Original Article

Influence of mononuclear cell therapy on disc degeneration in rabbits☆

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ABSTRACT

Objective: The objective of this research was to evaluate the influence of autologous mononuclear stem cells injections on histological changes of collagen in the fibrous annulus of the intervertebral disk after experimental injury.

Methods: 32 New Zealand rabbits were submitted to intervertebral disk puncture, followed by intradiscal injection of mononuclear cells from the iliac crest *versus* saline injection in the following time periods: two months after the injury (SC2M and SS2M), two weeks (SC2W and SS2W) immediately after injury (SCCP and SSCP), and without inducing degeneration (SCSP and SSSP). Two months after cell therapy, the animals were euthanized and collagen changes in the intervertebral discs were histologically evaluated.

Results: There were significant differences in ELAF between SS2W and SS2S groups ($p = 0.018$). This difference was due to an increase in type I collagen in SS2W group (56.7%) compared to SC2S (13.28%).

Conclusion: Treatment with mononuclear mesenchymal stem cells reduced changes in the type I and III collagen distribution in rabbits AF degenerated discs up to two weeks after the induction of degeneration.

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Influência da terapia celular mononuclear sobre a degeneração discal em coelhos

RESUMO

Objetivo: Avaliar a influência da injeção de células-tronco mononucleares autólogas sobre as alterações histológicas do colágeno no ânulo fibroso do disco intervertebral após lesão experimental.

Métodos: Foram submetidos 32 coelhos New Zealand a punção do discos intervertebrais lombares seguida de injeção intradiscal de células mononucleares provenientes da crista ilíaca *versus* injeção de solução salina nos seguintes períodos tempo: dois meses após a lesão (CT2M e SS2M), duas semanas (CT2S e SS2S), imediatamente após a lesão (CTCP e SSCP) e sem induzir a degeneração (CTSP e SSSP). Após dois meses da terapia celular, os animais foram submetidos a eutanásia e as alterações do colágeno nos discos intervertebrais foram avaliadas histologicamente.

Resultados: Houve diferença estatisticamente significativa na CEAF entre os grupos CT2S e SS2S (p = 0,018). Essa diferença decorreu de um aumento do colágeno do tipo I no grupo SS2S (56,7%) comparado com o CT2S (13,28%).

Conclusão: O tratamento com células mononucleares precursoras mesenquimais é capaz de reduzir as alterações na distribuição do colágeno do tipo I e III no AF de discos degenerados de coelhos até duas semanas após a indução da degeneração.

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Palavras-chave:
Colágeno
Disco intervertebral
Terapia celular
Histologia
Coelho

Introduction

Disk degeneration is part of the aging process and comprises the loss of structural, biological, and biochemical properties of the intervertebral disk (IVD).[1] It is characterized by the production of dysfunctional cells and a decrease of the intracellular components, leading to gradual loss of intradiscal fluid.[2] This causes disk dehydration, which entails a cascade of factors that can lead to symptoms and functional limitation. The main symptom of disk degeneration is low back pain.[3]

The etiology of disk degeneration is multifactorial; both constitutional and environmental factors play roles with varying degrees of importance.[4] Physical exertion, poor posture, obesity, professional occupation, smoking, alcohol, and diabetes are involved in the etiology of symptomatic disk degeneration.[5]

Low back pain is the second leading cause of medical consultation in the United States. Worldwide, approximately 60–80% of people will experience low back pain during their lifetime. According to US data, 20 billion dollars per year are spent on direct costs for the treatment of chronic low back pain; added to the indirect costs, this amount exceeds $100 billion.[6,7]

In order to alleviate this alarming picture, several therapeutic strategies have been attempted, ranging from non-invasive modalities – such as anti-inflammatory medication and physiotherapy – to surgical procedures such as vertebral fusion, intradiscal electrothermal therapy, and total disk replacement. However, current therapeutic methods are directed toward the treatment of the symptoms, not the interruption of and/or recovery from the degenerative process.[8] Various biological treatment models have been proposed as options for the application of the new technologies. The use of cell elements proposes a direct involvement in the modulation of the degenerative process, through the introduction of cells that are potentially able to reconstruct the damaged tissue.[9]

Based on an animal model previously studied and validated in this educational institution,[10] this study aimed to evaluate the influence of injection of autologous mononuclear cells on histological collagen changes in the annulus fibrosus (AF) of the IVD after experimental injury.

Material and methods

The experiments in this study were made in accordance with the rules and ethical principles set forth by the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal [COBEA]). The methods were based on previous studies conducted in this institution,[10–12] as well as on the studies by Lipson and Muir,[13] Masuda et al.,[14] and Rousseau et al.[15]

This study was approved by the Ethics Committee on Animal Use of the Pontifícia Universidade Católica do Paraná under No. 377 and was implemented in accordance with the rules of the Helsinki Declaration of the World Medical Association.

Male, white New Zealand rabbits (*Oryctolagus cuniculus*), weighing between 2.5 and 3 kg and aged approximately 8 months were used.

After standard anesthesia (10–12), rabbits were placed in the lateral position and submitted to a lumbotomy with exposure of the lumbar spine through the retroperitoneal access route (Fig. 1), which exposed the anterior surface of five consecutive lumbar IVDs (L2-L3 to L6-L7). The three experimental discs were punctured with a 40 mm × 12 mm (18 G 1½)

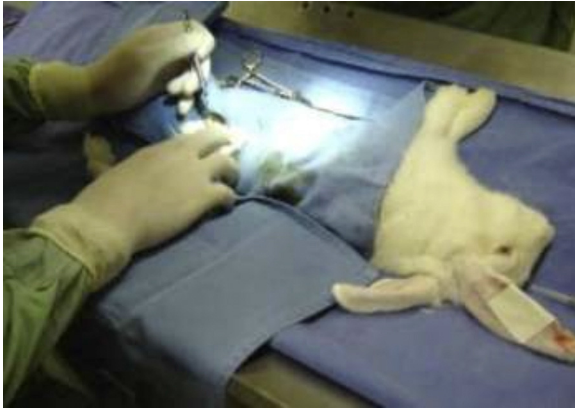

Fig. 1 – Surgical preparation.
Anesthetized rabbits were placed in right lateral decubitus position; the posterolateral retroperitoneal access route was used.

needle at a depth of 5 mm, which remained inside the IVD for 5 s (Fig. 2).

The process of isolation and collection of mononuclear stem cells (SC) was done by puncture-aspiration of bone marrow from the iliac crest, and is part of the protocol that has already been developed and consolidated in this educational institution.[16]

After cells were collected and isolated, they were introduced into the animal IVD with the same surgical technique as described above. The material was placed in the upper limit of the pre-drilled IVD, with a smaller-caliber needle (13 × 4.5; 26 G ½). Exactly analogous procedures were performed in animals that received only isotonic saline solution (SS); the amount injected was equal to the cell volume, following the abovementioned conditions.

The study included 32 animals, divided into eight groups: four groups that received SC and four control groups that received SS.

The groups were divided according to the moment of SC injection:

- SC2M (four animals): transplantation of autologous mononuclear SCs after two months of disk degeneration induction surgery;
- SC2W (four animals): transplantation of autologous mononuclear SCs after two weeks of disk degeneration induction surgery;

Fig. 2 – Needle preparation to perform the puncture.
The final 5 mm of a 40 mm × 12 mm (18 G1 ½) needle was delimited from its bevel and folded in an S-shape in order to standardize the depth of the punctures.

- SCCP (four animals): transplantation of autologous mononuclear stem cells immediately after the disk degeneration induction surgery.

This differentiation allowed for a periodic comparative critical analysis regarding the most appropriate period of implementation of cell therapy in light of the degenerative process.

- SCSP (four animals), the group that received mononuclear stem cells, but did not undergo disk degeneration induction surgery.

Similarly, the animals that were part of the control group were subdivided according to the pairing with respect to the experimental group and received isotonic SS injection, as follows: SS2M (four animals), corresponding to SC2M; SS2W (four animals), corresponding to SC2W; SSCP (four animals), corresponding to SCCP; and SSSP (four animals), corresponding to SCSP.

Of the 32 rabbits, six died during surgery or postoperatively. Thus, 26 rabbits were histologically examined, divided according to Tables 1 and 2.

Eight weeks after the injection (CS/SS) in the IVD, the rabbits were euthanized by injection of an overdose of pentobarbital (90 mg/kg) and their spines were harvested for histological analysis.

After the samples were processed, dehydrated, and embedded in paraffin, sections of 6 micron thickness were taken. The sections were stained with sirius red for collagen analysis. Through a microscope with polarized light, aspects related to the collagen arrangement in the IVDs were observed with 20-fold magnification.

From each slide prepared with an IVD, six fields of vision were photographed by the software DinoCapture® 2.0 v.1.2.7 (AnMo Electronics Corporation). The six fields had been predefined for all discs, starting from the most peripheral layer of the AF lamellae (Fig. 3). This convention allows for a panoramic and comprehensive sample of the AF structure.

The images were processed by the software Image Pro Plus® v.4.50 (Media Cybernetics, Silver Spring, MD), which quantified the green (collagen type III) and red dots (collagen type III) of the slide, and stratified them with their respective percentages (Fig. 4).

Results

In order to compare groups and find possible significant differences between them, the following variables were individually assessed: the inner layer of the AF (ILAF); the external layer of the AF (ELAF); and the entire AF.

For each variable and each type of collagen, in each application time, the null hypothesis that the collagen results in the SC group would be equal to those of the SS group was tested. Tables 3–5 shows the descriptive statistics and *p*-values of the statistical tests.

When comparing the ILAF of the group injected with SC with that of the groups injected with SS, no statistically significant difference was observed between the groups (Table 3).

Table 1 – Distribution of rabbits in groups injected with stem cells.

Group	Stem cells			
	SC2M	SC2W	SCCP	SCSP
Rabbits ($n = 14$)	4	3	4	3
Discs – intervention ($n = 37$)	12	9	7	9
Discs – control ($n = 60$)	16	14	16	14

SC2M, SC injection 2 months after puncture; SC2W, SC injection 2 weeks after puncture; SCCP, SC injection at the same time of puncture; SCSP, SC injection without puncture.

Table 2 – Distribution of rabbits in groups injected with saline solution.

Group	Saline			
	SS2M	SS2W	SSCP	SSSP
Rabbits ($n = 12$)	3	2	4	3
Discs – intervention ($n = 35$)	9	6	12	8
Discs – control ($n = 55$)	15	10	17	13

SS2M, saline injection 2 months after puncture; SS2W, saline injection 2 weeks after puncture; SSCP, saline injection at the same time of puncture; SSSP, saline injection without puncture.

When comparing the ELAF of the group injected with SC with that of the groups injected with SS, a statistically significant difference was observed between the SC2W and SS2W groups ($p = 0.018$). This difference was due to an increase in type I collagen in SS2W group (56.7%) when compared with SC2S (13.28%). Collagen type I and III values and group comparisons are shown in Table 4 and Fig. 5.

When comparing the AF of the group injected with SC with that of the groups injected with SS, a statistically significant difference was observed between the SC2W and SS2W groups ($p = 0.025$). Once again, this difference was due to an increase in type I collagen in SS2W group (76.1%) when compared with the SC2W (53.32%). Collagen type I and III values and group comparisons are shown in Table 5.

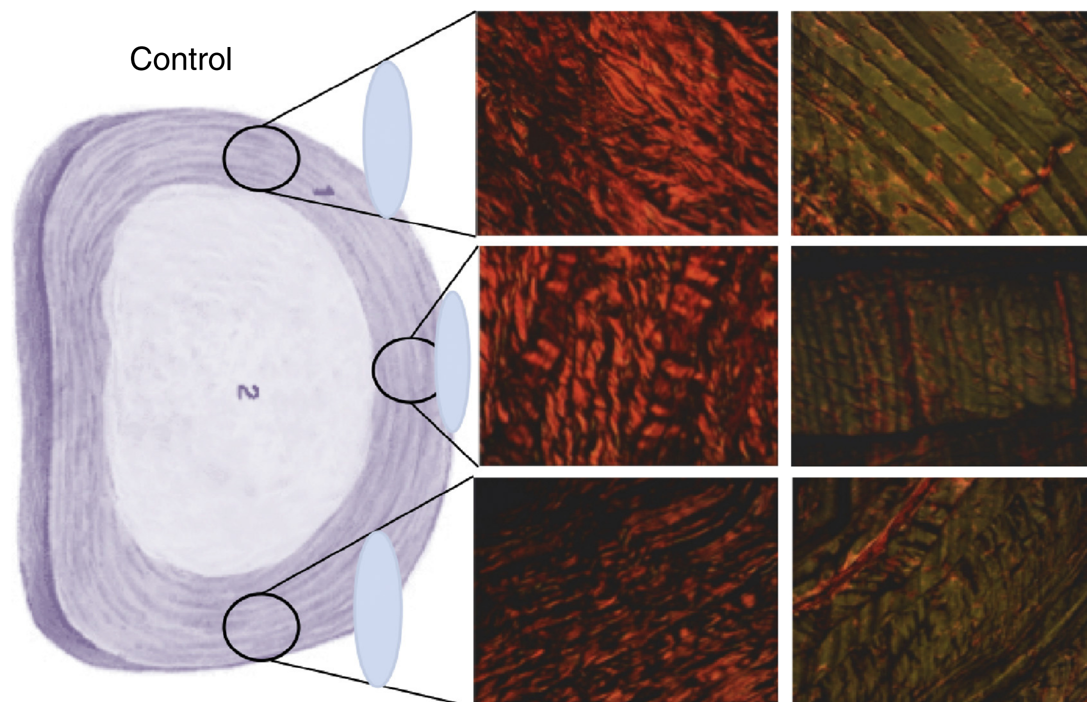

Fig. 3 – DinoCapture Tool.
Schematic illustration of the six fields photographed in a control disk. Two fields on the lateral right angle of the disk, one from the outer portion and one from the inner portion of the annulus; two fields on the lateral left angle of the disk, one from the outer portion and one from the inner portion of the annulus; and two fields on the center of the convex portion of the disk, one from the outer portion and one from the inner portion of the annulus. Note the predominance of green in the external layer of the annulus fibrosus (ELAF) denoting type III, and the predominance of red in the internal layer of the annulus fibrosus (ILAF), which indicates type I collagen.

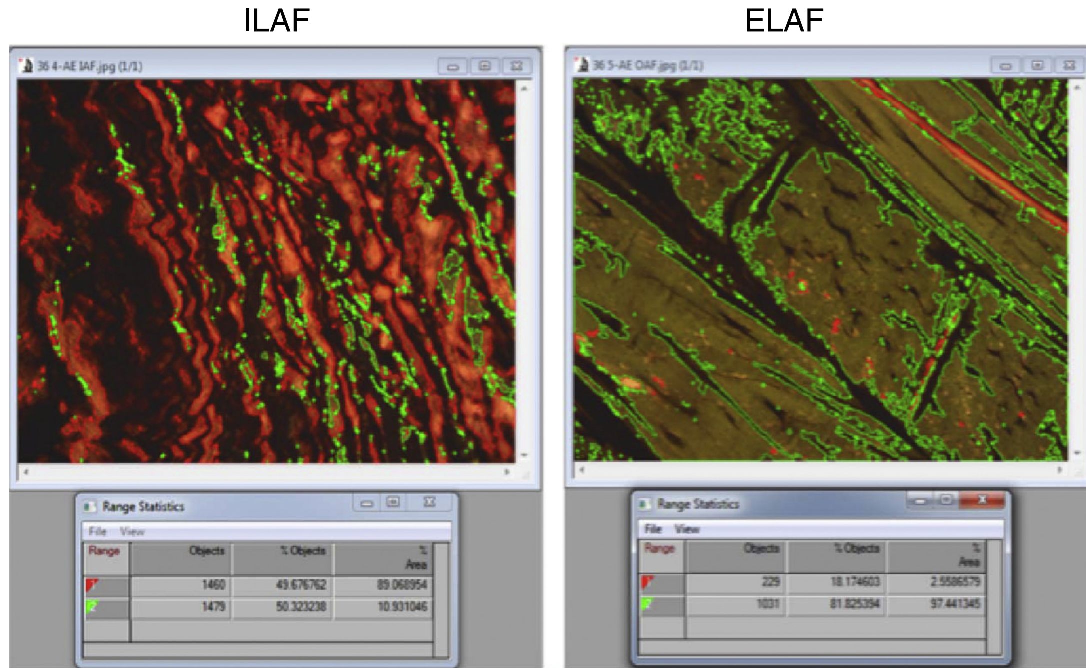

Fig. 4 – Image Pro Plus Tool.
Example of the software used to make the pixel count on a photograph of the inner layer of the annulus fibrosus (ILAF) and the external layer of the annulus fibrosus (ELAF). Three pieces of information are given for each image: 1, number of objects; 2, percentage of objects in the image; and 3, percentage of the total image area.

Discussion

Disk degeneration induction has been studied in depth in another branch of this research through slides stained in hematoxylin-eosin, fast green, and sirius red.[10] The present study used slides obtained from the same species and population of rabbits, and the induction of disk degeneration was performed in the same manner. The standardization of disk degeneration induction in rabbits in this experiment, through the needle puncture method, was effective and was reproduced in a similar manner to the results of other studies that used the same technique.[10,17–20]

Many contemporary studies used immunohistochemistry to assess different types of collagen.[1,21–23] The forerunners in

Table 3 – Comparison between groups injected with stem cells or saline regarding ILAF.

Variable	Col	Group	n	Mean	Minimum	Maximum	Standard deviation	p-Value[a]
ILAF	I	SC2M	12	73.37	2.39	98.65	29.18	0.286
		SS2M	9	85.5	42.5	97.5	18.2	
	III	SC2M	12	26.63	1.35	97.61	29.18	0.286
		SS2M	9	14.5	2.5	57.5	18.2	
	I	SC2W	9	93.36	88.93	98.33	3.28	0.157
		SS2W	6	95.6	87.7	99.6	4.2	
	III	SC2W	9	6.64	1.67	11.7	3.28	0.157
		SS2W	6	4.4	0.4	12.3	4.2	
	I	SCCP	7	96.61	92.56	99.56	2.40	0.800
		SSCP	12	96.9	90.4	99.4	2.8	
	III	SCCP	7	3.39	0.44	7.44	2.40	0.800
		SSCP	12	3.1	0.6	9.6	2.8	
	I	SCSP	9	91.16	78.9	98.52	6.7	0.149
		SSSP	8	94.8	85.9	98.7	4.4	
	III	SCSP	9	8.84	1.48	21.91	6.7	0.149
		SSSP	8	5.2	1.3	14.1	4.4	

There was no statistically significant difference between groups.
Col, collagen; SC2M, SC injection 2 months after puncture; SC2W, SC injection 2 weeks after puncture; SCCP, SC injection at the same time of puncture; SCSP, SC injection without puncture; SS2M, SS injection 2 months after puncture; SS2W, SS injection 2 weeks after puncture; SSCP, SS injection at the same time of puncture; SSSP, SS injection without puncture.
[a] Nonparametric Kruskal–Wallis test; $p < 0.05$.

Table 4 – Comparison between groups injected with stem cells or saline regarding ELAF.

Variable	Col	Group	*n*	Mean	Minimum	Maximum	Standard deviation	*p*-Value[a]
ELAF	I	SC2M	12	25.85	1.24	68.87	19.58	0.201
		SS2M	9	39.2	13.1	82.8	21.0	
	III	SC2M	12	74.15	31.13	98.76	19.58	0.201
		SS2M	9	60.8	17.2	86.9	21.0	
	I	SC2W	9	13.28	2.72	20.99	6.55	0.18
		SS2W	6	56.7	8.7	98.7	32.8	
	III	SC2W	9	86.72	79.1	97.28	6.55	0.18
		SS2W	6	43.3	1.3	91.3	32.8	
	I	SCCP	7	14.91	6.38	29.11	8.16	0.63
		SSCP	12	9.1	1.6	21.9	6.9	
	III	SCCP	7	85.9	70.89	93.62	8.16	0.63
		SSCP	12	90.9	78.1	98.5	6.9	
	I	SCSP	9	12.5	2.29	36.52	10.54	0.773
		SSSP	8	15.0	3.1	60.6	19.7	
	III	SCSP	9	87.95	63.48	97.71	10.54	0.773
		SSSP	8	85.0	39.4	96.9	19.7	

A statistically significant difference was observed between the SC2W and SS2W groups ($p = 0.018$).
Col, collagen; SC2M, SC injection 2 months after puncture; SC2W, SC injection 2 weeks after puncture; SCCP, SC injection at the same time of puncture; SCSP, SC injection without puncture; SS2M, SS injection 2 months after puncture; SS2W, SS injection 2 weeks after puncture; SSCP, SS injection at the same time of puncture; SSSP, SS injection without puncture.
[a] Nonparametric Kruskal–Wallis test; $p < 0.05$.

this area date back to the late 1990s.[1,21] This assessment has as a main advantage its specificity; however, it is still an expensive method in Brazil. In the present study, sirius red proved to be a simple and inexpensive method to assess type I and III collagen.

Regarding the number of cells injected into the treatment, current studies are not standardized. Some suggest that cell culture is essential to the success of treatment; however, most studies do not discuss considerations for optimal cell number. Serigano et al.[22] indicated that the optimum dose of autologous mesenchymal stem cells (MSCs) in dogs is 1×10^6 cells. In turn, Ghosh et al.[23] suggested that a lower dose of 0.1×10^6 may be more effective. According to the authors, an exaggerated number of cells in this environment with low nutrient supply may cause cells to compete for the supplement, which may be destructive to the NP due to the accumulation of dead cells and cellular degradation products.[23]

Furthermore, the discogenic differentiation of MSCs may also be stimulated by co-culture. MSCs can be directly cultivated in contact with IVD cells. During the co-culture of bone marrow-derived MSC and NP cells, it has been observed that they communicate in a bidirectional manner, which results

Table 5 – Comparison between groups injected with stem cells or saline regarding AF.

Variable	Col	Group	*n*	Mean	Minimum	Maximum	Standard deviation	*p*-Value[a]
Entire AF	I	SC2M	12	49.61	2.16	78.59	22.21	0.155
		SS2M	9	62.3	27.8	88.9	16.1	
	III	SC2M	12	50.39	21.41	97.84	22.21	0.155
		SS2M	9	37.7	11.1	72.2	16.1	
	I	SC2W	9	53.32	47.90	59.15	3.62	0.25
		SS2W	6	76.1	48.2	97.4	17.5	
	III	SC2W	9	46,68	40.85	52.10	3.62	0.25
		SS2W	6	23.9	2.6	51.8	17.5	
	I	SCCP	7	55.76	51.10	64.33	4.57	0.151
		SSCP	12	53.0	48.6	60.7	3.5	
	III	SCCP	7	44.24	35.67	48.90	4.57	0.151
		SSCP	12	47.0	39.3	51.4	3.5	
	I	SCSP	9	51.61	43.83	64.43	6.59	0.534
		SSSP	8	54.9	45.5	79.5	11.1	
	III	SCSP	9	48.39	35.57	56.17	6.59	0.564
		SSSP	8	45.1	20.5	54.5	11.1	

A statistically significant difference was observed between the SC2W and SS2W groups ($p = 0.025$).
Col, collagen; SC2M, SC injection 2 months after puncture; SC2W, SC injection 2 weeks after puncture; SCCP, SC injection at the same time of puncture; SCSP, SC injection without puncture; SS2M, SS injection 2 months after puncture; SS2W, SS injection 2 weeks after puncture; SSCP, SS injection at the same time of puncture; SSSP, SS injection without puncture.
[a] Nonparametric Kruskal-Wallis test; $p < 0.05$.

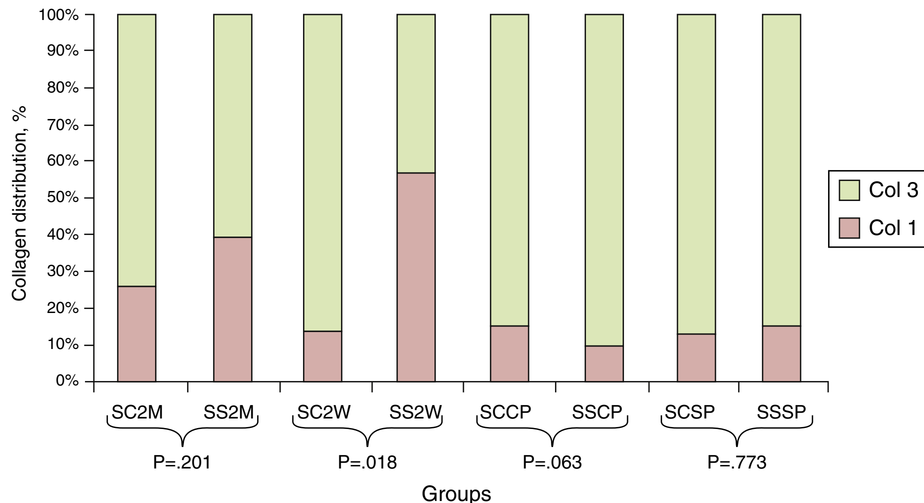

Fig. 5 – Comparison of percentage of collagen distribution in the external layer of annulus fibrosus (ELAF) between different groups injected with stem cells and saline.
A statistically significant difference was observed between the SC2W and SS2W groups ($p = 0.018$). Col 1, type 1 collagen; Col 3, type 3 collagen; SC2M, SC injection 2 months after puncture; SC2W, SC injection 2 weeks after puncture; SCCP, SC injection at the same time of puncture; SCSP, SC injection without puncture. SS2M, saline injection 2 months after puncture; SS2W, saline injection 2 weeks after puncture; SSCP, saline injection at the same time of puncture; SSSP, saline injection without puncture.

in an improvement in the phenotype of the NP cells and the differentiation of MSC cells.[24] This suggests that the implantation of MSCs can exert paracrine effects in degenerate NP cells that reside on the disk, and help restore normal cell function and the disk repair process.

In the present study, mononuclear stem cells were used. The fact that they are obtained through a simpler process than the MSC was taken into account, as they only need to undergo two processes of centrifugation and Ficoll-Hypaque density gradient, while the MSC need to undergo these same processes, in addition to culture and cell growth for approximately 14–16 days, thus presenting higher costs and greater risk of contamination.[16]

Injection of MSCs and mononuclear mesenchymal stem cells is typically safe, although there is a potential for formation of peripheral osteophytes, suggesting the importance of a proper and safe carrier to inject cells in this region.[25] In this regard, the carrier may allow the cell to receive axial loads, which are important to stimulate the synthesis of extracellular matrix and induce MSC differentiation without further exogenous stimulus.[26] Different carriers have been used in the literature, such as some hydrogels[26,27] and fibrin glue.[28]Although these studies advocate the use of a mobile carrier in the application of stem cells, the literature is controversial in this regard. Other studies have used only SS[29] or even no carrier.[22] In the present study, DMEM culture medium with 20% fetal bovine serum was used, a method that has also been used successfully in the literature.[30]

In the analysis of the present results, when assessing the entire AF, a difference between the groups that were injected two months after the puncture was observed. The SC2M showed a slight increase in type III collagen, divided into 49.61% type I and 50.39% type III. The SS2M showed 62.35% type I and 37.65% type III collagen. The group that received SC had a division closer to that of the groups in which disk degeneration was not induced, while the SS group showed a significant increase in the proportion of type I collagen. This disproportionality occurred in ELAF, indicating a disorganization of the AF structure. However, the difference was not statistically significant.

The same increase in collagen type I in ELAF and AF could also be observed between the groups injected two weeks after the puncture. The SC2W showed 53.32% type I and 46.68% type III collagen. The SS2W showed 76.15% type I and 23.85% type IIIcollagen. Once again, it was observed that the group that received SC had a division closer to that of the groups in which disk degeneration was not induced, while the SS group had a significant increase in the proportion of type I collagen, again at the expense of ELAF. In the statistical analysis, a statistically significant difference was observed both in ELAF ($p = 0.018$) as well as in AF ($p = 0.025$).

The present data suggest that mononuclear SC therapy was able to reshape the changes caused by the injury from the biopsy needle when the SC are injected after two weeks, but the same success was not achieved when SCs were injected two months after injury. Despite the fact that the data from SC2M was closer to the groups in which disk degeneration was not induced than were the values from the SC2W, this difference was not statistically significant.

However, when comparing the groups that were injected at the moment of puncture, SCCP showed 55.76% type I and 44.24% type III collagen. In turn, SSCP showed 53% type I and 47% type III collagen. This is the highest mismatch in the results, as it was expected that the SSCP would present

degeneration, with a change in the proportion of type I and type III collagen after puncture. This suggests that the damage caused in the discs of SSCP were healed.

When comparing the groups that were injected without puncture, the SCSP showed 51.61% type I and 48.39% type III collagen. In turn, the SSSP showed 54.9% type I and 45.10% type III collagen. As expected, no large differences were observed between these groups, as degeneration through puncture was not induced. No statistically significant difference was observed between groups. These data indicate that MSCs did not alter the structure of collagen in discs that were injected without degeneration.

Conclusion

Treatment with mononuclear mesenchymal stem cells is able to reduce changes in the distribution of type I and III collagen in the AF of degenerated rabbits discs until two weeks after degeneration induction.

Conflicts of interest

The authors declare no conflicts of interest.

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