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Original article

Serum and synovial lactate dehydrogenase levels after allogeneic mesenchymal stem cell implantation in rabbit cartilage defects

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Abstract

Cartilage defects have limited regenerative potential and remain a significant clinical challenge. Mesenchymal stem cell (MSC) therapy offers promise in tissue engineering, yet monitoring post-implantation responses remains difficult. Lactate dehydrogenase (LDH), a biomarker of cell damage and inflammation, may provide insight into systemic and local tissue reactions. This study aimed to assess the levels of serum and synovial fluid LDH following the implantation of allogeneic MSCs into cartilage defects in a rabbit model. A total of 56 New Zealand white rabbits were randomly divided into four groups: control (Group A), and groups receiving fibrin glue with adipose-derived stem cells (ASCs), bone marrow-derived stem cells (BMSCs), or synovial-derived stem cells (SDSCs). Serum and synovial fluid samples were collected at baseline and 12 weeks post-surgery to measure serum and synovial fluid LDH levels. Serum LDH levels were significantly elevated in all MSC-treated groups relative to controls ($p=0.019$), potentially due to high anaerobic metabolism or implantation-related toxicity, systemic response and apoptosis. However, no additional adverse effects were observed, making toxicity or further articular damage unlikely. No significant changes were detected in synovial fluid LDH across groups ($p=0.070$), suggesting limited value as a biomarker for cartilage repair.

Keywords: biomarker, cartilage, lactate dehydrogenase, rabbits, stem cells, synovial fluid

Introduction

Cartilage injuries remain a significant clinical challenge due to the absence of vascular, lymphatic and nerve systems combined with a low chondrocyte density (Mano and Reis 2007, Steinert et al. 2007), which severely limit its ability to self-repair. Traumatic injuries, degenerative conditions such as osteoarthritis (OA), and other joint disorders frequently lead to cartilage defects that, if left untreated, can result in the production of fibrous cartilage, which exhibits inferior biomechanical properties compared to hyaline cartilage (Hunziker et al. 2015) and progress to chronic pain and joint dysfunction (Moore et al. 2001, Johnson et al. 2020). Conventional treatment options, including microfracture, autologous chondrocyte implantation (Peterson et al. 2010, Dhillon et al. 2022), and osteochondral grafting, have shown varying degrees of success but often fail to fully restore cartilage structure and function (Toh et al. 2010, Im and Kim 2020). In recent years, mesenchymal stem cell (MSC) therapy has emerged as a promising alternative for cartilage regeneration due to its ability to differentiate into chondrogenic cells, modulate the inflammatory environment, and promote tissue repair (Friedenstein, et al. 1976, Caplan 2017, Oryan et al. 2017, Pittenger et al. 2019).

Lactate dehydrogenase (LDH) is an intracellular enzyme involved in anaerobic metabolism (Walter et al. 2007) and is widely used in veterinary medicine as a biomarker for tissue injury, inflammation, cell viability and neoplasia (Klein et al. 2020). Additionally, LDH appears to increase, at least intracellularly, in neoplastic and hematopoietic cells with significant proliferation (Pan et al. 1991a, Pan et al. 1991b). When cells undergo stress, necrosis, or apoptosis, LDH is released into extracellular fluids, including serum and synovial fluid, making it a useful indicator of cartilage damage and tissue response following therapeutic interventions. LDH4 and LDH5 are the predominant isoforms in articular cartilage (Yancik et al. 1987), making their assessment particularly relevant in evaluating cartilage injury and repair mechanisms. Additionally, the distribution of LDH varies depending on the layers of the articular cartilage. For instance, in the articular cartilage of healthy tibial condyles in dogs, LDH concentration was similar across all superficial layers and showed a slight increase in the deeper transitional zones (Dunham et al. 1986). Similar findings were reported by Walter et al. 2007, where LDH appeared to concentrate in the superficial layers, possibly due to increased metabolic activity. Monitoring LDH levels provides critical insights into the safety and effectiveness of MSC-based treatments, as increased LDH activity may indicate cell damage or inflammatory responses associated with the implantation process. Additionally, LDH has been extensively studied in various biological fluids of animals, including synovial fluid (Hurter et al. 2005, Walter et al. 2007, Proot et al. 2015), pericardial fluid (De Laforcade et al. 2005), peritoneal fluid (Levin et al. 2004) and milk (Gómez-Gascón et al. 2022).

Elevation of synovial fluid LDH has been detected in several joint diseases, including OA and septic arthritis, in both humans (Messieh 1996, Kopterides 2007) and horses (Yancik et al. 1987). However, the evaluation of LDH in dogs remains controversial. Some studies suggest that increased synovial LDH levels correlate with cartilage damage due to OA (Hurter et al. 2005, Walter et al. 2007) and septic arthritis (Proot et al. 2015), with LDH being released through damaged chondrocytes as a result of increased cell membrane permeability (Walter et al. 2007). Conversely, other studies argue that synovial LDH may not be a reliable indicator for immune-mediated arthritis or OA (Proot et al. 2015). Furthermore, a high concentration of LDH upon admission to the intensive care unit has been associated with a low survival rate in dogs (Lagutchik et al. 1998), further underscoring its relevance as a biomarker of systemic stress and injury.

Despite growing interest in MSC therapy for cartilage repair, limited research has focused on biochemical markers such as LDH to evaluate cellular responses post-implantation. Investigating LDH levels in both serum and LDHsf following allogeneic MSC implantation may offer a valuable assessment of cartilage healing, potential cytotoxic effects, and overall joint environment changes. Rabbits serve as an ideal preclinical model for cartilage defect studies due to their joint biomechanics and cartilage properties, which share similarities with human cartilage.

This randomized, controlled double-blind study aimed to estimate and analyse serum and synovial fluid LDH levels after the implantation of allogeneic MSCs in rabbit cartilage defects. By assessing LDH as a biomarker of tissue response, we seek to evaluate the impact of MSC therapy on cartilage healing, inflammation, and cellular viability. The findings of this study will contribute to a better understanding of MSC-mediated cartilage regeneration and may provide valuable insights into the clinical translation of stem cell-based therapies for joint disorders.

Materials and Methods

The animal study protocol used in this study was approved by the Veterinary Service of Athens, Greece, under ethical approval number 1155/13-03-2018. All animal experimental procedures were conducted

in accordance with international guidelines. The collection of BMSCs, ASCs and SDSCs was performed as reported in a previous study on female New Zealand white rabbits (Bami et al. 2020).

Isolation of BMSCs

Under anesthesia induced by intramuscular administration of xylazine (Xylapan, Vetoquinol UK) 5 mg/kg and ketamine (Imalgene, Merial, France) 35 mg/kg, 5ml of bone marrow were aspirated with a 15G gauge needle (KIMAL, UK) through penetration of the posterior superior iliac spine. The aspirate was centrifuged at 500 times gravity for 10 minutes and the isolated cell pellets were suspended in low glucose DMEM with 15% MSC qualified fetal bovine serum (FBS), 2mM L-glutamine, 1xAntibiotic-Antimycotic and 1xNonEssential Amino Acids (Life Technologies). After 48 h the detached cells were removed with phosphate-buffered saline (PBS). The media was changed every 3 days until the cells reached 90% confluency.

Isolation of ASCs

ASCs were obtained from the inguinal fat pads of rabbits after the animals' euthanasia by intramuscular pentobarbital (Thiopental, IFET, Greece) 150 mg/kg injection. The fat was washed with sterile PBS, was finely minced using scissors and then digested for 30-120 minutes at 37°C with collagenase type I. After centrifuging at 400xg for 5 minutes the cell pellets were resuspended in red blood cell lysis buffer, filtered and resuspended in low glucose DMEM, 10% MSC qualified FBS, 2mM L-glutamine, 1xAntibiotic-Antimycotic and 1xNon-Essential Amino Acids (Life Technologies). The cells were then plated in dishes for culture and the culture was continued until confluence was achieved.

Isolation of SDSCs

SDSCs were collected from the synovial membrane of rabbits after their euthanasia by intramuscular pentobarbital (Thiopental, IFET, Greece) 150 mg/kg injection. After being washed with sterile PBS 3% penicillin/streptomycin and minced synovial tissues were digested with 0.02% type I collagenase in PBS at 37°C for 6 hours. The cells were then filtered and the released cells were resuspended in low glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% MSC qualified FBS, 2mM L-glutamine, 1xAntibiotic-Antimycotic and 1xNonEssential Amino Acids (Life Technologies). The detached cells were removed with PBS and cultured for 6-10 days while the culture medium was replaced every 2-3days, until confluence.

As previously described, the in vitro proliferation and chondrogenic potential of rabbit MSCs isolated using this protocol were established (Bami et al. 2020). The cells used in this study were at passage 3.

Establishment of cartilage defects

A total of 56 mature female New Zealand rabbits, aged 8-12 months with a body weight ranging from 3.5 to 5.5 kg, were used in this study. The animals were housed in stainless-steel cages at 22-24°C, with humidity of 40-50% and a 12- hour light cycle. They had continuous access to standard rabbit food and tap water, and the cages were cleaned weekly. Prior to the experimental procedures, the rabbits were acclimatized for 1 week.

Before the induction of general anesthesia via intramuscular injection of xylazine (Xylapan, Vetoquinol UK) 5 mg/kg and ketamine (Imalgene, Merial, France) 35 mg/kg, blood samples (1ml) were drawn from the marginal ear vein of each rabbit using a 21- gauge needle into sterile tubes. After centrifugation of the samples for 15 min at 4000 rpm, the serum was separated into Eppendorf tubes and refrigerated.

The rabbits were placed in dorsal recumbency under aseptic conditions. A lateral approach to the stifle joint was performed and the patella was dislocated medially. Synovial fluid samples (0.5 ml) were collected using a 21-gauge needle into non-anticoagulated tubes. The samples were centrifuged at 4000 rpm for 15 minutes and stored in a refrigerator. Care was taken to avoid contamination of the samples with blood. Serum and synovial fluid levels were evaluated within 24 hours of collection. Osteochondral defects with a depth of 3mm were then created on the patellar groove using a surgical drill equipped with a drill stopper (Veterinary Instrumentation). The defects were thoroughly lavaged with normal saline.

Study design

All rabbits were randomly divided into four groups:

- Group A (control group, n=8 rabbits): The defects were left untreated.
- Group B (n=16 rabbits): The defects were implanted with ASCs ($2x10^7$ cells/ml) and sealed with fibrin glue (50 μL/defect).
- Group C (n=16 rabbits): The defects were implanted with BMSCs ($2x10^7$ cells/ml) and fibrin glue (50 μL/defect).
- Group D (n=16 rabbits): The defects were implanted with SDSCs ($2x10^7$ cells/ml) and fibrin glue (50 μL/defect).

Fibrin glue (Tisseel, Baxter, USA) was prepared according to manufacturer instructions and formed

a sealant over the defect within 5 min of application. The surgical site was then thoroughly lavaged with sterile saline and the patella was relocated. The joint capsule was sutured using 4/0 Monocryl sutures (Ethicon, Johnson & Johnson Medical, Somerville, NJ) and the surgical incision was closed in layers.

Postoperatively, meloxicam (Meloxidyl, Ceva, France) 0,6 mg/kg and enrofloxacin (Enrotron, ani Medica GmbH, Germany)10 mg/kg were administered subcutaneously for 7 days. A standard rabbit diet and fresh water were offered daily. Although most animals began bearing weight shortly after surgery, indicators of inflammation, pain or infection were closely monitored and assessed frequently.

Serum and synovial fluid LDH evaluation

Twelve weeks postoperatively, blood samples were collected from the rabbits, centrifuged at 4000 rpm for 15 minutes, and stored in Eppendorf tubes at 4°C.

Anesthesia was induced with an intramuscular injection of xylazine (Xylapan, Vetoquinol UK) at 5 mg/kg and ketamine (Imalgene, Merial, France) at 35 mg/kg. Following anesthesia, synovial fluid samples from the operated limbs were collected into non-anticoagulated tubes, centrifuged at 4000 rpm for 15 minutes, and refrigerated. LDH levels were measured in a blinded manner. LDH was quantitatively measured (ADVIA 1800, Siemens, Germany). Finally, the animals were euthanized by intraperitoneal overdose of pentobarbital (Thiopental, IFET, Greece) 150 mg/kg.

Statistical analysis

The values of the variables were presented either as the mean and standard deviation or as the median and interquartile range (IQR) when the data had high dispersion and did not follow a normal distribution. The normality of the distribution of measurements was assessed using the Kolmogorov-Smirnov test and the normal probability plot. A mixed two-factor analysis of variance model was used to examine the interaction between the factors (intervention and time) on the variables of serum and synovial fluid LDH. For comparisons of these variables, paired t-tests or the Wilcoxon test were applied when the data did not follow a normal distribution. To analyze the differences in serum and synovial fluid LDH levels over time within groups (before and after the intervention), the percentage change of these variables was calculated. Comparisons of these percentage changes in LDH across groups were performed using a one-way analysis of variance model, and pairwise comparisons were made using the Bonferroni test. When normality assumptions were not met, non-parametric Kruskal-Wallis and Dunn tests were applied, adjusted using the Benjamini-Hochberg FDR method. All statistical analyses were performed using SPSS version 21.00 (IBM Corporation, Somers, NY, USA), and all tests were two-sided. A p-value of <0.05 was considered statistically significant. Due to lack of reference values and multiple comparisons a statistical analysis without outliers and after Bonferroni correction (the significance level was adjusted to p=0.01) was performed for serum LDH.

Results

Serum sample analyses revealed no statistically significant difference of LDH values among groups at the baseline (p=0.01). Also, there was no significant difference noted between the absolute [group A (p=0.345), B (p=0.221), C (p=0.496), D (p=0.239)] or percentage change (p=0.214) of serum LDH from baseline among groups.

However, the final LDH values of group A were significantly lower (p=0.01) compared to group B (p=0.001), C (p=0.002) and D (p=0.009) (Table 1). No statistically significant difference was observed between the synovial fluid LDH values among the groups at the baseline (p=0.136), nor at the end (p=0.070) of the procedures. Additionally, no statistically significant change in the values from the baseline to the end of the study was found for any of the groups: A (p=0.889), B (p=0.679), C (p=0.469), and D (p=0.255), nor for the percentage change in LDH values (p=0.677) (Table 2).

Discussion

In the present study, several outliers in serum LDH values were observed. Their presence may be attributed to both direct and indirect factors. Direct factors include potential subclinical, coexisting pathological conditions in the individual animal, stress or random, isolated events (e.g., accidental injuries). According to previous studies, rabbits that received high doses of vitamin C tend to have lower serum LDH levels (Mumtaz et al. 2019). Additionally, stress induced by extreme environmental conditions during transportation has been shown to elevate serum LDH levels (Najyinsige et al. 2013). Indirect factors include technical errors, such as hemolysis during sample collection or transportation (e.g., poor refrigeration, exposure to sunlight, etc.) (Yu et al. 1979).

According to the existing literature, there is no consistent reference range for serum LDH values in rabbits. Nakyinsige et al. (2013) reported a mean normal serum LDH value of 309.70 U/L, while Gasco

Table 1. Mean values ± standard deviations of serum LDH levels for the different rabbit groups.

Groups	Baseline	End	p-value $_{\text{within group}}$	% change baseline-end
Group A (n=5)	7.89 (12.68)	7.10 (10.17)	0.345	-59.15 (100.96)
Group B (n=14)	19.30(12.08) *	21.55(23.53) *	0.221	21.36 (141.20)
Group C (n=15)	21.59(8.63) *	23.68(14.87) *	0.496	-3.28 (118.15)
Group D (n=12)	18.18 (5.72)	21.44(16.16) *	0.239	18.59 (127.52)
p-value $_{\text{within group}}$	p=0.054	p=0.019		0.214

* $p<0.05$

Table 2. Medians and interquartile ranges (IQR) of synovial fluid LDH for the different rabbit groups.

Groups	Baseline	End	p-value $_{\text{within group}}$	% change baseline-end
Group A	29.95 (28.35)	30.33 (32.18)	0.889	-15.71 (249.32)
Group B	43.95 (19.85)	42.20 (28.23)	0.679	-11.48 (133.20)
Group C	43.69 (29.46)	47.45 (28.69)	0.469	-16.51 (70.69)
Group D	49.94 (26.62)	61.68 (68.94)	0.255	18.21 (178.78)
p-value $_{\text{within group}}$	p=0.136	p=0.070		0.677

et al. (2019) suggested a value of 143 U/L as the mean normal serum LDH concentration for healthy rabbits. Other studies presented a mean normal serum LDH value of 385.20 U/L for male rabbits and 304.55 U/L for female rabbits (Özkan and Pekkaya 2018). Additionally, Hein and Hartmann (2003) indicated a range of normal values for the same variable, spanning from 0 to 571 U/L. Therefore, based on the significant variability in the results of previous studies, the presence of outliers in this study may have been justified. However, due to the lack of specific reference values, the statistical analysis was conducted after Bonferroni correction and removing the outliers to ensure the reliability of the results.

LDH is an intracellular, cytoplasmic enzyme that is released following the destruction of the cell membrane, as occurs during the processes of apoptosis and necrosis (Kumar et al. 2018). Consequently, after surgical procedures, an increase in serum LDH levels is expected (So et al. 2016). Similar findings were reported following temporary occlusion of the thoracic aorta (Nielsen et al. 1994) and experimentally induced mesenteric ischemia (Zhang et al. 2011). In both experimental models, the rise in serum LDH was indicative of tissue damage, but did not specify the extent or the potential for reversal of the damage. Finally, the administration of general anesthesia led to an increase in serum LDH levels, with the most significant rise being caused by the administration of diazepam, likely due to its direct effect on hepatic cells (Gil et al. 2004). Therefore, the increase in serum LDH levels observed in this study during the postoperative phase was expected for all groups. However, according to our results LDH values were significantly higher for groups that underwent MSCs transplantation compared to the controls. This suggests that LDH release into systemic circulation may serve as a surrogate marker for biological processes occurring within the joint microenvironment following MSC implantation.

Various inflammatory processes as well as intense cellular proliferation have been associated with increased LDH levels. Specifically, the inoculation of Diplococcus pneumoniae in rabbits (Leise et al. 1968), as well as conditions such as ulcerative colitis, fibrocystic mastopathy and gastric mucosal metaplasia, led to an increase in the enzyme concentration intracellularly (Pan et al. 1991b). Additionally, a similar rise was observed in patients with various neoplasms, due to the heightened glycolytic activity of cancer cells (Forkasiewicz et al. 2020). Moreover, anaerobic glycolysis is the primary source of glucose and intracellular ATP production for healthy cartilage cells through the Embden-Meyerhof-Parnas pathway (Lane et al. 2015). Under hypoxic conditions, chondrocytes metabolize glucose exclusively through anaerobic mechanisms (Khan et al. 2009). However, even under normal oxygen concentrations, chondrocytes prefer anaerobic energy production (Warburg-like effect) (Suits et al. 2008). Consequently, the anaerobic metabolism in chondrocytes also leads to LDH production (Lane et al. 1977). However, the enzyme remains intracellular due to the limitations in the permeability of the chondrocyte cell membrane. LDH is released into the synovial fluid when chondrocytes undergo apopto-

sis, rupture, or continuous influx of water molecules, from where it diffuses into the bloodstream (Walter et al. 2007). For the first time in this study, serum LDH elevation was presented after MSCs transplantation into ODs. The elevated enzyme levels observed may indicate an increased anaerobic metabolism, likely due to the intense proliferation of chondrocytes, and consequently serve as evidence of articular cartilage regeneration.

Another potential contributor to elevated LDH could be hypoxic stress at the implantation site. MSCs embedded in a dense extracellular matrix may experience limited oxygen diffusion, especially in the avascular environment of cartilage. This metabolic stress could upregulate anaerobic glycolysis, increasing intracellular LDH production and subsequent leakage upon cell lysis or necrosis. Previous references to serum LDH values after MSCs transplantation are rare. Specifically, a significant increase in serum LDH was observed in humans after the transplantation of autologous HSCs (Bolwell et al. 1999). A similar rise was reported in patients with Hodgkin's lymphoma following the administration of BMSCs and HSCs (Kojouri et al. 2005). These findings were associated with chemotherapy toxicity, haemolysis, or the mass death of MSCs within the vascular system. Consequently, it is not possible to clearly differentiate whether the rise in serum LDH observed in this study is due to intense glycolytic activity due to chondrocyte proliferation, potential toxicity of the procedure, further damage to the articular cartilage or apoptosis of the implanted MSCs. However, no additional adverse effects were observed, making toxicity or further articular damage unlikely.

The present biochemical findings can be further interpreted in light of our previously published macroscopic and histological evaluation, in which improved cartilage repair was observed in MSC-treated groups, particularly in the SDSCs group, supporting the interpretation of the elevated LDH levels observed in the present work. Specifically, MSC-treated defects demonstrated significantly improved International Cartilage Repair

Society (ICRS) scores compared with untreated controls, while histological O'Driscoll scores were significantly higher in the SDSCs group than in the ASCs and BMSCs groups, indicating superior structural organization and tissue integration (Anatolitou et al. 2023). Furthermore, our previously published immunohistochemical study (Anatolitou et al. 2024) demonstrated that, although no statistically significant differences were detected among the MSC-treated groups regarding collagen type II expression, a trend toward higher expression in the SDSCs group was observed, reaching levels comparable to native cartilage. In contrast, aggrecan expression was significantly higher in the BMSCs and SDSCs groups compared with the ASCs group, indicating enhanced extracellular matrix synthesis. These findings reflect enhanced cellular activity and matrix synthesis within the defect area. When considered together, these results suggest that the elevated serum LDH levels observed in the present study are associated with metabolically active repair tissue and enhanced regenerative processes. Such metabolically demanding regeneration is likely supported by increased anaerobic glycolysis in both chondrocytes and transplanted MSCs, providing a plausible explanation for the observed biochemical changes. Importantly, in the absence of adverse clinical signs and in conjunction with improved extracellular matrix composition and histological repair, increased serum LDH appears to reflect active regeneration rather than implantation-related toxicity or cartilage degeneration.

From a clinical perspective, the observed elevation of serum LDH following MSC implantation appears to reflect increased metabolic activity associated with active cartilage regeneration rather than pathological tissue damage. Accordingly, increased LDH levels may serve as a supplementary, non-invasive indicator of biological activity within the repair site. Therefore, serum LDH could potentially be useful for monitoring the regenerative response after MSC-based cartilage repair. However, due to its limited tissue specificity, LDH should be interpreted in combination with clinical, imaging and histological findings.

Interestingly, in the present study the synovial fluid LDH did not show significant changes throughout the duration of the research across the different rabbit groups. According to the existing literature, the measurement of synovial fluid LDH in both healthy rabbits and rabbits subjected to OD induction and MSC transplantation has not been repeated, as far as the authors are aware. Therefore, the reference range for synovial fluid LDH values for this species has not yet been established. In the present study, an attempt was made for the first time to measure this in order to draw relevant conclusions.

Regarding humans, the normal synovial fluid LDH values range between 105-333 IU/L (Pejovic et al. 1992). In veterinary medicine, reference values have been documented for horses (50-109 IU/L, mean value 80 IU/L) (Rejnö 1976), dogs (61 ± 9 IU/L) (Walter et al. 2007), and camels (mean ± standard deviation 12.7 ± 14.8 IU/L) (Al-Rukibat and Isamil 2014). In the present study, the median (interquartile range) synovial fluid LDH values ranged from 29.95 (28.35) to 49.94 (26.62) U/L at the start of the procedures, which could potentially serve as a reference

range for this species. However, since LDH is a sensitive enzyme influenced by various exogenous and endogenous factors, more studies with a larger number of animals should be conducted to confirm the reproducibility of the above findings.

Additionally, there have been reports of increased synovial fluid LDH levels in various arthropathies of immunological or infectious origin, both in horses (Rejnö 1976, Yancik et al. 1987) and in humans (Messieh 1996). In dogs, an increase in synovial fluid LDH levels has been suggested as an early indicator of OA (Hurter et al. 2005, Walter et al. 2007), as well as a diagnostic tool for septic arthritis (Proot et al. 2015). Furthermore, the measurement of synovial fluid LDH has been used in humans since 2007 for the diagnosis of microbial arthritis (Kopterides 2007). However, Hunter et al. (2005) concluded that serum LDH measurements cannot serve as a reliable marker for OA in dogs. Similarly, in this study, no statistically significant difference was found in synovial fluid LDH values following MSC implantation, and therefore no further correlation could be made. Several explanations can be considered. First, the half-life of LDH in synovial fluid may be relatively short due to enzymatic degradation or clearance mechanisms within the joint space. Also, the localized release of LDH from cartilage or transplanted cells may be too low in magnitude to overcome the dilution effect in the synovial compartment. Furthermore, serum LDH may be a more sensitive or earlier marker of systemic response to joint injury compared to local measurement.

This study had several limitations. First, despite removing outliers and applying Bonferroni correction, the variability in baseline LDH values remains a challenge due to the lack of consistent reference ranges in rabbits. Second, the measurement of total LDH activity, rather than specific isoenzymes, limits the specificity of the results for cartilage-related metabolic activity. Because LDH is widely distributed in multiple tissues, elevated serum levels cannot be attributed exclusively to chondrocyte metabolism or cartilage repair processes. Finally, biochemical sampling was performed only at baseline and at 12 weeks postoperatively. Serial pre- or postoperative LDH measurements were not performed in order to minimize repeated anaesthesia and procedural stress to the animals. This limited temporal resolution may have missed early, transient, or peak changes in LDH levels that could occur during the acute inflammatory or early regenerative phases following MSC implantation. Nevertheless, future studies incorporating multiple intermediate time points would allow a more detailed and comprehensive evaluation of dynamic biochemical changes during cartilage healing.

Author Declarations

Ethics approval

The experimental protocol was approved by the Veterinary Services of Region of Attica, Hellenic Republic (1155/13-03-2018) and all procedures in animals were carried out following the recommendations of the international guidelines.

Use of generative artificial intelligence

The authors confirm that they did not use any generative artificial intelligence methods or AI-assisted methods in the preparation of this manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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