applied biosciences

Systematic Review

Characterization of the Joint Microenvironment in Osteoarthritic Joints for In Vitro Strategies for MSC-Based Therapies: A Systematic Review

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Abstract: Osteoarthritis is a joint disease that causes pain, stiffness, and reduced joint function because the protective cushioning inside the joints, called cartilage, gradually wears away. This condition is caused by various factors and complex processes in the joint's environment, involving different types of cells producing factors that can either maintain the joint health or contribute to osteoarthritis. This study aimed to understand the factors influencing both healthy and diseased joints in DDD strategies for the in vitro preconditioning of MSCs. An electronic search in the PubMed, Scopus, and Web of Science databases was carried out using the terms (cartilage OR chondr*) AND (repair OR regeneration OR healing) AND (niche OR microenvironment)) AND ("growth factor" OR GF OR cytokine). Researchers used various methods, including macroscopic examinations, histology, immunohistochemistry, and microCT. Molecules associated with joint inflammation were identified, like macrophage markers, MMP-13, TNF, apoptotic markers, and interleukins. Chondrogenesis-related factors such as aggrecan GAG, collagen type II, and TGF beta family were also identified. This study suggests that balancing certain molecules and ensuring the survival of joint chondrocytes could be crucial in improving the condition of osteoarthritic joints, emphasizing the importance of chondrocyte survival and activity. Future preconditioning methods for MSC- and EV-based therapies can find suitable strategies in the described microenvironments to explore co-culture systems and soluble or extracellular matrix factors.

Keywords: musculoskeletal disease; niche; tissue repair

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Citation: Silvestrini da Silva, A.; Hertel, F.C.; Valente, F.L.; Voorwald, F.A.; Borges, A.P.B.; Sabino, A.d.P.; Sepulveda, R.V.; Reis, E.C.C. Characterization of the Joint Microenvironment in Osteoarthritic Joints for In Vitro Strategies for MSC-Based Therapies: A Systematic Review. *Appl. Biosci.* **2024**, *3*, 450–467. https://doi.org/10.3390/applbiosci3040029

Academic Editors: Alessandro Pitruzzella, Alberto Fucarino and Fabio Bucchieri

Received: 16 November 2023
Revised: 9 October 2024
Accepted: 10 October 2024
Published: 17 October 2024

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1. Introduction

Osteoarthritis (OA), a degenerative joint disease characterized by progressive inflammation [1], is an example in which MSCs have been successfully used for treatment [2,3]. OA is a highly prevalent musculoskeletal disorder that affects approximately 250 million people worldwide [4]. OA can affect any joint but most commonly affects the knee, hands, hip, and spine. In addition to this evident harm to the individual's health, the economic burden of OA for patients and society is considerable [3,5,6], with large expenses related to long-term treatments and the number of people affected, a number that has increased by 48% between 1990 and 2019 [5,6].

Mesenchymal stem/stromal cells (MSCs) have been proven effective for OA treatment [1,2,7]. These cells are defined as those adult cells capable of self-renewing and differentiation, giving rise to mesenchymal tissues [1]. However, cell therapy still carries the

concerns of foreign cell transplant, related to adverse immune responses and challenges of the logistics of live cell therapy, together with regulatory and ethical considerations regarding consistency and quality [7–9]. Since MSCs mostly exert their therapeutic effects through secreted paracrine factors, recently, extracellular vesicles (EVs) have been studied in an effort to develop cell-free therapeutic strategies, including preconditioning methods [10–12]. It has been shown that EVs contain numerous bioactive molecules, including lipids, proteins, mRNAs, transfer RNAs (tRNAs), long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and mitochondrial DNA (mtDNA) [13], which can influence local tissues with regenerative and anti-inflammatory effects [14]. D'arrigo et al. (2019) [15] clearly addressed this promising field in the systematic review, showing 20 studies on extracellular vesicles for OA treatment. Results showed the reduction in inflammatory factors and improvement in cell proliferation in in vitro works and the prevention of cartilage destruction, improvement of subchondral bone integrity, and decrease in M2 macrophage infiltration and inflammatory cytokines in animal models.

Regarding their benefits, MSCs are not quiescent in the adult tissues where they reside, but exert functions related to that tissue microenvironment [15,16]. The microenvironment is a key point in cell therapy, as MSCs receive information from it, responding with the right bioactive factors both when considering where they are harvested and where these cells are implanted [16–19]. Therefore, the therapeutic effects of EVs are probably related to the microenvironment in which MSCs cells came from or the conditions in which they were cultured [10,20]. Indeed, Gupta (2022) [21] and Duan (2021) [22] have shown differences in MSC secretome and its effect as a consequence of different in vitro preconditioning methods. Currently, several characteristics of the OA microenvironment are known; it is clear, however, that are still factors to be better understood in an osteoarthritic joint in order to unravel factors influencing MSC-based therapies. An example is the influence that the inflammatory microenvironment can exert when cell therapy is applied to an osteoarthritic joint. Cartilage regeneration has been documented, but also fibrocartilage or hypertrophied cartilage, tissues that are not the goal of an adequate regeneration for joint function [23], and the factors driving this process are still unknown. Similarly, the microenvironment of an osteoarthritic joint in the process of recovery (reduction in inflammation and/or cartilage regeneration) is still the result of many studies seeking the basis to favor this process. Thus, with this systematic review, we aimed at analyzing the articular microenvironment in an osteoarthritic joint and in those undergoing different treatments as approached by the studies, seeking to detail the factors involved both in the disease and regeneration. We believe that preconditioning methods are the key for successful therapies with MSCs and their derived EVs; thus, detailing the factors driving the process specifically for cartilage regeneration is essential for developing in vitro methods.

2. Materials and Methods

2.1. Search Strategy

This systematic literature review was carried out by two independent authors [ASS and ECCR] and conducted following the guidelines of preferred reporting items for systematic reviews and meta-analysis (PRISMA). The authors searched the database independently and were blinded for organizations, financial assistance, researchers' information, and conflicts of interest. An initial protocol was defined to guide the research as follows:

(a) Population: animal model for induction of articular osteoarthritis;
(b) Intervention: treatment protocols applied directly to the lesion;
(c) Results: description of the microenvironment of a damaged joint in the process of repair.

The search was carried out in PubMed, Web of Science, and Scopus databases. The databases were accessed in January 2024, and included studies in the last 10 years. To achieve the maximum sensitivity of the search strategy, we combined the following keywords (strings) with Boolean operators (AND or OR): (cartilage OR chondr*) AND (repair

OR regeneration OR healing) AND (niche OR microenvironment)) AND ("growth factor" OR GF OR cytokine). For data extraction, the software StArt 2.0 (State of the Art through Systematic Review) (UFSCAR), was used.

2.2. Selection Criteria

The studies were selected in three consecutive steps and according to the eligibility criteria chosen by the authors. First, after eliminating duplicates, the titles were selected to assess their eligibility. Then, the articles that met, or were considered to meet, the criteria had their abstracts evaluated. Sequentially, the selected articles were retrieved as full texts. In the final step, all studies that reported the outcome of interest were selected. Details are shown in Figure 1.

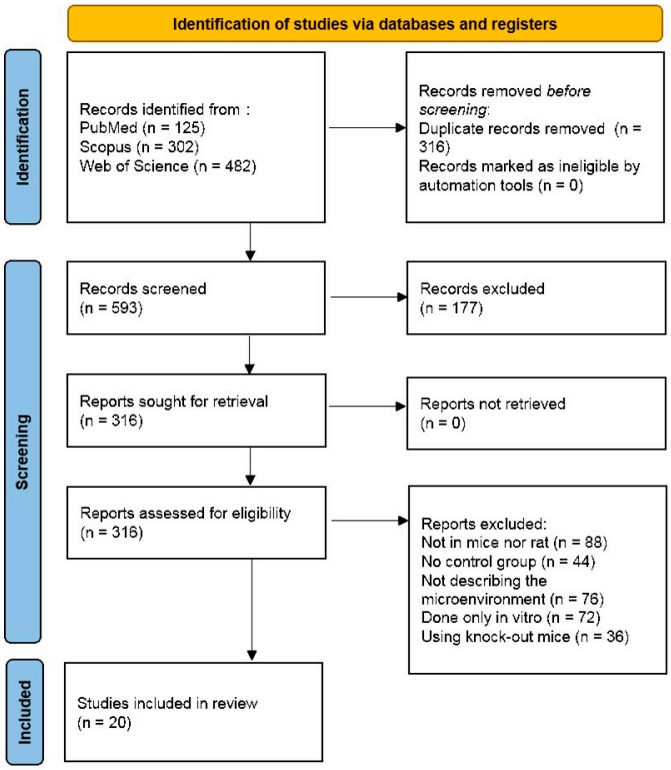


Figure 1. Flowchart of the systematic review mechanism.

Studies were considered eligible if they met the following criteria:

(a) Experimental studies with induction of articular osteoarthritis in mice and rat models only;
(b) Only studies in English;
(c) Studies that addressed the description of the in vivo microenvironment/niche for the development of osteoarthritis;
(d) Studies that had a negative control group.

The following exclusion criteria were defined:

(a) Studies only in vitro;
(b) Studies that addressed the microenvironment/niche only in in vitro evaluations;
(c) Studies that addressed cartilage that was not of the hyaline type;
(d) Studies that did not have a negative control group;
(e) Studies using knock-out animals.

3. Results

Of the 67 studies selected for the extraction phase (full-text articles reviewed), 47 were excluded mostly for using species other than mice or rat and for not addressing the joint microenvironment. Table 1 summarizes the findings [24–43].

Regarding the experimental animal model, the- majority used the rat as an animal model (15 studies). As for the induction of OA, most studies used the ligament rupture associated with medial meniscus excision, and the anterior cruciate ligament rupture. In all included studies, OA was induced in the femorotibiopatellar joint uni- or bilaterally, except for Forrester et al. (2022) [35] using the scapuloumeral joint, and for Zhang et al. (2022)a [36] using the temporomandibular joint, both due to focusing on a particular dysfunction of these joints. Time-points of euthanasia for the collection of joints for analysis varied considerably among studies, with a minimum observation time-point of days [29] and a maximum of 10 months [35]. Complementary analyses and tests varied greatly, but for histology, a grading system such as ELISA or PCR were the most common. Most of the studies evaluated upregulated negative regulators of chondrogenesis in the experimental and control groups. Table 2 summarizes the findings in OA microenvironments.

Table 1. Results observed after induced OA in each of the included studies.

Article Number	Authors and Model	Analysis	Time-Points (Weeks or as Described)	General Results
[24]	Dai et al. (2018) Rat Ligament rupture and partial bilateral medial meniscus excision	Gait observation. Toluidine blue staining histology for GAG labeling. Immunohistochemistry: MMP13, CD68, MR, and TUNEL. Synovial fluid ELISA: TGF-β1 and TGF-β3.	6	Control group still presented a painful gait, unlike treated groups. The articular cartilage surface exhibited structural damage, including discontinuities and fractures. Weak tagging for GAG. Strong tagging for MMP13, CD68, and TUNEL analysis. Weak tagging for MR. Significantly decreased levels of TGF-β1 and TGF-β3.
[25]	Chen et al. (2019) Rat Bilateral osteochondral defect	Macroscopic evaluation. Histology: ICRS score [36]. Immunohistochemistry: collagen type II and MMP13.	12	Worst indices in the ICRS macroscopic score among the study groups. Worst indices in the ICRS histological score among the study groups. Weak type II collagen labeling on the defective joint surface and high MMP13 expression.
[26]	Hu et al. (2019) Rat Ligament rupture and partial bilateral medial meniscus excision	Histology: OARSI score [38] with toluidine blue staining and safranin O fast green (GAG). Immunohistochemistry: TUNEL, type II collagen, aggrecan, MMP-13, CD68, and MR. Synovial fluid ELISA (TGF-β1 and TGF-β2).	7	Worst indices in the OARSI histological score among the study groups. Reduced GAG content. Low labeling of aggrecan, collagen type II, and MR High marking of MMP-13, CD68, and TUNEL. Low levels of TGF-β1 and TGF-β2.
[27]	Cheng et al. (2021) Rat Medial collateral ligament and the medial meniscus transection	Pain. Histology (damage score). Micro-CT. RT-PCR (IL-1 b, IL-6, TNF-a).	4 and 12	Cartilage destruction, joint swelling, and bone erosion. Increased IL-6, TNF-a.

Table 1. *Cont.*

Article Number	Authors and Model	Analysis	Time-Points (Weeks or as Described)	General Results
[28]	Cong et al., (2021) Rat Papain-induced	Histology: Mankin scoring. TEM (transmission electron microscopy). Synovial fluid ELISA (MMP-13, IL-6, TNF-a). RT-qPCR and immunohistochemistry (β-catenin, MMP13, and GSK-3β).		Mankin score: worst in OA model. Chondocytes: enlarged vacuole-like structures. Collagen fibers loose and broken. High levels of MMP-13, IL-6, and TNF-a. High levels of β-catenin and MMP13, and low levels of GSK-3β.
[29]	Fukui et al. (2021) Mice Anterior cruciate ligament rupture	OARSI. qRT-PCR (IL-1b, IL-6). IVIS Spectrum imaging system (fluorescent for MMP activity).	4 h 1, 3, and 7 day	Increased OARSI score. Increased IL-1b, IL-6: 4 h up to 1 day. Increased MMP activity: 3 and 76 days. Sinovite.
[30]	Jiang et al. (2021) Rat Osteochondral defect	Immunohistochemistry: IL-1 and IL-10; TNF-α, CD206, and CD68; CD86, CD73, and CD105	10 days	Weak labeling for IL-10, CD206, and CD68. Strong tagging for CD86. There was no difference in the labeling of TNF-α, IL-1, CD73, and CD105 between groups.
			20 days	Weak labeling for IL-10, CD206, and CD68. Strong tagging for CD86. There was no difference in the labeling of TNF-α, IL-1, CD73, and CD105 between groups.
		ELISA: TNF-α, IL-1b, IL-6, IL-17.	2	High expression of TNF-α, IL-1b, IL-6, and IL-17.
			12	High expression of TNF-α, IL-1b, IL-6, and IL-17.
		Histology: Kikuchi score with safranin O fast green (GAG) color.	2	Worst indices in Kikuchi histological score among study groups. Weak scoring for GAG.
			12	Worst indices in Kikuchi histological score among study groups. Weak scoring for GAG.
		Immunohistochemistry: TNF-a, IL-1b, IL-6, IL-17.	2	High expression of TNF-α, IL-1b, IL-6, and IL-17.
			12	High expression of TNF-α, IL-1b, IL-6, and IL-17.
		Behavior and pain analysis: WBI.	2	Low WBI value.
			12	Low WBI value.
[31]	Liu et al. (2021) Rat Anterior cruciate ligament transection with medial meniscectomy	Weight-bearing distribution of the hind limbs. Knee joint width. ELISA for inflammatory cytokines (IL-6, IL-1β, and TNF-α).	12	Decreased weight bearing. Higher knee joint width. Increased serum levels of IL-6, IL-1β, and TNF-α.

Table 1. *Cont.*

Article Number	Authors and Model	Analysis	Time-Points (Weeks or as Described)	General Results
[32]	Mou et al. (2021) Rat Intra-articular injection of monoiodoacetic acid	ELISA: TNF-α, IL-1b, IL-6, IL-17.	2	High expression of TNF-α, IL-1b, IL-6, and IL-17.
			12	High expression of TNF-α, IL-1b, IL-6, and IL-17.
		Histology: Kikuchi score [40] with safranin O fast green (GAG) color.	2	Worst indices in Kikuchi histological score among study groups. Weak scoring for GAG.
			12	Worst indices in Kikuchi histological score among study groups. Weak scoring for GAG.
		Immunohistochemistry: TNF-a, IL-1b, IL-6, IL-17.	2	High expression of TNF-α, IL-1b, IL-6, and IL-17.
			12	High expression of TNF-α, IL-1b, IL-6, and IL-17.
		Behavior and pain analysis: WBI.	2	Low WBI value
			12	Low WBI value
[33]	Qian et al. (2021) Mouse Anterior cruciate ligament transection surgery	Histology: HE and safranin O. Mankin score.	1	Cartilage superficial destruction. Mankin score increased in comparison with sham control.
			2	Cartilage destruction. Decreased safranin O staining. Increased Mankin score compared with previous week.
			4	Cartilage destruction up to the calcified cartilage layer. Increased Mankin score compared with previous week.
			8 and 12	Full-thickness cartilage defect. Increased Mankin score compared with previous weeks.
[34]	Arakawa et al. (2022) Mice Destabilization of medial meniscus	OARSI score. Meniscus histology. Immunohistochemistry (TNF-a and MMP-13).	8, 12	OARSI score higher. Meniscus score higher. TNF-a and MMP-13 higher.
[35]	Forrester et al. (2022) Mice Botulinum toxin injection (paralysis model)	Gait. MicroCT. Histology: MSAS. RT-PCR (Ihh, Acan, Runx2, Dkk1, Col2A1, Col10A1, BGLAP, ALPL, and BMP2).	24 and 40	Abnormal gait. Increased MSAS. Decreased expression Runx2, Dkk1, and BMP2. Increased expression Col10A1 and BGLAP.
[36]	Zhang et al. (2022) a Rat Malloclusion stress	Histology: safranin O and HE. OARSI-modified Mankin score. Immunohistochemistry (ADAMTS5, MMP13, HIF2 alpha, apoptosis factor Caspase3).	2	Cartilage degeneration: irregular surfaces, superficial clusters, areas of loss of proteoglycan. Scores increased when compared with normal cartilage.
			4	Cartilage degeneration: irregular surfaces, superficial clusters, areas of loss of proteoglycan. Scores increased when compared with previous time-point. Increased ADAMTS5, MMP13, HIF2 alpha, apoptosis factor Caspase3
			8	

Table 1. *Cont.*

Article Number	Authors and Model	Analysis	Time-Points (Weeks or as Described)	General Results
[37]	Zhang et al. (2022) b Rat Modified Hulth method (excision of anterior cruciate ligament + lateral meninscus)	Weight bearing. RT-qPCR (Autophagy genes: p62, Atg3, Atg7, Atg12). TEM.	4	Increased expression of p62, Atg3, Atg7, Atg12.
			10	Increased expression of p62, Atg3, Atg7, Atg12, but lower than 4 weeks.
[38]	Fukui et al. (2023) Rat Medial meniscus extrusion (MME) Medial meniscus transection (MMT)	Gait. OARSI. Histology. RT-PCR (IL-1β, TNFα, MMP-3, ADAMTS-5, MMP-13, and NGF).	8	Gait (2, 4, 6, and 8 weeks): decreased stand time, maximum contact area, intensity and swing speed in MME. Increased OARSI score (MME and MMT). Increased IL-1β, MMP-3, MMP-13, and NGF (MME and MMT). TNFα (MMT > MME, but not different from control).
[39]	Huang et al. (2023) Rat Anterior cruciate ligament transection	Histology. qRT-PCR (NEAT1, miR-374b-5p, and PGAP1).	4	Narrow joint space, thin hyaline cartilage, increased thickness of calcifed cartilage, and macrophage hyperplasia was observed. Decreased expression of NEAT1 and PGAP1. Increased expression of miR-374b-5p.
[40]	Long et al., (2023) Rat Destabilization of medial meniscus	OARSI. Histology: synovitis. RT-qPCR (Matn3, IL-17). Western blotting (IL-17, MMP-13, ADAMTS5, aggrecan, Col2A1, LC3I, LC3I, and Beclin 1). ELISA (IL-6 and TNF-a).	4	Increased synovitis and OARSI score. Increased IL-6, TNF-a, MMP-13, ADAMTS5, and IL-17. Decreased aggrecan, Col2A1, LC3I, LC3I, and Beclin 1.
[41]	Valerio et al. (2023) Rat Intra-articular fracture mediated by 5 joule blunt impact	Histology. Micro-CT (BV/TV, Tb.N, Tb.Th, TB.Sp, BMD and morphology). Multiplex immunoassay (IL1A, IL17, CCL2, CCL3, CCL11, CCL7, CXCL1, COMP, NTX). Immunohistochemistry.	2 and 8	Increased synovitis and fibrosis. Micro-CT: qualitative differences in morphology. Increased IL1A, IL17, CCL2, COMP, NTX.
[42]	Wang et al. (2023) Rat Papain-induced	Mankin score. ELISA (IL-1 b, IL-6, TNF-a) in serum. RT-qPCR and Western blotting (mRNA and proteins of the TLR4/MyD88/NF-kappa B signaling pathway).	4	Increased Mankin score. Increased IL-1 b, IL-6, TNF-a. Increased TLR4, ofMyD88 mRNA, NF-kB mRNA.
[43]	Wu et al., (2023) Mice Anterior cruciate ligament transection	OARSI. Micro-CT. ELISA (COMP, CTX-II, PIINP). Immunohistochemistry (aggrecan, COLII, ADAMTS4, and MMP13).	8	OARSI score higher. Micro-CT: lower BMD, BV/TV, Tb.Th, Tb.N and higher Tb.Sp. Higher COMP, CTX-II, PIINP. Lower aggrecan, COLII. Higher ADAMTS4 and MMP13.

Table 2. List of expressed and unexpressed factors in joints submitted to OA.

Article Number	Authors	Increased Factors in OA	Decreased Factors in OA	Factors That Increased in Chondrogenesis
[24]	Dai et al. (2018)	MMP-13 CD68 TUNEL	GAG MR TGF-β1 TGF-β3	GAG MR TGF-β1 TGF-β3
[25]	Chen et al. (2019)	MMP-13	Type II collagen	Type II collagen
[26]	Hu et al. (2019)	MMP-13 CD68 TUNEL	Aggrecan, Type II collagen MR TGF-β1 TGF-β2	Aggrecan, Type II collagen MR
[27]	Cheng et al. (2021)	IL-6, TNF-a	-	-
[28]	Cong et al. (2021)	MMP-13 IL-6 TNF-α β-catenin	GSK-3β	GSK-3β
[29]	Fukui et al. (2021)	IL-1b, IL-6, MMP		
[30]	Jian et al. (2021)	CD86	GAG Type II collagen IL-10 CD206 CD68	GAG Type II collagen IL-10 CD206 CD68
[31]	Liu et al. (2021)	PARP-1, iNOS COX-2	-	-
[32]	Mou et al. (2021)	TNF-α IL-1b IL-6 IL-17	GAG	GAG
[33]	Qian et al. (2021)	ADAMTS5 (1 o 4 weeks) COX-2 iNOS CD31-positive cells VEGF-A VEGFR2 (weeks 4 to 12)	ADAMTS5 (total cartilage destruction at 8 and 12 weeks) Type II collagen VEGFR2 (weeks 1 and 2)	-
[34]	Arakawa et al. (2022)	TNF-α MMP-13	-	-
[35]	Forrester et al. (2022)	Col10A1 BGLAP	Runx2, Dkk1, and BMP2	-
[36]	Zhang et al. (2022) a	ADAMTS5 MMP13 HIF2 alpha Apoptosis factor Caspase3	Aggrecan Type II collagen HIF1 alpha	
[37]	Zhang et al. (2022) b	p62 Atg3 Atg7 Atg12 (autophagy genes)	-	-

Table 2. *Cont.*

Article Number	Authors	Increased Factors in OA	Decreased Factors in OA	Factors That Increased in Chondrogenesis
[38]	Fukui et al. (2023)	IL-1β MMP-3 MMP-13 NGF	-	-
[39]	Huang et al. (2023)	miR-374b-5p	NEAT1 PGAP1	-
[40]	Long et al. (2023)	IL-6 TNF-a MMP-13 ADAMTS5 IL-17	Aggrecan Col2A1 LC3I LC3I Beclin 1	Aggrecan Col2A1 LC3I LC3I Beclin 1
[41]	Valerio et al. (2023)	IL1A IL17 CCL2 COMP NTX	-	-
[42]	Wang et al. (2023)	IL-1 b IL-6 TNF-a TLR4 ofMyD88 NF-kB mRNA		TLR4, ofMyD88 NF-kB mRNA
[43]	Wu et al. (2023)	ADAMTS4 MMP13	Aggregan COLII	-

Regarding the quality of studies and risk of bias, Figure 2 shows the main characteristics of their assessment. The "unclear" score indicates inadequate reporting of most analyzed items.

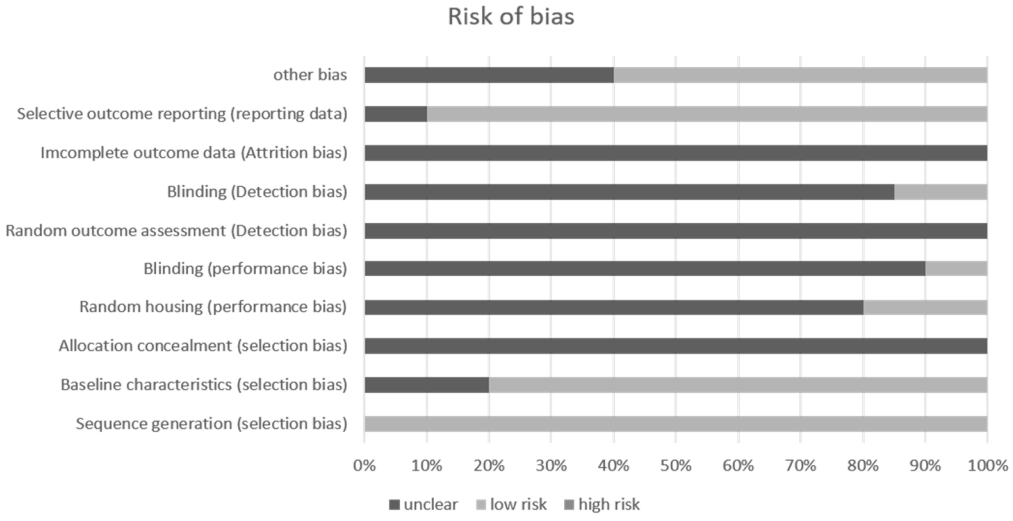


Figure 2. Risk of bias in assessing the methodological quality of the 20 articles included in this systematic review.

Regarding the generation of random sequences, a "low risk" score was identified in all studies. The method used to conceal the allocation was not reported, but all studies reported that the allocation was performed in a randomized manner. As for baseline characteristics, 80% of the studies were identified as having a "low risk" score for providing

complete information regarding animal characteristics and distribution between groups. The others were identified as "unclear" because they concealed some information regarding the characteristics of the animals or did not describe whether they carried out an equal distribution in the groups regarding the gender of the animals when using animals of both sexes. As for allocation concealment, random housing, blinding of participants, and evaluation of random outcomes, most studies were identified with a score of "unclear" because they did not provide information that would allow this assessment to be made. As for the blinding of the results, 15% of the studies were identified with an "unclear" score because they did not report whether the evaluators were blinded, while the majority, 75%, did so, being classified as "low risk". All studies were identified with a "low risk" score for the incomplete data of the results, since they provided the results of all evaluated groups. A "low risk" score was also assigned to selective reports, as 90% of the studies provided information on the protocols. As for other sources of bias, 40% of the studies were identified with an "unclear" score as they did not provide quantitative or descriptive data regarding the findings in immunohistochemistry, providing only microscopic images.

4. Discussion

Before discussing the main objective of the present study, the OA microenvironment and the methodological quality of the studies must be assessed. The risk of bias analysis showed an overall low risk, with no high risk reported. On the methodological variations among the studies, we considered animal models and evaluation methods to have a greater probability to interfere on the reported results, thus we focused on the two species more related to each other and mostly used as animal models, i.e., rat and mice [44–53].

Smaller animal models are much easier to handle, allow for quick experimental studying, generate lower maintenance costs, and are more easily accessible than larger animal models (horses, pigs, and dogs) [54–56]. However, they may not allow an adequate collection of tissue samples due to their small size and tend to differ to a greater extent in their anatomical and histological structure when compared with humans [57]. Large animal models such as swine or sheep allow for a slower progression of the disease and more time to assess the stages of OA, similar to what is observed in humans [54]; however, they are more costly and their studies take longer to conduct.

Also, evaluation methods must be consistently considered once results are highly dependent on their accuracy. All studies in this review performed histological analysis of joint tissue samples, importantly relating the microenvironment factors to the stage of OA evaluated. Several histological scoring systems for staging and classifying OA were found to be applied universally in any OA induction model, mainly joint instability, enzymatic-induced degradation, or critical osteochondral defect [58]. The most widely used histological scoring systems was OARSI.

Based on the findings of the studies in this review, it was clear upon reviewing the reported triggering factors that the joint is unable to recover its structural and functional integrity if it does not receive adequate therapy. This is evident by the findings of fibrocartilage, tissue disorganization, joint surface irregularity, fibrosis, poor score in histological OA staging scores, increased expression of negative chondrogenesis regulators, and decreased expression of positive chondrogenesis regulators or of those that should be expressed in a normal joint. In such microenvironments, the negative regulators of chondrogenesis that had high expression can be seen in Table 2. Of these, the main ones are the pro-inflammatory cytokines, which are fundamental mediators of the onset and progression of OA, many related to macrophages. For instance, macrophages are the main producers of TNFα and, interestingly, are also highly responsive to TNFα [49], a factor shown to be increased in OA by five studies [28,32,34,40,42]. IL-1β is an important macrophage-derived pro-inflammatory cytokine that acts primarily through the induction of a cytokine network [59,60]. IL-6 plays an important role in macrophage polarization [61] and IL-17 is involved in macrophage activation [62]. IL-1β and IL-6 were shown to be increased in the OA microenvironment by five [29,32,38,41,42] and six studies [27–29,32,42], respectively.

These showed the importance of macrophages and their products for the OA microenvironment and should be taken into account when designing an in vitro preconditioning method.

Macrophages are among the variety of immune system cells included in the regulation of the inflammatory process. Macrophages are phagocytic cells that can be found in almost every tissue. There is growing evidence to suggest that macrophages are strongly involved in modulating inflammation in osteoarthritic joints through the secretion of inflammatory mediators [63], as clearly shown in this review. Depending on the signaling, macrophages can present two phenotypes: (a) M1 type macrophages that are pro-inflammatory; (b) M2 type macrophages that are anti-inflammatory and pro-resolving. The imbalance between these two phenotypes can lead to chronic inflammation and has been identified as essential in the development of OA because it can differentially modulate the anabolic or catabolic responses of different cell types with the onset or progression of OA [63,64]. Type M1 macrophages secrete, in addition to inflammatory cytokines, cartilage matrix degradation enzymes, including MMPs, leading to cartilage degeneration [65]. MMPs, especially MMP-13, were the factors mostly evaluated among the included studies, shown to be increased in all 10 of these works [24–26,29,30,34,36,38,40,43]. Macrophages with M2 anti-inflammatory phenotypes promote responses that fight inflammation and are subdivided into M2a (induced by IL-4 and IL-13), M2b (induced by TLR agonists), and M2c (induced by IL-10) [66]. Therefore, when designing an in vitro system for MSC preconditioning aiming at obtaining extracellular vesicles to favor cartilage repair, a suitable strategy would be the co-culture of MSCs with M2 macrophages, always avoiding the pro-inflammatory M1 macrophages and their factors such as IL-1b and IL-6. Indeed, numerous co-culture systems with macrophages have been described [67–69], although not for the purposes of MSC preconditioning as we explore in this review, which shows the successful methods for this co-culture.

Importantly, there are a large number of commonly used macrophage markers such as CD14, CD16, CD64, CD68, CD71, and CCR5. The exact marker to use will depend on the macrophage subset and conditions in your local environment. CD80, CD68, and iNOS markers are expressed in M1 macrophages; the markers CD163 and CD200r are expressed in the M2a phenotype; CD86 and MHCII markers are expressed by both M1 and M2b macrophages; the M2c macrophages express the markers CD163, TLR1, and TLR8. There are a few unique macrophage markers, and often multiple markers will be needed to identify your cell type [70]. In this review, we could observe that some studies included in their analysis the evaluation of the expression of macrophage surface markers (CD68), M1 macrophage markers (CD86), and M2 macrophage markers (MR); the first two increased in OA and MR decreased in OA and increased in chondrogenesis. They also evaluated the expression of macrophage and cytokine products (TNF-α, IL-1β, IL-6, IL-17, and MMP13); all increased in OA (detailed information in Table 2). CCL2 [41], a chemokine chemotactic for monocytes, was studied and can be discussed regarding these considerations about macrophages. This information is essential for us to understand the mechanisms involved in the progression of OA and the evolution of the repair process of articular cartilage tissue exposed to an inflammatory microenvironment. However, most studies used a small number of markers to characterize the macrophage phenotype, although multiple markers are needed to identify cell type. Therefore, with the complexity of co-culture systems and macrophage phenotyping, when designing an in vitro microenvironment for MSC preconditioning, a suitable strategy to be explored is the direct use factors produced by M2 macrophages.

In addition to cytokine and macrophage analysis, TUNEL analysis was also performed by Dai et al. (2018) [24] and Hu et al. (2019) [26] and shown to be increased in OA, as it allows the detection of apoptotic cells, thus helping to assess the evolution of the disease [48]. Evidence indicates that cell death may be an important factor in the early stages of OA development and its perpetuation, which may also contribute to the microenvironment signaling. Accordingly, a strategy to be explored for OA treatment

would be the prevention of cell death using the manipulation of the microenvironment, including MSCs and their products.

Therapeutic modalities analyzed by the studies included in this review were highly variable, including exercise, biomaterials, cell therapy with MSCs, growth factors, collagen, flavonoids, and exosomes [24–28,30,32,40,42]. These treatments, despite being very promising, did not achieve complete regeneration of the articular cartilage, a fact mainly observed when compared with the sham/normal group. However, these results are analyzed here not only for the treatment itself but from another perspective, i.e., assessing the microenvironment of these repair processes, and identifying the factors related to regeneration to mimic this successful microenvironment in vitro for the development of preconditioning methods for MSCs.

Many studies included in this review evaluated the expression of aggrecan, GAG, and type II collagen in normal joints, untreated osteoarthritic joints, and osteoarthritic joints treated with therapies that were shown to be chondrogenic. In the presence of OA, these factors were found decreased or not expressed, contrary to what has been reported in normal and treated joints, in which these factors are highly expressed. Aggrecan, GAG, and type II collagen constitute the majority of the extracellular matrix (ECM) components of whole articular cartilage [71,72], with some of the ECM component proteins being regulated by Sox9, including type II collagen. Type II collagen expression is necessary for chondrocyte differentiation [73–75]. Aggrecan is a large proteoglycan that gives articular cartilage the ability to support loads and possesses a central protein with covalently linked GAG chains [76,77]. These results clearly demonstrate cartilage regeneration to some extent, but most importantly, for the present review, these factors must be highly expressed in a healthy joint microenvironment.

Also, among the important factors for the activity of chondrocytes is the transforming growth factor β (TGFβ) superfamily, which regulates their activity [78] in addition to controlling the development of articular cartilage tissue [78,79]. The three types of TGFβs found in mammals are TGFβ1, TGFβ2, and TGFβ3, each encoded by a different gene [80], and all forms are produced and secreted by chondrocytes [80]. Furthermore, their signaling is directly associated with the synthesis and maintenance of ECM of the cartilage tissue, increasing the synthesis of proteoglycans [81] and the neutralization of pro-inflammatory signaling mediated by IL-1 [82]. Another important role played by TGFβ is that of regulating and blocking hypertrophy in chondrocytes [83]. These data corroborate the results found by Dai (2018) [24] and Hu (2019) [26], included in the present review. In both studies, in the presence of an osteoarthritic joint in which there was mostly formation of fibrotic tissue, expressions of TGFβ1, TGFβ2, and TGFβ3 were very low, in contrast with what is found in sham/normal joints (significant expression of TGFβ1, TGFβ2, TGFβ3, and type II collagen).

All these positive regulators of chondrogenesis and chondrocyte activity may be potential targets for the development of therapeutic strategies regarding the development of treatments with MSCs. These markers can be studied to mimic in vitro a successful microenvironment for cartilage regeneration, i.e., the precondition factor in order to obtain adequate MSCs directed to cartilage repair for both MSC implantation or to isolate EVs from MSCs. Different approaches have been applied to regulate EV composition and function, such as the preconditioning of parental cells, for example. A study using MSCs loaded with curcumin revealed that EVs derived from this preconditioned microenvironment reduced the expression of pro-inflammatory mediators including IL-6, TNF-α, MMP1, and PGE2 [84]. Similar findings were observed with the use of kartogenin, which, moreover, increased the expression of chondrogenic genes (aggrecan, Collagen II, and SOX9) [85]. Other factors extensively described, like the preconditioning of parent cells, are the ones of the TGF beta family [86–88].

Physical activity and joint mobility play a crucial role in preserving joint health and preventing or managing OA [89]. Eight studies [24,27,30–32,35,37,38] conducted behavioral and pain posture analyses, important for the contextualization and evaluation of the

degree of OA induced. The restoration of function in the affected limb is one of the main treatment goals for OA, making it essential to analyze these parameters in OA therapies.

Interestingly, not many articles staged their animal model, e.g., by separating time-points by early-onset inflammation, mid-stage, and late-stage. However, using time-points of days for early-onset and more than 12 weeks for late stage, we could see that not many differences were seen on the factors present, but on their degree within a given study. As shown by Cheng et al. (2021) [27], Jiang et al. (2021) [30], Mou et al. (2021) [32], Forrester et al. (2022) [35], and Valerio et al. (2023) [41], most factors increased in the later stages, except for autophagy genes that showed increased expression at 4 weeks in comparison with 10 weeks (Zhang et al. (2022)) [37]. Apart from that, only Qian et al. [33] showed a difference on a factor between time-points: ADAMTS5 was upregulated in OA up to 4 weeks and downregulated later, and the opposite occurred for VEGFR2.

Numerous works showed how MSCs respond to the microenvironment and, for cartilage, a great example was the work from Sarugaser et al. (2009) [19]. Upon a single MSC implantation in an osteochondral defect in vivo model, MSCs adhering to the methaphysis favored bone formation, and the ones adhering to the growth plate favored new chondroblast. Therefore, it is to expect that MSCs receiving the signaling of positive regulators of chondrogenesis in the culture medium, like TGF-β1 and TGF-β3, or as a matrix, like type II collagen or aggrecan, or even as a co-culture with M2 macrophages, would then be directed to produce extracellular vesicles favoring chondrogenesis. Not only this, preconditioning methods can be developed targeting the increase in these factors in MSC cultures. However, many aspects may affect MSC preconditioning and extracellular vesicle production, not only the soluble factors but their combination, dosage, and period of treatment, for example. All these must be the objectives of future studies, aiming at promoting the best standardized method for MSC commitment and extracellular vesicle production for OA treatment.

Our study is limited on proposing methods to use for the preconditioning of MSCs, since each method must be evaluated. Here, we intended to demonstrate the possibilities and perspectives for future researchers on recreating the discussed microenvironments in vitro. Also, for future therapies in humans, further differences in OA and joint regeneration between species may also be addressed.

5. Conclusions and Future Perspectives

After this extensive evaluation, we can conclude that controlling the inflammatory process, considering in particular the mechanisms of chondrocyte survival and maintenance of their activity, is the key point for further research on OA treatment strategies. This is a direct consequence of the fact that the negative regulatory factors of chondrogenesis present in inflammation (especially MMP13, CD68, CD86, TUNEL, TNF-α, IL-1b, IL-6, and IL-17) are intimately and inversely associated with the expression of factors shown to be indicative of a good evolution in regeneration (aggrecan, GAG, type II collagen, TGF-β1, TGF-β3, IL-10, and CD206). Upon the findings of our systematic review, chondrocyte survival and the maintenance of their activity is the central point involved in the regulation of the articular cartilage microenvironment. Therefore, when considering future preconditioning methods for MSC- and EV-based therapies, strategies to be explored can be (i) the co-culture of MSCs with active chondrocytes or M2 macrophages or (ii) the soluble or extracellular matrix factors related to a good evolution in regeneration (aggrecan, GAG, type II collagen, TGF-β1, TGF-β3, IL-10, and CD206). Indeed, these strategies are currently under evaluation by different research groups, with promising possibilities for OA treatment.

Author Contributions: Conception and design of the systematic review, A.S.d.S.; data analysis, synthesis, and interpretation, F.C.H.; synthesis of results from the included studies, F.L.V.; initial stages of the systematic review, assisting in the development of the research protocol and the formulation of search strategies, F.A.V.; critical appraisal of the included studies, assessing their methodological quality and risk of bias, A.P.B.B.; providing valuable expertise in the field under investigation, A.d.P.S.; actively involved in the literature search process, identifying and retrieving relevant articles

from various databases, R.V.S.; substantial contributions to the conception of this study, organization and synthesis of the data, and review, E.C.C.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded and supported by CAPES financial code 001, FAPEMIG and CNPq.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data generated, analyzed, and presented in this study are available upon request to the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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