LITERATURE REVIEW

Exploring bovine three-dimensional chondrocyte culture models in osteoarthritis research: A systematic review

Explorando los modelos tridimensionales de cultivo de condrocitos bovinos en la investigación de la osteoartritis: Una revisión sistemática

Explorando modelos de cultura de condrócitos tridimensionais bovinos na pesquisa em osteoartrite: uma revisão sistemática

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Abstract

Background: The use of different animal species for chondrocyte culture has been employed to investigate the diseases that affect cartilage, including osteoarthritis. Bovine cartilage and chondrocytes can be used to establish three-dimensional cell cultures, which offer a more dependable *in vitro* model when compared to conventional monolayer cultures. However, bovine chondrocytes in three-dimensional cultures have not been widely implemented, losing a potential source of mammal tissue that could prove valuable for preclinical studies on osteoarthritis. **Objective:** The objective of this study was to conduct a comprehensive review of the existing scientific literature that employs three-dimensional cultures of bovine cartilage to investigate osteoarthritis. **Methods:** A systematic search was performed using the electronic databases PubMed and Scopus, to identify clinical studies using 3D cell culture for osteoarthritis. Search terms included: ´3D culture', '3D cell culture', 'bovine cartilage' and 'chondrocyte'. A total of 59 articles were gathered, and after screening, 12 articles were included in the final analysis. Risk of bias assessment was conducted categorizing each of the studies as having a 'low,' 'medium,' or 'high' risk of bias. **Results:** Analysis of the articles included in this review highlighted the increased variability in harvesting sites involving carpal, metacarpal, and knee joints, as well as variation in culture methods utilizing cell passages ranging from passage zero to passage nine. Moreover, medium, and high risk of bias were detected in all the articles probably due to challenges in randomization and blinding of the studies. In summary, this review critically examines three-dimensional cell culture for the investigation of cartilage disorders, with a particular emphasis on bovine cartilage. **Conclusions:** Future studies using chondrocyte culture in 3D or tissue-engineered constructs, should include consistent methods across the *in vitro* phase of the study. Factors such as chondrocyte harvest site, donor age, and passage number can significantly impact biological characteristics and cartilage regeneration potential. Therefore, it is suggested that the comparison of relevant translational models should include age-matched conditions to avoid further confounding factors.

Keywords: *cartilage; cell culture; hydrogel; musculoskeletal tissues; osteoarthritis; risk of bias; tissue harvest; tissue procurement.*

Resumen

Antecedentes: El uso de diferentes especies animales para el cultivo de condrocitos se ha empleado para investigar las enfermedades que afectan al cartílago, incluida la osteoartritis. El cartílago bovino y los condrocitos se pueden utilizar para establecer cultivos celulares tridimensionales, que ofrecen un modelo *in vitro* más fiable en comparación con los cultivos monocapa convencionales. Sin embargo, los condrocitos bovinos en cultivos tridimensionales no se han implementado ampliamente, perdiendo una fuente potencial de tejido proveniente de mamíferos, que podrían ser útiles para estudios preclínicos sobre la osteoartritis. **Objetivo:** El objetivo del presente artículo fue realizar una revisión exhaustiva de la literatura científica existente que emplea cultivos tridimensionales de cartílago bovino para investigar la osteoartritis. **Métodos**: Se realizó una búsqueda sistemática utilizando las bases de datos electrónicas PubMed y Scopus, para identificar estudios clínicos utilizando cultivo celular 3D para la artrosis. Los términos de búsqueda incluyeron: ´3D culture', '3D cell culture', 'bovine cartilage' y 'chondrocyte'. Se recolectaron un total de 59 artículos y, tras la selección, se incluyeron 12 artículos en el análisis final. La evaluación del riesgo de sesgo se llevó a cabo categorizando cada uno de los estudios como riesgo de sesgo "bajo", "medio" o "alto". **Resultados:** Se encontró que en los artículos incluidos en esta revisión existía una alta variabilidad en los sitios de aislamiento que incluyen las articulaciones del carpo, del metacarpo y de la rodilla, así como una alta variación en los métodos de cultivo, utilizando pasajes celulares que van desde el pasaje cero hasta el pasaje nueve. Además, se detectó un riesgo medio y alto de sesgo en todos los artículos, probablemente debido a las dificultades en la aleatorización y el cegamiento de los estudios. En resumen, esta revisión examina críticamente el cultivo celular tridimensional para la investigación de trastornos del cartílago, con un énfasis particular en el cartílago bovino. **Conclusiones:** Los estudios futuros que utilicen el cultivo de condrocitos en 3D o construcciones de ingeniería de tejidos deben incluir métodos coherentes en toda la *fase in vitro* del estudio. Factores como el lugar de recolección de condrocitos, la edad del donante y el número de deposiciones pueden afectar

significativamente las características biológicas y el potencial de regeneración del cartílago. Por lo tanto, se sugiere que la comparación de los modelos traslacionales relevantes debe incluir condiciones ajustadas a la edad para evitar factores de confusión adicionales.
Palabras clave: *cartílago; cultivo celular; cultivo de tejido; hidrogel; obtención de tejido: osteoartritis; riesgo de sesgo; tejidos musculoesqueléticos.*

Resumo

Antecedentes: O uso de diferentes espécies animais para a cultura de condrócitos tem sido empregado para pesquisar doenças que afetam a cartilagem, incluindo osteoartrite. Cartilagem bovina e condrócitos podem ser usados para estabelecer culturas de células tridimensionais, que oferecem um modelo *in vitro* mais confiável em comparação com culturas convencionais de monocamadas. No entanto, condrócitos bovinos em culturas tridimensionais não foram amplamente implementados, faltando uma fonte potencial de tecido de mamíferos, o que poderia ser útil para estudos pré-clínicos de osteoartrite. **Objetivo:** Consequentemente, nosso objetivo foi realizar uma revisão abrangente da literatura científica existente empregando culturas tridimensionais de cartilagem bovina para investigar osteoartrite. **Métodos:** Foi realizada uma busca sistemática, utilizando as bases de dados eletrônicas PubMed e Scopus, para identificar estudos clínicos utilizando cultura de células 3D para osteoartrite. Os termos de pesquisa incluíram: ´3D culture', '3D cell culture', 'bovine cartilage' e 'chondrocyte'. Foram resgatados 59 artigos e, após seleção, 12 artigos foram incluídos na análise final. A avaliação do risco de viés foi realizada classificando-se cada um dos estudos em "baixo", "médio" ou "alto" risco de viés. **Resultados:** Verificamos que nos artigos incluídos nesta revisão houve alta variabilidade nos sítios de isolamento, incluindo as articulações do carpo, metacarpo e joelho, bem como alta variação nos métodos de cultura, utilizando passagens celulares que variam da passagem zero à passagem nove. Além disso, detectamos um risco médio e alto de viés em todos os artigos, provavelmente devido a dificuldades de randomização e cegamento dos estudos. Em resumo, esta revisão examina criticamente a cultura de células tridimensionais para a pesquisa de distúrbios da cartilagem, com ênfase particular na cartilagem bovina. **Conclusões:** Estudos futuros usando cultura de condrócitos em construções 3D ou de engenharia de tecidos devem incluir métodos

consistentes em toda a *fase in vitro* do estudo. Fatores como lugar de coleta dos condrócitos, idade do doador e número de passagens podem afetar significativamente as características biológicas e o potencial de regeneração da cartilagem. Portanto, sugere-se que a comparação de modelos translacionais relevantes inclua condições pareadas por idade para evitar variáveis de confusão adicionais.

Palavras-chave: *cartilagem; colheita de tecidos; cultura celular; hidrogel; obtenção de tecidos; osteoartrite; risco de viés; tecidos musculoesqueléticos.*

Introduction

Musculoskeletal disorders rank highly as one of the most prevalent causes of physical disabilities worldwide (Li *et al.*, 2021). Approximately 1.71 billion individuals suffer from conditions associated with the musculoskeletal system, encompassing ailments such as arthritis (including osteoarthritis, rheumatoid arthritis, and psoriatic arthritis), gout, and osteopenia (WHO, 2022). Osteoarthritis (OA) is characterized by cartilage degradation caused by dysregulated anabolic and catabolic responses affecting normal chondrocyte biological cues.

Traditionally, monolayer (2D) chondrocyte culture has been used *in vitro* to study cellular and pharmacological interactions with candidate molecules. However, 2D culture models, exhibit a limited representation of the in vivo environment mainly due to inadequate cell-cell and cell-extracellular matrix interactions, which are crucial for maintaining chondrocyte phenotype (Fiederlein and Evans, 2020). Three-dimensional (3D) culture provides a better model of the *In Vivo* milieu compared to 2D culture, allowing for a deeper understanding of OA progression.

Chondrocytes and other cell types can be cultured in 3D to mimic the *In Vivo* environment while maintaining phenotypic characteristics closely related to the native tissue. Nonetheless, the substantial array of alternatives for modeling OA, involves different cell sources obtained from distinct animal species, mainly mammals. Common cell sources comprise those obtained from common laboratory animal species such as rodents and rabbits, that, although

it has demonstrated importance for orthopaedic research, may have important biological and morphological limitations (Meng *et al.*, 2020; Cardona-Ramirez *et al.*, 2022). Additionally, tissues obtained from larger animals such as dogs, and sheep have also been used, revealing important morphological similarities to the human species (Mancuso *et al.*, 2010; Oh *et al.*, 2021; Soontararak *et al.*, 2022). However, due to different ethical and cultural concerns, the aforementioned species may not be widely available for investigators with an interest in cartilage diseases (Liguori *et al.*, 2017; Swatland, 2010). Conversely, bovine meat and milk industry has led to a wide offer of products for human consumption. Moreover, slaughterhouses also process a considerable quantity of tissues that may be of interest for the academia and the scientific industry, arising as a potential source of tissues and organs to study diverse musculoskeletal diseases including the potential effect of orthobiologics and cartilage preservation strategies (Camacho and Mardones, 2021; Solanki *et al*., 2021). Additionally, the bovine species represents an attractive model to study OA due to the similarity in cartilage thickness and anatomy (Bascuñán *et al.*, 2019). Therefore, the objective of this paper is to systematically analyze most recent publications using bovine chondrocytes as a source of cellular material for OA studies.

Materials and Methods

Search strategy

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Page *et al.*, 2021). The computer-assisted literature search was performed using PubMed and Scopus electronic databases to identify clinical studies using 3D cell culture for osteoarthritis. The following search terms and Boolean operators were used: ´3D culture', '3D cell culture', 'bovine cartilage', and 'chondrocyte' (Table 1). Databases were exported to bibliographic manager software files (.RIS) containing all relevant information such as author name, year of publication, title, keywords, and abstract. Bibliographic files were then imported into R studio (R version 4.1.2), to consolidate information on one single database. Duplicated references were removed using the package *´litsearchr´* (Grames *et al.*, 2019). The remaining articles were screened by two authors

(M.R.J and M.P.C.) and independently reviewed, the title for relevance and the materials and methods section to include only articles that used bovine chondrocytes for 3D culture.

Table 1. Search terms and boolean operators were used for the inclusion of the articles.

Electronic database	Query	Results
PubMed	"3D culture" OR "3D cell culture" AND bovine cartilage OR chondrocyte	20
Scopus	"3D culture" OR "3D cell culture" AND bovine cartilage OR chondrocyte	39

Study risk of bias assessment

The risk of bias assessment was conducted using the Quality Assessment Tool For *in vitro* Studies (QUIN) (Sheth *et al.*, 2022). Briefly, the assessment of the quality used a predefined set of bias domains. The final assessment involved categorizing each of these study features as having a 'low,' 'medium,' or 'high' risk of bias. Two investigators (M.P.C. and M.R.J) independently conducted the assessment.

Results

Study selection and characteristics

A total of 59 studies were identified; 17 records were removed due to duplication, and 24 were removed because their title suggested that information was not relevant for analysis. During the screening process, three (3) records were excluded because chondrocytes were not obtained from bovine tissues, and one (1) record was not accessible for retrieval. Two (2) records were excluded because the methodology did not include cell culture details. Lastly, a total of 12 articles were included in the analysis (Figure 1).

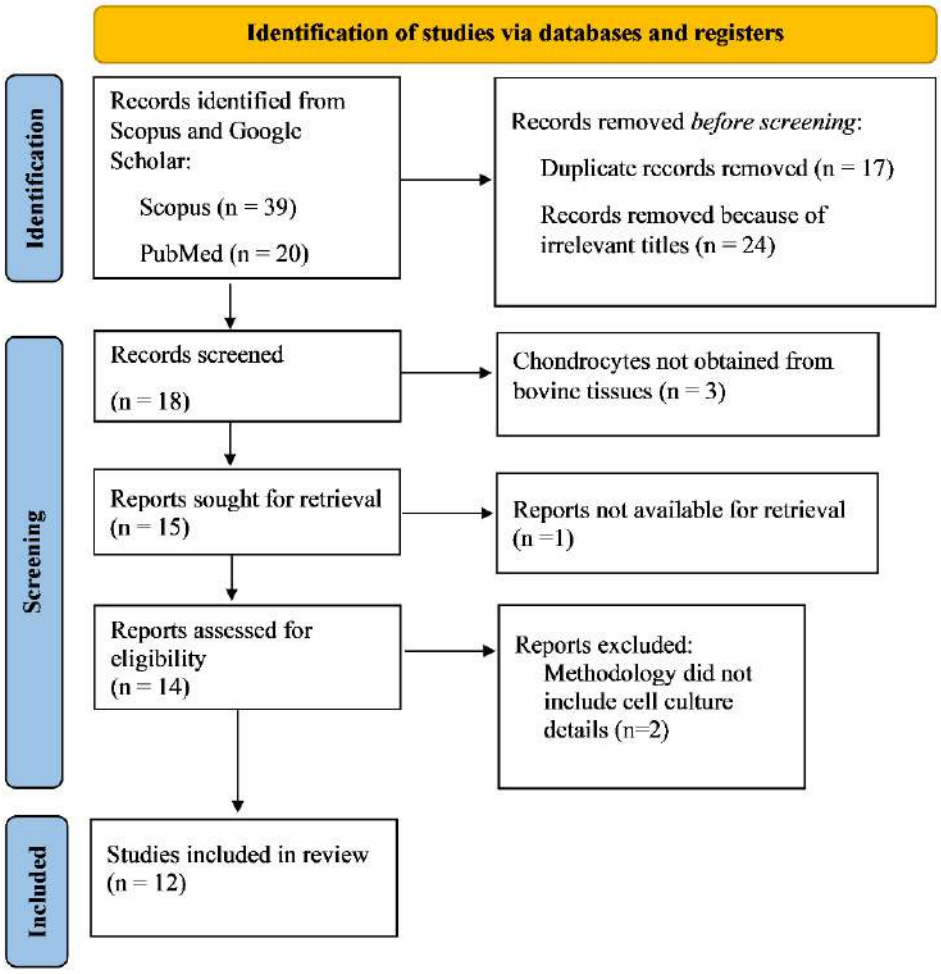

Figure 1. Identification and study selection according to the PRISMA guidelines.

Cartilage harvest and chondrocyte culture

Chondrocyte sources varied significantly among studies. While a variety of studies used adult chondrocytes, other studies utilized chondrocytes obtained from skeletally immature animals ranging from nine-week-old calves to twelve-old month steers (Ahmed *et al.*, 2014; Çelik *et al.*, 2016; Li *et al.*, 2016; Lee *et al.*, 2019; Antunes *et al.*, 2020; Gawri *et al.*, 2022).

Additionally, the site of harvest was not consistently described at the time of procurement. While the most common site of harvest was the carpal-metacarpal joint, other authors used the stifle (knee) and the fetlock joint (Lee *et al.*, 2017; Lee *et al.*, 2019; Antunes *et al.*, 2020). Furthermore, there was also variation in the chondrocyte passage used for the experiments. Although most authors used a variety of passages, ranging from P0 to P4, others studies

208 included chondrocyte passages ranging from P4 to P9 (Pizzolatti *et al.*, 2018). Furthermore,
209 many studies, did not mention what passages were used for the experiments (Table 2).

210 **Table 2.** Chondrocyte culture characteristics

Reference	Chondrocyte culture	Passage
Heywood *et al.* (2022)	Adult metacarpophalangeal cartilage	P0-P4
Gawri *et al.* (2022)	Metacarpal-carpal cartilage from 9 to 12-month-old steers.	N. A
Antunes *et al.* (2020)	Full-thickness fetlock joint cartilage of 4-8-month-old calves.	N. A
Müller *et al*. (2020)	Chondrocytes isolated from the metacarpal joint of 1-2-year-old cattle.	P1
Pizzolatti *et al*. (2018)	Bovine carpal joints	P4-P9
Li *et al*. (2016)	Chondrocytes were isolated from articular cartilage from the knees of a nine-week-old calf.	P2
Çelik *et al*. (2016)	Cartilage isolated from the knee joint of young calves.	N.A.
Mellor *et al*. (2014)	Hooves from 18–24-month-old steers using the metacarpophalangeal joints.	P2
Farnsworth *et al*. (2014)	Metacarpophalangeal joints of 2–3-year-old steers.	N. A
Ahmed *et al*. (2014)	Cartilage harvested from bovine metacarpo-phalangeal joints (6–9 months old).	P2
Lee *et al*. (2017)	Cartilage harvested from the patellofemoral groove of a bovine leg.	N. A

211 Passage number; N.A: Not available

212

213 *Risk of bias assessment*

214 The Quality Assessment Tool For *in vitro* Studies (QUIN Tool) analyzed 12 criteria to grade
215 the *in vitro* studies as high, medium, or low risk depending on the summed scores (Sheth *et*

al., 2022). The first criteria which consisted of the clarity of the objectives, was adequately specified in all the articles (Table 3). However, all the other categories showed different grades of bias. Six (6) studies included in the analysis exhibited a high risk of bias according to the QUIN tool assessment (Çelik *et al.*, 2016; Li *et al.*, 2016; Pizzolatti *et al.*, 2018; Lee *et al.*, 2019; Antunes *et al.*, 2020; Heywood *et al.*, 2022). Criteria that were more prone to the risk of bias were the operator details, randomization, outcome assessor details, and blinding. Moreover, sample size was not adequately described in seven articles (Farnsworth *et al.*, 2014; Li *et al.*, 2016; Lee *et al.*, 2019; Antunes *et al.*, 2020; Müller *et al.*, 2020; Gawri *et al.*, 2022; Heywood *et al.*, 2022) and only one study included a detailed explanation of sample size calculation (Lee *et al.*, 2017). Conversely, seven articles provided a detailed description of the comparison groups (Ahmed *et al.*, 2014; Farnsworth *et al.*, 2014; Mellor *et al.*, 2014; Çelik *et al.*, 2016; Lee *et al.*, 2017; Gawri *et al.*, 2022; Müller *et al.*, 2020), six papers included a detailed description of the methodology (Ahmed *et al.*, 2014; Farnsworth *et al.*, 2014; Mellor *et al.*, 2014; Li *et al.*, 2016; Lee *et al.*, 2017; Gawri *et al.*, 2022), and only three articles provided a clear description of the sampling technique (Ahmed *et al.*, 2014; Mellor *et al.*, 2014; Gawri *et al.*, 2022).

234 **Table 3.** Risk of bias assessment using QUIN tool (Sheth *et al.*, 2022).

Ref.	Aims	Sample size	Sampling technique	Comp. group	Methods	Operator details	Rand.	Outcome measure	Outcome assessor	Blind	Statist	Result	Total score	Final score %	Risk of bias
Heywood *et al.* (2022)	2	1	1	1	1	0	0	1	0	0	2	2	11	45.8	High
Gawri *et al*. (2022)	2	1	2	2	2	0	0	1	0	0	2	2	14	58.3	Medium
Antunes *et al*. (2020)	2	1	0	1	1	0	0	2	0	0	1	1	9	37.5	High
Lee *et al*. (2019)	2	1	0	1	1	0	0	2	0	0	2	2	11	45.8	High
Müller *et al*. (2020)	2	1	1	2	1	0	1	2	0	1	1	1	13	54.2	Medium
Pizzolatti *et al*. (2018)	2	0	0	1	1	0	0	1	0	0	1	2	8	33.3	High
Li *et al*. (2016)	2	1	0	0	2	0	0	2	0	0	1	2	10	41.7	High
Çelik *et al*. (2016)	2	0	0	2	1	0	0	1	0	0	2	1	9	37.5	High
Mellor *et al*. (2014)	2	0	2	2	2	0	0	1	2	0	1	2	14	58.3	Medium
Farnsworth *et al*. (2014)	2	1	1	2	2	0	1	2	0	0	2	2	15	62.5	Medium
Ahmed *et al*. (2014)	2	0	2	2	2	0	0	2	0	0	2	2	14	58.3	Medium
Lee *et al*. (2017)	2	2	0	2	2	0	0	2	0	0	2	2	14	58.3	Medium

235 **0** = Not Specified; **1** = Inadequately specified; **2** = Adequately specified

236 **Aims:** Clearly stated aims/objectives; **Sample size:** Detailed explanation of sample size calculation; **Sampling technique:** Detailed
237 explanation of sampling technique; C**omp. group:** Details of comparison group; **Methods:** Detailed explanation of methodology; **Rand:**
238 Randomization; **Outcome measure:** Method of measurement of outcome; **Outcome assessor:** Outcome assessor details; **Blind:**
239 Blinding; **Statist:** Statistical analysis; **Result**: Presentation of results.

Discussion

Cellular sources for *in vitro* evaluation of musculoskeletal tissues vary depending on the intended application, either for basic science or translational purposes. Furthermore, animal tissues may offer advantages to human cell lines mainly by reducing costs and facilitating the availability for researchers with an interest in cartilage diseases.

In vitro pre-clinical research plays a crucial role in the development of new materials and techniques, providing essential information for further testing in clinical trials. Chondrocyte harvest site and age of donor may affect biological characteristics of studies. In the current study, variation of the harvest site ranged from carpus, metacarpus, and the knees from both, adult and young animals. Isogai *et al*. (2006) compared bovine chondrocytes from different animal locations for tissue-engineered cartilage modeling and found that chondrocytes from different sources showed variations in cell proliferation rates, gene expression, and extracellular matrix production (Isogai *et al.*, 2006). Interestingly, authors found that Collagen-I and Aggrecan relative gene expression was highest in costal chondrocytes compared to chondrocytes isolated from articular cartilage. Similarly, Maličev *et al*. (2011) evaluated cell viability, proliferation, morphology, and collagen expression from chondrocytes harvested from the debrided edge of a chronic lesion of the articular surface compared to the edge of the lesion. Authors found differential expression and cell yield between the two harvest sites and suggested that cultivation of chondrocytes solely from the edges of the lesion cannot be recommended for use in autologous chondrocyte implantation (Maličev *et al.*, 2011). Hence, confirming the importance of considering the specific characteristics of chondrocyte types in the design of tissue-engineered cartilage models.

Different studies analyzing the effect of chondrocyte passages on cartilage formation have found that serial cell passages can cause loss of differentiated phenotype (Brodkin *et al.*, 2004; Hamilton *et al.*, 2005; Kang *et al.*, 2007). Kang *et al*. (2007) found that chondrocytes cultured through various passages showed decreased growth rate, viability, and increased apoptosis. Additionally, authors also showed that passage 2 chondrocytes expressed high levels of collagen type II, while passage 5 chondrocytes showed dedifferentiation with low collagen type II expression. Furthermore, when using chondrocytes for 3D culture or tissue

engineered constructs, using passage 1 chondrocytes exhibited mature cartilage while tissues engineered with passage 5 chondrocytes did not have chondrocyte morphology or cartilage-specific matrices (Kang *et al.*, 2007). Similarly, Nam *et al*. (2014) compared the effects of cryopreservation and passaging on cell viability, proliferation of chondrocytes and synovium-derived mesenchymal stem cells (MSCs) used as sources for Autologous Chondrocyte Transplantation (ACT). Authors found that passaging and cryopreservation significantly affected the ability of chondrocytes to maintain their morphology, express chondrogenic genes, and differentiate compared to synovium-derived cells that were not affected by passaging and cryopreservation (Nam *et al.*, 2014).

Moreover, age of donor is also an important factor on ECM production capability. Son and Levenston (2015) evaluated phenotypic changes of juvenile and adult articular chondrocytes and fibrochondrocytes across multiple passages and subsequent 3D culture and found that Col-1 expression increased with passage for adult cells, but decreased for juvenile cells, and 3D gel culture reversed this increase for adult cells (Son and Levenston, 2017). Therefore, beside considering factors such as place of harvest, chondrocyte passage and age of donor, using cell therapy for surgical treatments may require additional experimental conditions to direct cell phenotype, such as the use of growth factors in the culture medium various cell sources for tissue-engineering strategies.

When analyzing the summed scores for risk of bias, we found that most papers reached a grade of medium or high risk of bias, probably due to lack of complete description of procedures, specifically in the randomization and blinding criteria, leading to poor grade on the risk of bias assessment tool. Whether the grading instructions for each criterion on the assessment tool were clear is still not known. It would be worth to compare different grading strategies to see which of those may achieve the better consensus. Consequently, various authors have developed different guidelines for reporting *in vitro* studies based on the CONSORT checklist for reporting randomized clinical trials (Faggion, 2012; Krithikadatta *et al.*, 2014). Additionally, good standards for reporting preclinical research are necessary for improving efficiency and ensuring the reliability of study findings. However, ongoing refinement of the current risk of bias reporting tools are still needed. The QUIN tool utilized

in this paper provided adequate information about the descriptions of relevant characteristics of *in vitro* studies, including randomization and blinding processes. It is important to note that the twelve criteria evaluated by the QUIN tool could only tell if the authors of the articles included such descriptions.

Importantly, the use of animal tissues for *In Vitro* studies can enhance the understanding of cellular behavior in cartilage integration. Research has shown that bioengineered cartilage derived from bovine chondrocytes can effectively migrate and integrate with native cartilage when treated with platelet-rich plasma (PRP) **(**Wu *et al.*, 2022). Furthermore, mechanical properties of engineered cartilage constructs may be tuned by modifying the osmolarity of the culture medium (Oswald *et al.*, 2011). Additionally, the expression and secretion of appropriate extracellular matrix (ECM) may also be affected by the viscosity of the cell culture medium, showing higher levels of cartilaginous gene expression in lower viscosity medium (Zheng *et al.*, 2023). Hence, providing evidence of the importance of culture conditions for mechanical properties of cartilage constructs.

In conclusion, having examined the most relevant evidence for the use of bovine chondrocytes in 3D culture, authors suggest future studies to include consistent methods across the *in vitro* phase of the study, such as uniform harvest sites (based on previous molecular analysis of ECM yield), as well as maintaining chondrocyte passages between passage zero (P0) and passage four (P4) to preserve cellular phenotype, especially for cartilage transplantation purposes. Furthermore, comparison of relevant translational models should include age-matched conditions (either pediatric or adult cartilage diseases) to provide a precise model and avoid further confounding factors.

Declarations

Conflicts of interest

The authors declare they have no conflicts of interest regarding the work presented in this report.

Author contributions

Cardona-Ramírez S, Conceived and designed the manuscript, contributed with data analysis, wrote, and edited the manuscript. Ramírez-Jaramillo M, and Currea-Gómez MP wrote and prepared the manuscript, Collected the data, and contributed to data analysis. All authors provided critical feedback during writing and editing.

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Use of artificial intelligence (AI)

No AI or AI-assisted technologies were used during the preparation of this work.

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