they had been exposed to a 5% (w/v) blocking solution made of non-fat milk for a whole night at 4°C. Then goat secondary antibodies were coupled with horseradish peroxidase. Protein detection was performed using a chemiluminescence kit (Sigma Aldrich, Shanghai, China). The GAPDH served as a positive control, whereas β-actin served as a negative control. The western blot assay was carried out to assess the expression levels of JNK, ERK and p38 mRNA in rat tissue homogenates, using the modified protocol described by Nito *et al.*[11].

RT-PCR: The RT-PCR was followed by the method of Zwickl *et al.*[12]. In the study, total RNAs were extracted using the Trizol chemical reagent and subsequently converted into complementary DNAs (cDNAs) through reverse transcription. The levels of Sox9, Col2a1, aggrecan and Col10a1 gene expression were assessed in BMSCs using the SYBR Green gene expression assessment. The expression levels were standardized using GAPDH as a reference gene. The assessment was conducted using a TaqMan microRNA assessment kit.

Assessment of cytokine levels using ELISA: The concentrations of IL-1β, IL-6 and TGF-β were assessed by the ELISA technique kit (Quansys Bioscience, Logan, Utah, USA) using the modified protocol described by Greenberg *et al.*[13].

Statistical analysis: All the data were assessed twice and signified as the Mean±SD. Tukey's t-test was assessed to employed to measure the statistical importance of the variations among the groups. The threshold level was maintained at $p<0.05$.

RESULTS

The technique of flow cytometry was employed to assess the characteristics of BMSCs, as depicted in Fig. 1(a-d). The cellular samples exhibited positive expression of the CD29, CD90 and CD105 markers while showing negative expression of the CD45 marker. This pattern of marker expression strongly indicates that the cells under investigation can be classified as BMSCs.

Migration and proliferation of BMSCs were assessed in the FAS-treated cells as revealed in Fig. 2(a-b). On days 1, 3 and 7, the viability of BMSCs in FAS-treated cells was determined using the CCK-8 test. On days 1 and 3, there was no discernible variation in the viability of FAS-treated cells. On day 7, the viability of cells was enhanced in FAS-treated 100 nM compared to 0 nM cells (Fig. 2a). The migration of BMSCs in cells treated with FAS was assessed using a transwell assessment, as depicted in Fig. 2b. There was a noticeable rise ($p<0.01$) in cell migration observed in cells treated with FAS at a concentration of 100 nM as compared to cells treated with 0 nM.

The impact of FAS on the process of chondrogenic differentiation in BMSCs was evaluated through the analysis of mRNA levels of Sox9, Col2a1, aggrecan and Col10a1 in the BMSCs at various time durations: Day 3, 7 and 14 (Fig. 3a-d). The mRNA levels of Sox9, Col2a1, aggrecan and Col10a1 remained unchanged on the third day of treatment with FAS in BMSCs. In contrast, the levels of mRNA expression for Sox9, Col2a1, aggrecan and Col10a1 were found to be substantially increased in the group treated with FAS at concentrations of 10 and 100 nM, as compared to the group treated with 0 nM, on both days 7 and 14.

The levels of JNK, p38 and ERK proteins were assessed in the BMSCs treated with FAS through the use of a western blot (Fig. 4a). The JNK, p38 and ERK protein levels were considerably greater ($p<0.01$) in the control group than in FAS group. The FAS-treated group of BMSCs exhibited a considerably similar ($p<0.01$) in the expression levels of p-JNK/JNK, p-p38/p38 and p-ERK/ERK proteins than the control group.

The assessment of the impact of FAS was conducted by employing an ELISA technique to quantify the levels of inflammatory cytokines within the cartilage tissue of rats afflicted with OA (Fig. 4b). The levels of IL-1β, IL-6 and TGF-β

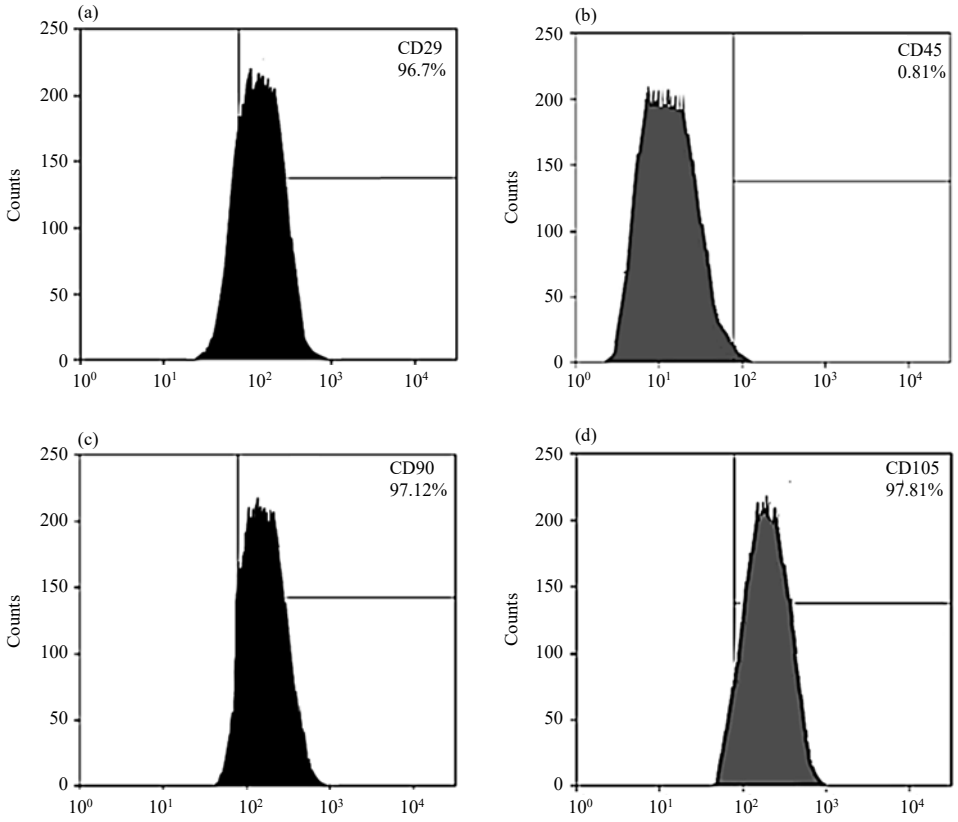

Fig. 1(a-d): Quality control of BMSCs, Cells were incubated with antibodies against, (a) CD29, (b) CD45, (c) CD90 and (d) CD105

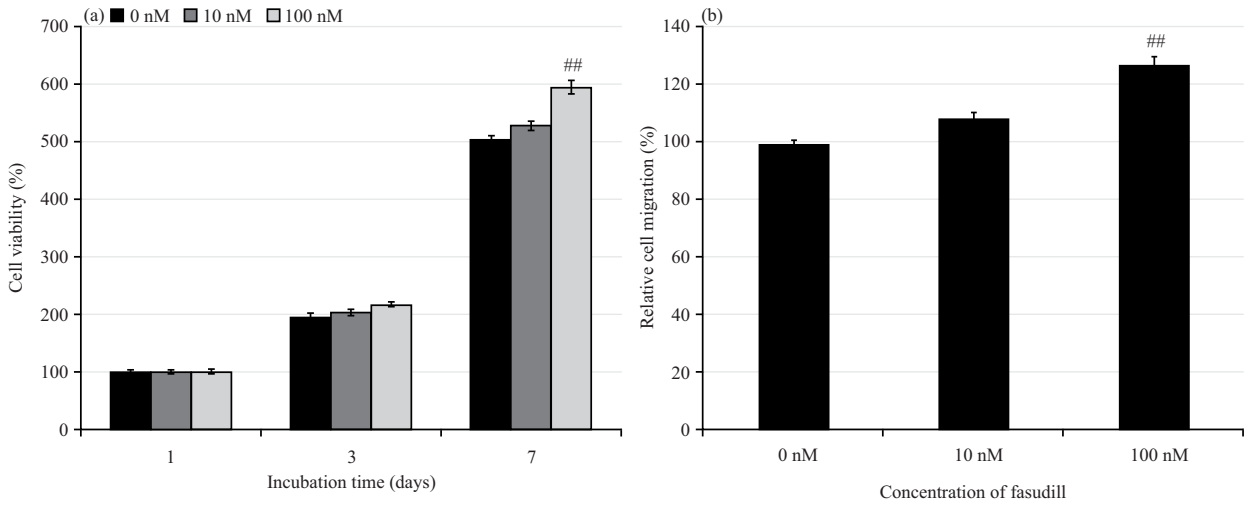

Fig. 2(a-b): Effect of FAS on the migration and proliferation of BMSCs
Mean±SEM and $^{##}p<0.01$ compared to 0 nM

were observed to be increased in the cartilage tissue of the group of rats diagnosed with OA than the control group. The group that received FAS treatment exhibited a statistically significant decrease ($p<0.01$) in the levels of IL-1β, IL-6 and TGF-β in the cartilage tissue than the group with OA.

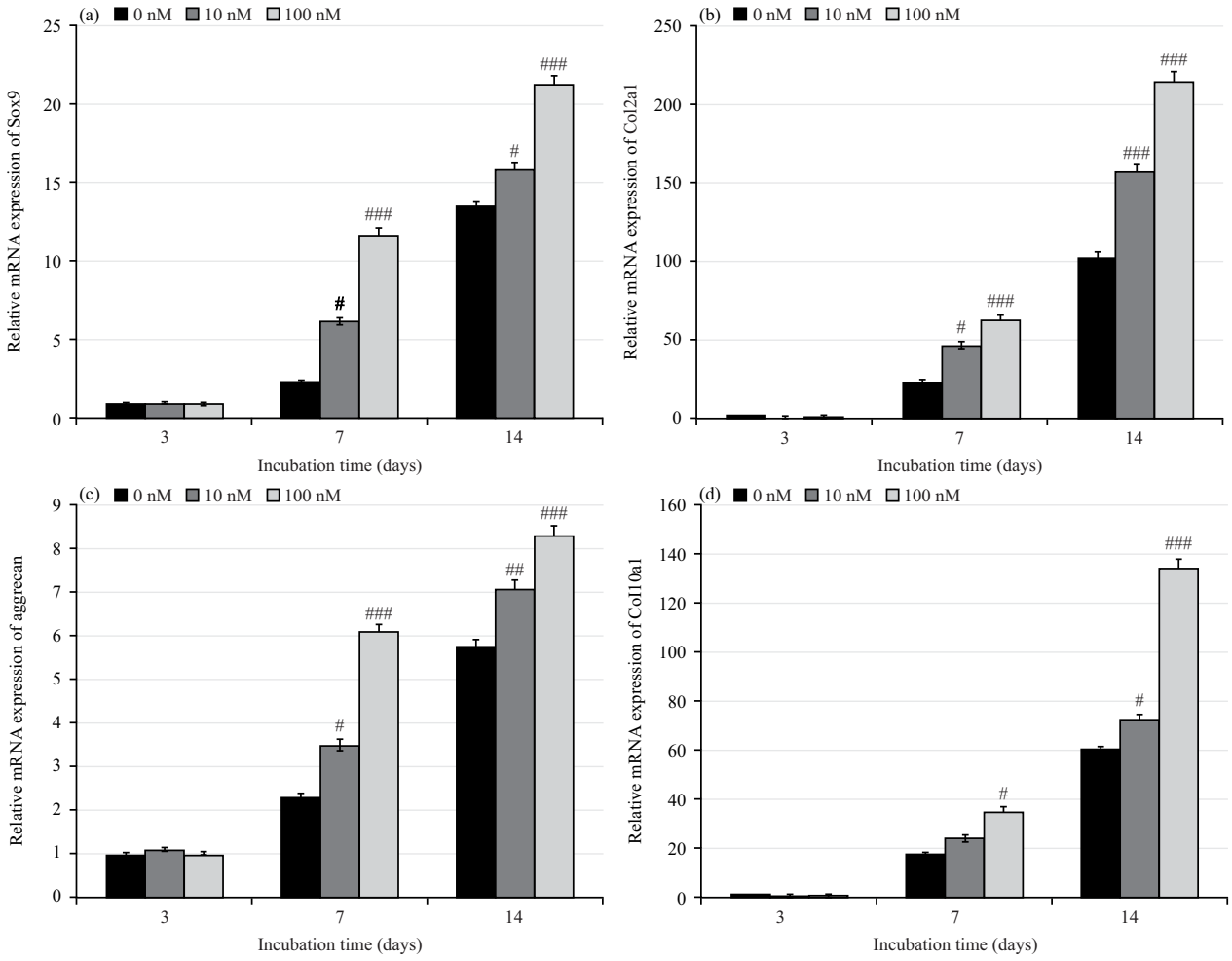

Fig. 3(a-d): Effect of FAS on the chondrogenic differentiation of BMSCs, (a) mRNA expression of Sox9, (b) mRNA expression of Col2a1, (c) mRNA expression of aggrecan and (d) mRNA expression of Col10a1

Mean±SEM, #p<0.1 compared to 0 nM, ##p<0.01 compared to 0 nM and ###p<0.05 compared to 0 nM

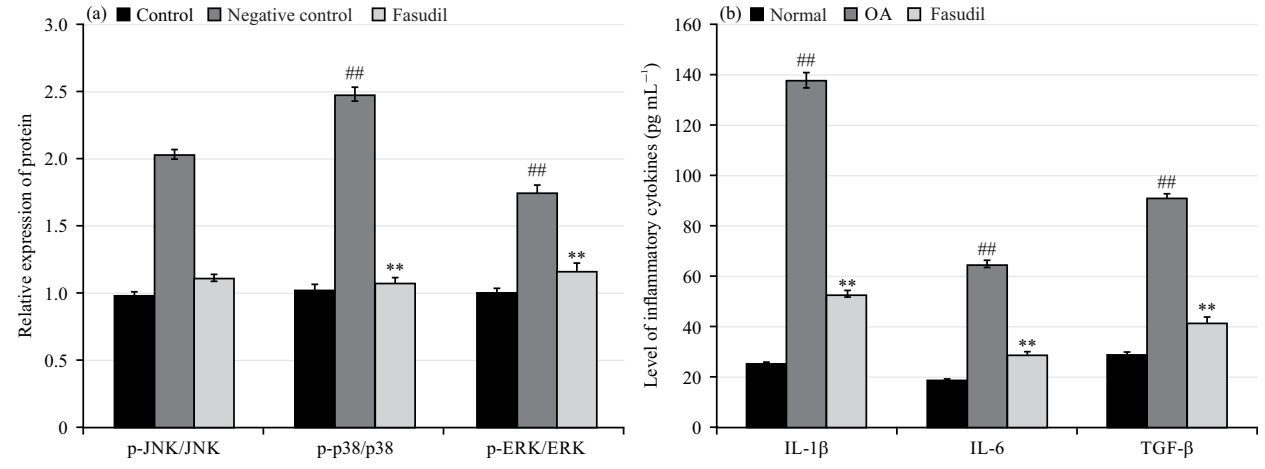

Fig. 4(a-b): Effect of FAS on the expression of protein, inflammatory cytokines and pathways, (a) Expression of JNK, p38 and ERK protein in the BMSCs and (b) Level of inflammatory cytokines in OA rats

Mean±SEM, ##p<0.01 compared to control or normal group and **p<0.05 compared to control or normal group

DISCUSSION

The OA represents a significant manifestation of arthritis and the prolonged utilization of pharmaceutical interventions for its treatment is associated with various constraints. Excessive joint loading, foreign body intrusion and inflammation are the major causes of damage to chondrocytes in OA. Cellular density is reported to be reduced, which inhibits the proliferation of chondrocytes and chondrocytes cannot regenerate by themselves[14]. Although, many of the treatments concentrate on cartilage engineering, insufficient regeneration can be achieved. The SOX family is a transcription factor involved in cellular development and differentiation. Multiple proliferation and transcription factors impact the development of BMSCs into cartilage cells. The Sox9 is reported to be a marker of osteogenesis and chondrogenesis in BMSCs by regulating their differentiation and condensation[15]. Upregulating the expression of Sox9, which activates the Col2a1 transcription and the activation of the expression of the collagen (Col10a1) gene enhances the development of chondrogenesis. Moreover, chondrocytes synthesize proteoglycans by regulating aggrecan. The existing body of literature indicates that the process of chondrogenic differentiation can be stimulated by upregulating the expression of Sox9[16]. Chen *et al.*[17] discovered that FAS enhances the process of bone formation in BMSCs via controlling the MAPK-P38 pathway, hence expediting the recovery of femoral fractures in animals. Furthermore, the findings of this study provide evidence that the administration of FAS has an impact on the expression levels of Sox9, Col2a1, Col10a1 and aggrecan. In addition, the group treated with FAS demonstrates enhanced proliferation and migration of BMSCs than the control group. The deterioration of the cartilage in OA can be ascribed to elevated levels of cytokines. Cytokines possess the ability to reduce the levels of MMP-13, Col II and TGF-β expression, all of which are involved in facilitating chondrocyte differentiation. The administration of FAS resulted in a decrease in cytokine levels and TGF-β, while simultaneously promoting the upregulation of MMP-13 and Col II expression within the cartilage tissue of rats with OA. The MAPK pathway encompasses various signaling cascades, namely JNK, p38 and ERK, which play crucial roles in regulating normal physiological processes. These pathways are responsible for the activation of mitochondrial pathways, among other cellular events[18]. Moreover, several categories of tumors elicit autophagic cell death by triggering the JNK pathway[19] and the chondrogenic differentiation involves the triggering of JNK/MAPK/ERK signaling pathways[20]. The administration of FAS demonstrates improvement in the modified JNK/MAPK/ERK signaling pathways in both BMSCs and the cartilage tissue of rats with OA.

CONCLUSION

The FAS administration has been discovered to protect against OA by promoting the chondrogenic differentiation process in mesenchymal stem cells obtained from bone marrow. Additionally, it has been observed that FAS exhibits a capacity to decrease the concentration of cytokines and inhibit the activation of JNK/MAPK/ERK signaling pathways within the cartilage tissue of rats with OA.

SIGNIFICANCE STATEMENT

The OA is a chronic condition that affects the joints and is marked by progressive deterioration and enlargement of the articular cartilage. This study investigates the impact of FAS on the differentiation of BMSCs into chondrocytes and its potential as a treatment approach for OA. Based on the results obtained from the research, FAS treatment has been revealed to protect against osteoarthritis by augmenting the chondrogenic differentiation process in mesenchymal stem cells obtained from bone marrow. Furthermore, it has been noted that FAS can reduce the levels of cytokines and hinder the activation of JNK/MAPK/ERK signalling pathways in the cartilage tissue of rats with OA.

ACKNOWLEDGMENT

The authors would like to convey their appreciation to the higher authorities for providing the necessary facilities.

REFERENCES

1. Loeser, R.F., S.R. Goldring, C.R. Scanzello and M.B. Goldring, 2012. Osteoarthritis: A disease of the joint as an organ. Arthritis Rheumatism, 64: 1697-1707.
2. Li, Z., Z. Zhang, Y. Ren, Y. Wang and J. Fang *et al.*, 2021. Aging and age related diseases: From mechanisms to therapeutic strategies. Biogerontology, 22: 165-187.
3. Zha, K., Z. Sun, Y. Yang, M. Chen and C. Gao *et al.*, 2021. Recent developed strategies for enhancing chondrogenic differentiation of MSC: Impact on MSC-based therapy for cartilage regeneration. Stem Cells Int., Vol. 2021. 10.1155/2021/8830834.
4. Akkiraju, H. and A. Nohe, 2015. Role of chondrocytes in cartilage formation, progression of osteoarthritis and cartilage regeneration. J. Dev. Biol., 3: 177-192.

5. Shi, J. and L. Wei, 2013. Rho kinases in cardiovascular physiology and pathophysiology: The effect of fasudil. J. Cardiovasc. Pharmacol., 62: 341-354.
6. Zhou, X., J. Yang, Y. Liu, Z. Li and J. Yu *et al*., 2019. Observation of the effect of bone marrow mesenchymal stem cell transplantation by different interventions on cirrhotic rats. Braz. J. Med. Biol. Res., Vol. 52. 10.1590/1414-431X20187879.
7. Murata, K., N. Fujita and R. Takahashi, 2022. *Ninjinyoeito* ameliorated cigarette smoke extract-induced apoptosis and inflammation through JNK signaling inhibition in human lung fibroblasts. BMC Complementary Med. Ther., Vol. 22. 10.1186/s12906-022-03574-5.
8. Ma, J., M. Shen, D. Yue, W. Wang, F. Gao and B. Wang, 2022. Extracellular vesicles from BMSCs prevent glucocorticoid-induced BMECs injury by regulating autophagy via the PI3K/Akt/mTOR pathway. Cells, Vol. 11. 10.3390/cells11132104.
9. Hu, Y., X. Li, G. Huang, J. Wang and W. Lu, 2019. Fasudil may induce the differentiation of bone marrow mesenchymal stem cells into neuron-like cells via the Wnt/β-catenin pathway. Mol. Med. Rep., 19: 3095-3104.
10. Xin, W., S. Yuan, B. Wang, Q. Qian and Y. Chen, 2021. Hsa_circ_0066523 promotes the proliferation and osteogenic differentiation of bone mesenchymal stem cells by repressing PTEN. Bone Joint Res., 10: 526-535.
11. Nito, C., H. Kamada, H. Endo, P. Narasimhan, Y.S. Lee and P.H. Chan, 2012. Involvement of mitogen-activated protein kinase pathways in expression of the water channel protein aquaporin-4 after ischemia in rat cortical astrocytes. J. Neurotrauma, 29: 2404-2412.
12. Zwickl, H., E. Niculescu-Morzsa, F. Halbwirth, C. Bauer and V. Jeyakumar *et al*., 2016. Correlation analysis of SOX9, -5, and -6 as well as COL2A1 and aggrecan gene expression of collagen I implant-derived and osteoarthritic chondrocytes. CARTILAGE, 7: 185-192.
13. Greenberg, A.K., S. Basu, J. Hu, T.A. Yie, K.M. Tchou-Wong, W.N. Rom and T.C. Lee, 2002. Selective p38 activation in human non-small cell lung cancer. Am. J. Respir. Cell Mol. Biol., 26: 558-564.
14. Chen, H., X.N. Tan, S. Hu, R.Q. Liu, L.H. Peng, Y.M. Li and P. Wu, 2021. Molecular mechanisms of chondrocyte proliferation and differentiation. Front. Cell Dev. Biol., Vol. 9. 10.3389/fcell.2021.664168.
15. She, Z.Y. and W.X. Yang, 2015. SOX family transcription factors involved in diverse cellular events during development. Eur. J. Cell Biol., 94: 547-563.
16. Lefebvre, V. and M. Dvir-Ginzberg, 2017. SOX9 and the many facets of its regulation in the chondrocyte lineage. Connect. Tissue Res., 58: 2-14.
17. Chen, M., D. Luo, J. Zhan, Y. Hou, S. Chen, X. Li and D. Lin, 2020. Fasudil enhanced differentiation of BMSCs *in vivo* and *vitro*, involvement of P38 signaling pathway. Chem. Biol. Interact., Vol. 317. 10.1016/j.cbi.2020.108944.
18. Wang, C. and R.J. Youle, 2009. The role of mitochondria in apoptosis. Annu. Rev. Genet., 43: 95-118.
19. Towers, C.G., D. Wodetzki and A. Thorburn, 2020. Autophagy and cancer: Modulation of cell death pathways and cancer cell adaptations. J. Cell Biol., Vol. 219. 10.1083/jcb.201909033.
20. Ma, N., X. Teng, Q. Zheng and P. Chen, 2019. The regulatory mechanism of p38/MAPK in the chondrogenic differentiation from bone marrow mesenchymal stem cells. J. Orthop. Surg. Res., Vol. 14. 10.1186/s13018-019-1505-2.