Exosomes Derived from Mesenchymal Stem Cells Inhibit Catabolism in Human Chondrocytes by Activating Autophagy Via Inhibition of the NF-Kb Pathway

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Abstract

Objective: We aimed to determine the significance of MSC-derived exosomes (MSC-Exos) in chondrocyte autophagy under normal and inflammatory conditions.

Design: Human umbilical cord-derived MSCs (hMSCs) were cultured in vitro. hMSC-Exos (EX) were isolated by an ultracentrifugation method. Transmission electron microscopy and western analysis were used to identify exosomes. Human chondrocytes were extracted from five adult males with OA undergoing total knee arthroplasty. Primary cultures of chondrocytes from OA patients were stimulated with 50 ng/ml tumor necrosis factor-α (TNF-α) in the presence or absence of hMSC-Exos. Autophagy levels were determined based on expression of autophagic marker LC3, StubRFP-SensGFP-LC3 analysis, and electron microscopy. Catabolic gene and chemokine expression were evaluated using quantitative PCR. The NF-κB inhibitor NS398 was used to analyze the role of the NF-κB pathway in autophagic activation.

Results: hMSC-Exos increased LC3-II levels as well as autophagosome number in chondrocytes. hMSC-Exos inhibited TNF-α–induced expression of MMP-3, -9, and -13; ADAMTS5; CCL-2 and -5; and CXCL1. NF-κB inhibition activated autophagy in TNF-α–treated chondrocytes. These results indicate that hMSC-Exos might suppress the levels of catabolic and inflammatory factors in chondrocytes by promoting autophagy via NF-κB pathway inhibition.

Conclusions: Our data support the interest in hMSC-Exos to develop new therapeutic approaches for joint conditions.

Keywords: Exosome; Chondrocyte; Autophagy; OA; NF-κB pathway

Introduction

Osteoarthritis (OA) is the most prevalent musculoskeletal disorder and a major cause of joint pain and disability. Damage to joints as a result of trauma, obesity, aging, and genetic background are the major risk factors for OA [1]. Chondrocytes are the only resident cells in cartilage and are responsible for both synthesis and turnover of the abundant extracellular matrix (ECM). Therefore, maintenance of healthy chondrocytes appears to be an important factor in maintaining cartilage and preventing degeneration of cartilage [2]. Mesenchymal stem cells (MSCs)—multipotent precursors of connective tissue cells that can be isolated from many adult tissues, including those of the diarthrodial joint—have emerged as a potential therapy [3]. MSC therapies have demonstrated efficacy in cartilage repair in animal and clinical studies.
The efficacy of MSC-based therapies, which were previously predicated on the chondrogenic potential of MSCs, is increasingly attributed to paracrine secretion, particularly of exosomes [4]. The crucial role of MSC-derived exosomes for the regulation of cell migration, proliferation, differentiation, and ECM synthesis has been increasingly supported by recent findings [5]. A report in 2016 showed that adipose-derived stem cells (ADSCs) are able to activate autophagy and inhibit catabolism in chondrocytes during inflammation, and the mTOR pathway might be involved in activation of autophagy [6]. Autophagy, a mechanism of organelle recycling that promotes cell survival, has been previously implicated in osteoarthritis (OA) [7]. Currently, researchers suggest that autophagy may increase as an adaptive response to protect chondrocytes from various environmental changes, whereas failure of the adaptation may lead to progression of cartilage degradation [8]. Djavaheri-Mergny M demonstrated that the decline in autophagy during aging creates problems in cellular housekeeping functions that stimulate NF-κB signaling in order to, directly or via inflammasomes, trigger an age-related proinflammatory phenotype. Moreover, there are indications that inflammatory signaling can repress autophagy and thus induce this destructive interplay between autophagy and inflammasomes. For instance, tumor necrosis factor-α (TNF-α), an inflammatory cytokine, can induce or repress autophagy in an NF-κB–dependent manner [9]. Yi et al suggested that inhibiting the NF-κB pathway can promote autophagy and decrease apoptosis and the inflammatory response in lipopolysaccharide (LPS)-induced nucleus pulposus cells. Meanwhile, autophagy triggered by NF-κB inhibition plays a protective role against apoptosis and inflammation [10].

Materials and Methods

Human Umbilical-Cord-Derived Mscs (Hmscs)

hMSCs were isolated from umbilical cords of five healthy donors. The experimental design was approved by the Institutional Ethical Committee (Zhengzhou Central Hospital, Henan, China). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013. Samples were washed with phosphate-buffered saline (PBS) and minced and digested at 37°C for 1 h with 2% type I collagenase (Gibco, Life Technologies, Madrid, Spain). Digested tissue was filtered through a 100-µm cell strainer (BD Biosciences, Durham, NC, USA). Cells were then washed with Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded into tissue culture flasks (1–2 × 10^4 cells/mL) in DMEM/Ham’s F12 medium with penicillin and streptomycin (1%), supplemented with 15% extracellular vesicle-free human serum, and incubated at 37°C in a humidified atmosphere of 5% CO₂. To eliminate the extracellular vesicle fraction, serum was centrifuged for 18 h at 120,000 × g and 4°C using a SW-28 swinging-bucket rotor (Beckman Coulter, Brea, CA, USA). When cells reached semiconfluence, culture plates were washed and the MSC phenotype confirmed by flow cytometry (Cytoflex, Beckman Coulter) using the specific antibodies anti-CF73-PE, anti-CD90-APC, anti-CD105-APC-A750, anti-CD34, anti-CD45, anti-CD11b, anti-CD19, and anti-HLA-DR-FITC (Biolegend, San Diego, CA USA), and measuring cell viability with propidium iodide staining. Finally, conditioned medium (CM) was collected from cultured cells at passage 0 every 48 h of culture. CM was pooled, centrifuged, and stored under sterile conditions at -80°C prior to further use.

Isolation of exosomes

Exosomes were obtained from hMSC CM using a filtration/centrifugation-based protocol [34]. Cellular debris was eliminated by centrifugation at 300 × g for 10 min. Vesicles were then collected from the supernatant through differential centrifugation steps. CM was filtered through an 800-nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200 × g for 20 min at 4°C to pellet microvesicles. Then, supernatants were filtered through a 200-nm filter (Merck) and centrifuged at 100,000 × g for 90 min at 4°C. Pellets were washed once with sterile PBS, resuspended in 2 mL PBS, and stored at -80°C until further use.

Human chondrocyte cultures

The knee specimens were obtained from three females and two males, 69.4±7.2 years of age (mean ± standard error of the mean [SEM]), with diagnosis of advanced OA undergoing total knee arthroplasty. Diagnosis was based on clinical, laboratory, and radiological evaluation. The study design was approved by the Institutional Ethical Committee (Zhengzhou Central Hospital, Henan, China). Samples were obtained with patient’s consent according to the Declaration of Helsinki. Knee articular cartilage samples were obtained and cut into about 1 mm³ pieces. Tissues were digested with 0.25% trypsin-EDTA for 30 min and collagenase II for 4 h and then filtered. After rinsing, the chondrocytes were cultured in DMEM with high-dose (4.5 g/L) glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37°C with 5% CO₂. Second-passage chondrocytes were used in the experiments to eliminate the influence of dedifferentiation on experimental results.

Toluidine blue staining for morphological differentiation of chondrocytes

The chondrocytes were inoculated into a six-well plate and, when the cells reached 50%–60% confluence, the culture medium was discarded. The chondrocytes were then fixed in 4% paraformaldehyde for 30 min, stained with 1% toluidine blue at room temperature for 10–30 min, washed with absolute ethyl alcohol until the cells were colorless, and observed under an inverted microscope (Olympus, Tokyo, Japan).
The H+ -ATPase inhibitor bafilomycin A1 (0.1 µM) was used to measure the role of hMSC-Exos or hMSC-Exos combined with 50 ng/mL TNF-α in autophagic flux. Then, the NF-κB pathway involved in autophagic activation was investigated. When cells were treated with 50 ng/mL TNF-α and hMSC-Exos or specific COX-2 inhibitor NS398 (10 µM), hMSC-Exos and NS398 were always added to the medium 1 h prior to TNF-α addition. In all experiments, chondrocytes were treated with TNF-α for 24 h. During the autophagic flux assay, 0.1 µM bafilomycin A1 was added to the medium 1 h prior to addition of hMSC-Exos or hMSC-Exos combined with 50 ng/mL TNF-α, followed by co-incubation for 24 hours.

RNA extraction and quantitative (q)PCR

Total RNA was isolated from tissues or cell lines using Trizol reagent (Invitrogen, USA). RNA was reversed transcribed into cDNA using the PrimeScript™ one step RT-PCR kit (TaKaRa, China) according to the manufacturer’s protocol. The mRNA level was measured using the SYBR® Premix DimmerEraser™ kit (TaKaRa, China) and the ABI7500 system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was calculated using the 2-ΔΔCT method and normalized to β-actin. The primer sequences are listed in Additional file 1: Table 1.

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Western blotting

Chondrocytes in 10-cm dishes were washed with cold PBS and incubated with RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, followed by cell scraping and centrifugation. Nuclear protein was isolated as recommended by the Beyotime (Shanghai, China). After measuring protein concentration with the bicinchoninic acid (BCA) assay, 40 µg total protein in loading buffer were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were immersed in 5% nonfat milk for 2 h to block nonspecific binding and then incubated with primary antibody (anti-LC3, 1:1000; anti-NF-κB, 1:500, abcom, USA) overnight at 4°C. After incubation with secondary antibody (1:2000), an enhanced chemiluminescence (ECL) detection system (Perkin Elmer, USA) with an ECL reagent was used to visualize proteins on the membrane. A semi-quantitative analysis of protein bands was performed using AlphaEaseFC 4.0 software (Alpha Innotech, San Leandro, CA, USA).

Stable expression of stubRFP-sensGFP-LC3 and StubRFP-SensGFP-LC3-mut in chondrocytes

Lentiviral vector containing the stubRFP-sensGFP-LC3 and StubRFP-SensGFP-LC3-mut reporter was purchased from Genechem (Shanghai, China). Cells stably expressing stubRFP-sensGFP-LC3 were selected by treatment with puromycin (2 µg/ml). After different treatments, cells were fixed and analyzed by fluorescence microscopy (Olympus BX51, Japan).

Table 1: Primer sequences for MMPs, ADAMTSs, chemokines, and GAPDH.
Transmission electron microscopy (TEM)

Chondrocytes cultured in 10-cm dishes were treated with TNF-α and/or hMSC-Exo for 24h and then harvested by manual scraping. After centrifugation, chondrocytes were fixed with 2.5% glutaraldehyde overnight and post-fixed with 1% osmium tetroxide for 2h at 4°C. After staining with 2% uranyl acetate, chondrocyte pellets were dehydrated through an acetone series and then embedded in Epon 812. After semi-thin sectioning, chondrocytes were stained with toluidine blue and observed under a light microscope. Finally, ultrathin sections were prepared based on light microscopic observations. Cellular ultra-structures were visualized using a transmission electron microscope (Hitachi, Japan)

Statistical analysis

Statistical assay was performed using GraphPad Prism 7.0 (GraphPad, San Diego, CA USA). All data in this study are shown as the mean ± standard error of the mean (SEM) of three independent experiments. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan’s multiple range test. P value < 0.05 was considered statistically significant.

Results

Characterization of exosomes derived from hMSCs

Exosome fractions were isolated as indicated in Materials and Methods. NTA2.3 (Nanosight LM10, UK) analysis indicated a mean diameter of 30–150 nm and a concentration of 0.97 × 10^7 particles/mL. Figure 1a shows a representative NTA analysis of exosome fractions. Exosome morphology was studied using TEM (Figure 1b). Expression of proteins in exosomes, including CD9, CD81, and transforming growth factor β1 (TGF-β1) was assessed by western blotting (Figure 1c).

Successful isolation and identification of chondrocytes

Chondrocytes were successfully isolated from OA cartilage tissue and stained with toluidine blue. As shown in Figure 2, toluidine blue staining illustrated that the chondrocytes were long-fusiform or irregular in shape. Overall, the above-mentioned findings demonstrate the successful isolation of chondrocytes from normal and OA cartilage tissue.

MSC-derived exosomes activate autophagy in TNF-α-treated chondrocytes

LC3 is a classic marker of autophagy. Western blotting of LC3 and stubRFP-sensGFP-LC3, therefore, were used to demonstrate the stimulatory effects of exosomes on autophagy. Exosomes significantly enhanced LC3-II expression in chondrocytes treated with TNF-α for 24h (Figure 3a, b). Interestingly, we found that LC3-II expression was increased in chondrocytes treated with exosomes alone and was lower than that in cells co-treated with both exosomes and TNF-α, indicating that inflammation enhances the stimulatory effect of exosomes on autophagy. stubRFP-sensGFP-LC3 is also widely used to monitor accumulation of the cytoplasmic LC3-positive puncta and autophagosomes. We established chondrocytes stably expressing stubRFP-sensGFP-LC3 using a lentiviral vector and assessed the autophagic flux. We used stubRFP-sensGFP-LC3-mut lentiviral vector as negative control. sensGFP is sensitive to pH change owing to the fusion of autophagosomes and lysosomes, whereas stubRFP is stable. Consistent with the results described above, we observed enhanced autophagosome-lysosome fusion in chondrocytes using a laser scanning confocal microscope. The number of yellow dots (LC3-positive puncta) in exosome-treated chondrocytes under inflammatory conditions induced by TNF-α was higher than that in chondrocytes treated with TNF-α alone (Figure 3c), suggesting accumulation of autophagosomes in the chondrocytes. TEM is the gold standard by which activation of autophagy is confirmed. Autophagosomes and autolysosomes with double membranes appeared in the chondrocytes treated with TNF-α and hMSC-Exos for 24h (Figure 3d). hMSC-Exos significantly enhanced the number of autophagosomes in TNF-α–treated chondrocytes compared with hMSC-Exos-treated cells. We further investigated autophagic flux by treatment with bafilomycin A1 to inhibit the fusion of lysosomes with autophagosomes during the late phase of autophagy, leading to LC3-II accumulation in the cytoplasm. Bafilomycin A1 significantly elevated the LC3-II/β-actin level in hMSC-Exos-treated chondrocytes, suggesting that hMSC-Exos increased autophagic flux (Fig. 3e, f). Furthermore, the stimulatory effect of hMSC-Exos combined with TNF-α on autophagic flux was also demonstrated by the increase in LC3-II/β-actin ratio following the addition of bafilomycin A1 (Figure 3g, h).

Autophagy mediates the inhibitory effect of hMSC-Exos on catabolic genes under inflammatory conditions

In order to investigate the involvement of autophagy in hMSC-Exos inhibition of TNF-α–induced catabolic genes, the levels of catabolic gene mRNA, including MMP-3, -9, and -13, ADAMTS4 and 5, CCL-2 and -5, and CXCL1 in chondrocytes were investigated by real-time PCR. Interestingly, hMSC-Exos significantly attenuated the TNF-α–induced increases in MMP-3, -9 and -13; ADAMTS5; CCL-2 and -5; and CXCL1 (Figure 4).

Involvement of NF-κB pathway in hMSC-Exos-induced autophagic activation

Inhibition of the NF-κB pathway involved in autophagic activation was investigated using NS398, an NF-κB inhibitor. NF-κB expression was enhanced after NS398 treatment in chondrocytes treated with TNF-α for 24hours, suggesting
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Discussion

The crucial role of MSC-derived exosomes for the regulation of cell migration, proliferation, differentiation, and ECM synthesis has been increasingly supported by recent findings[11-13]. A report in 2010 showed that exosomes are

Figure 1: Exosome characterization. (a) Representative results of NTA analysis of exosome fractions. (b) Transmission electron micrograph of MSC exosomes. Round structures, 30–100 nm in diameter, were identified as exosomes. Scale bar, 200 nm. (c) Representative western blot of the exosome markers CD9, CD81, and TSG101 in MSC exosomes.

Figure 2: Successful isolation and culture of chondrocytes. OA cartilage tissues stained with toluidine blue (× 200).

inhibition of the NF-κB pathway. Importantly, LC3-II levels were increased by NS398 in TNF-α–induced chondrocytes (Fig. 5a, b). stubRFP-sensGFP-LC3 analysis showed that the number of yellow puncta in the cytoplasm was also increased after treatment with NS398 (Fig. 5c), indicating activation of autophagy.
Figure 3: Effects of hMSC-Exos on autophagy in chondrocytes treated with TNF-α for 24 hours. Representative images are shown. (a) Chondrocytes were treated with TNF-α and/or hMSC-Exos for 24 hours, and LC3-II expression was analyzed by western blotting. (b) The optical density of the LC3-II/β-actin bands was analyzed. Data represent mean±95% confidence interval (CI), *P<0.01, n=5. (c) stubRFP-sensGFP-LC3 assay was used to detect the number of autophagosomes in chondrocytes. (d) Chondrocyte ultrastructure was examined by TEM. The white arrow indicates autophagosomes and autolysosomes in the cytoplasm. (e, f) Western blotting of LC3 following treatment with hMSC-Exos and/or bafilomycin A1 (Baf; 0.1 μM) for 24 hours, followed by determination of the optical density of LC3-II/β-actin bands. The values represent mean±95% CI, *P<0.05, n=5. (g, h) Treatment with hMSC-Exos combined with TNF-α and/or Baf (0.1 μM) for 24 hours was followed by western blotting of LC3 and analysis of the optical density of LC3-II/β-actin bands. The values represent mean±95% CI, *P<0.05, n=5.
secreted as active factors by MSCs responding to damage caused by myocardial ischemia reperfusion (I/R) [14]. Moreover, reports have demonstrated that MSC exosomes contribute to the repair and regeneration of cartilage via regulating immune reactivity, diminishing apoptosis, and increasing proliferation [15-17]. Exosomes, which function as intercellular communication vehicles, are small lipid-bilayer membrane-bound vesicles between 50 and 150 nm in diameter. Exosomes are able to transfer cargos of nucleic acids (mRNAs and microRNAs), proteins, and bioactive lipids [18]. Exosomes can produce biological responses in recipient cells [28]. Some findings have shown ambiguous effects of exosomes on the immune response or possible tumorigenicity, which may be considered unfavorable properties of exosomes[19, 20]. Nevertheless, there have been few studies conducted to investigate the precise molecular mechanisms by which MSC exosomes can promote chondrogenesis[16,20-23]. In the present study, we demonstrated that MSC-derived exosomes activate autophagy and enhance autophagic flux in TNF-α–treated chondrocytes, as demonstrated by western and mRFP-GFP-LC3 analysis. The NF-κB pathway was inhibited in chondrocytes treated with exosomes, suggesting that the NF-κB pathway might be involved in autophagic activation. Finally, the anti-catabolic effect of exosomes on chondrocytes was shown by real-time PCR. Autophagy is a self-degradative process that is important for balancing sources of energy at critical times during development and in response to cell stress. Recent data support the idea that autophagy can occur in combination with apoptosis in OA [24]. Indeed, Almonte-Becerril and

Figure 4: Inhibition of catabolic genes in chondrocytes treated with exosomes under inflammatory conditions for 24 hours. Representative images are shown. (a) Levels of MMP-3, -9, and -13; ADAMTS-4 and -5; CCL-2 and -5; and CXCL1 mRNA in TNF-α–and/or hMSC-Exo-treated chondrocytes were analyzed by real-time PCR. Data represent mean±95% CI, *P<0.05, **P<0.01, n=5.
collaborators demonstrated that, in early stages of OA, chondrocytes from the superficial zone showed increased expression of both apoptotic cell death and autophagic markers, even though the authors suggested that autophagy is activated as an adaptive response to sublethal conditions, with the aim of avoiding cell death [25]. Researchers have demonstrated that autophagy is a constitutively active and apparently protective process for the maintenance of homeostasis in normal cartilage. In contrast, human OA, OA and aging-related and surgically-induced OA in mice are associated with reduction and loss of ULK1, Beclin1, and LC3 expression in articular cartilage, suggesting that autophagy was decreased in the surgical OA mouse model. Increased expression of proinflammatory cytokines in cartilage, synovial membrane, and subchondral bone are believed to be linked to the development and progression of structural changes in the OA joint [26]. In this study, the mechanisms involved in the effects of MSC-derived exosomes on catabolism and inflammation in chondrocytes were investigated. Autophagy was found to mediate the effects of exosomes in chondrocytes. The stimulatory role of exosomes in autophagy was demonstrated by western analysis of LC3-II and mRFP-GFP-LC3 assay, TEM, and autophagic flux assay using bafilomycin A1. OA is a joint disorder identified by ECM degradation initiated by abnormal joint tissue metabolism followed by anatomic, and/or physiologic derangements. It is the end result of a combination of genetic, metabolic, biochemical, inflammatory, and mechanical predispositions and insults, but the loss of articular cartilage is certainly a consequence of this [27] The loss of articular cartilage can, in turn, aggravate inflammation, joint misalignment, and bony remodeling (subchondral bone destruction and osteophyte development), loss of muscular and ligamentous joint support, and ultimately the defining clinical symptoms of joint pain, instability and stiffness [28]. Chondrocytes are sensitive to physical injury, and extreme mechanical forces alter the chondrocyte balance of anabolic and catabolic factors, compounding injury and inducing inflammation [29]. Catabolic enzymes, such as matrix metalloproteinase-13 (MMP-13), the dominant factor in collagen type II degradation, and disintegrin and metalloproteinases with the thrombospondin motifs (ADAMTS4 and 5) that degrade the predominant proteoglycan, aggrecan, are upregulated in chondrocytes and synovial cells during OA. In our study, catabolic genes, including MMP-3, -9, and -13 and ADAMTS5 and the proinflammatory cytokines, including CCL-2 and -5 and CXCL1, were also inhibited by hMSC-Exos treatment, suggesting the potential role of hMSC-Exos in maintaining cellular homeostasis and treating OA. Therefore, the effect of hMSC-Exos on catabolism requires further investigation. The NF-κB transcription factor family is ubiquitously expressed in all cell types and regulates essential cellular responses including survival, differentiation, apoptosis, and autophagy [30]. It is clear that chronic and low-grade inflammation is involved in the progression of OA that leads to catabolic responses in chondrocytes via upregulation of factors such as nuclear NF-κB [31]. Li-Bo J [32] suggested that ADSCs activate autophagy and enhance autophagic flux in chondrocytes treated with IL-1β or LPS, and the mTOR pathway might be involved in the activation. Activation of NF-κB and autophagy are two processes involved in the regulation of cell death, but whether there is crosstalk between these two signaling pathways is largely unknown. Rapamycin can inhibit the overexpression of inflammatory catabolic genes by activating autophagy, and can suppress the NF-κB signaling pathway in chondrocytes to break the positive feedback loop with inflammatory factors and reduce the rate and level of inflammation progression [33]. Although the inhibitory role of MSCs on NF-κB has been extensively studied, the association between NF-
κB and autophagy in chondrocytes is unclear. It has been reported that LPS can induce inflammation by promoting secretion of various inflammatory factors, including IL-1β and activating the COX-2/PGE2 pathway. In our study, the NF-κB inhibitor NS398(COX-2 inhibitor) was shown to increase LC3-II levels and autophagosome number in TNF-α–treated chondrocytes. In porcine primary granulosa cells, Gao et al. found that NF-κB activated autophagy via activation of the JNK pathway. The JNK pathway might also be associated with upregulation of autophagy in cancer cells. However, the detailed mechanisms underlying NF-κB inhibitory effects on autophagic activation require further investigation. In conclusion, the present study demonstrates that MSC-derived exosomes induce autophagic activation and inhibit the expression of TNF-α–induced catabolic genes and chemokines. hMSC-Exos suppresses the activation of the NF-κB pathway stimulated by TNF-α. Finally, the NF-κB pathway inhibitor NS398 activates autophagy in TNF-α–treated chondrocytes. Our findings further our understanding of hMSC-Exos-induced autophagic activation via the NF-κB pathway.

**Data Availability Statement**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**Author Contributions**

All listed authors have made substantial contributions to the following aspects of the manuscript:

1. The conception and design of the study, or acquisition of data, or analysis and interpretation of data.
2. Drafting the article or revising it critically for important intellectual content.
3. Final approval of the version to be submitted.

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**Conflict Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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