Transplantation of limbal derived MSCs grown on contact lenses in dogs with dry eye syndrome - can stem cells help?

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ABSTRACT
Keratoconjunctivitis Sicca (KCS), also known as “dry eye syndrome”, is a common ocular disease in dogs, caused by inflammation of the lacrimal gland, resulting in decreased tear production. Efforts are being made to develop alternative therapies in order to prevent lifelong use of drugs for patients with KCS. Mesenchymal stem cells (MSCs) are known to be effective in the treatment of many immune-mediated diseases in human and animal models due to their immunoregulatory properties. The aim of this study was to transplant limbal mesenchymal stem cells (LMSCs) to the ocular surface on contact lenses and to evaluate the therapeutic effects of the LMSCs by clinical examination findings. The animals were randomly divided into study and control groups. The LMSC group (n = 10) received LMSCs (at least 2×10^6 cells) cultured on contact lenses. The conventional treatment group (n = 10) received artificial tears, topical 0.05% Cs A, and antibiotic eye drops, 3 times a day for 4 weeks. The Schirmer test, tear break-up time, impression cytology, Rose Bengal staining, and tear osmolarity were measured in all patients. The findings of the pre-treatment, two weeks and four weeks after the treatment, were evaluated statistically. In both groups, significant improvement was present compared to the pre-treatment findings. However, there was no significant difference between the groups. KCS treatment using LMSCs produced on contact lenses is promising, with its ease of application, non-immunogenic properties and single dose administration.

Key words: dog; contact lens; limbal mesenchymal stem cells; KCS; transplantation
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Lifelong application of immunosuppressive drugs, such as cyclosporine and tacrolimus, are included in the treatment of KCS (SALL et al., 2006). Some researchers have reported that some dogs are resistant to cyclosporine treatment (WILLIAMS, 2017). Efforts are being made to develop alternative therapies in order to prevent lifelong use of drugs that inhibit immune response and inflammatory processes for treatment of patients with KCS (BITTENCOURT et al., 2016).

Mesenchymal stem cells (MSCs) are known to be effective in the treatment of many immunemediated diseases in human and animal models due to their immunoregulatory properties. Previous studies have shown that allogeneic MSC transplantation in dogs is a safe treatment method (VILLATORO et al., 2015). In this study, limbal MSCs grown on contact lenses were transplanted to dogs with KCS, and the aim was to investigate their effects on tear production and changes to the clinical findings of dogs.

Materials and methods

All procedures performed in studies involving animals were in accordance with the ethical standards of the Local Animal Ethics Committee of Ankara University, approval no: 2015-3-52.

Animals. Twenty dogs of various breeds and genders over 1 year of age suffering from discharge, redness, itching, and discomfort in the eye were included in the study. Patients undergoing dry eye treatment, patients with corneal ulceration, and severe ocular infection were excluded from the study. Dogs with dry eye were divided into two groups according to the treatment as the LMSC group (n = 10) or the control group (n = 10).

Tear osmolarity measurement, Schirmer tear test I, impression cytology, TBUT measurement, and the Rose Bengal staining values of the dogs were recorded at pre-treatment, and after 2 and 4 weeks.

Tear osmolarity measurement. Tear osmolarity was measured in each eye from the lateral lower conjunctival sac, using the TearLab™ osmolarity system (TearLab Corp.) and the values were recorded in mOsm/L.

Schirmer tear test. Schirmer tear test I was performed on each eye. A standard Schirmer filter paper (Bio-Schirmer Strips, Biotech Vision Care, India) was placed in ventral conjunctival fornix for 60 s, without the use of topical anesthesia. Patients with values of 15 mm/1 min or less were accepted as dry eye.

Impression cytology. Impression cytology was performed at pre-treatment and 4 weeks after the treatment. Samples for corneal impression cytology were collected from the eyes, using a cellulose acetate filter paper (Millipore® Corp., Type GS, USA) with 0.4 μm pore diameter and 3 x 5 mm dimensions under local ophthalmic anesthesia, with 0.5% proparacaine hydrochloride (Alcain 0.5% Ophthalmic Solution, Alcon, USA). The opaque surface of cellulose filter paper was placed on the upper temporal limbus, a soft impression was made for 5 sec, and then it was removed. The samples were fixed with 95% ethanol, then stained with Periodic Acid Shiff (PAS) and Hemalun, evaluated under a light microscope, and goblet cells were counted.

TBUT measurement. The cornea was stained using standard paper strips containing 1 mg sodium fluorescein (Fluorescein Sodium Strip, ERC, Turkey). The time at which the tear film was first broken was determined under a cobalt blue light on the biomicroscope. Dry eye diagnosis was confirmed by examining the other findings of patients with values less than 20 seconds.

Rose Bengal staining. The cornea was stained using 1.3 mg Rose Bengal impregnated paper strips (Rose Bengal Ophthalmic Strips, Biotech Vision Care, India) and evaluated with a biomicroscope. The presence of dye was accepted as a positive result.
Isolation of dog fetal limbus-derived MSCs (LMSCs). Fetal tissues were obtained during cesarean section to terminate pregnancy in dogs with an unwanted pregnancy. The tissues obtained from the fetuses were transferred fresh to the laboratory. Fetal limbus tissues were mechanically dissected into small pieces using a sterile scalpel. Then, the tissues obtained by the explant culture method were placed in T25 flasks and incubated at 37°C in a 5% CO₂ humidified atmosphere. 77% DMEM (Lonza, Belgium), 20% fetal bovine serum-FBS (Lonza, Belgium), 2% L-Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin, Amphotericin (Biological Industries, Israel) were added into the medium. The medium was changed once every 2-3 days to remove non-adherent cells. When about 70% adhesions were present, the adherent cells were passaged. The cells were grown to the 3rd passage (P3) and then separated into groups for immunochemistry staining, for differentiation (adipocytes, chondrocytes, osteocytes), and for real-time quantitative PCR analysis, and the remainder was used for transplantation.

Differentiation of MSCs. The LMSCs were seeded at a density of 5×10⁴ cells/cm² in six-well plates at P3 and differentiated into adipogenic, chondrogenic or osteogenic differentiation medium for 21 days, as previously described (WILLIAMS and TIGHE, 2018). Von Kossa staining, Oil red staining, and Alcian blue staining were used to reveal osteogenic, adipogenic and chondrogenic differentiation, respectively (Fig. 1).

Immunohistochemical analysis. P63a and ABCG2 expressions were investigated by immunostaining, to determine whether limbal stem cells retained their phenotype. Cells grown in flasks were washed with 4% paraformaldehyde solution, removed from the surface with Trypsin-EDTA C (0.025%), centrifuged at 2000 rpm for 10 minutes, and then made into cytoblocks. 2 micrometer thick sections were taken from each block. Immunohistochemical staining was performed with anti-ABCG2 (Abcam/ab3380) and Anti-p63 (Abcam/ab735) primary antibodies using the streptavidin-biotin peroxidase method.

Real-time quantitative PCR analysis. Total RNA was isolated from the cultured limbal mesenchymal stem cells (LMSCs) and adipose tissue-derived mesenchymal stem cells (Ad-MSCs) to examine p63a and ABCG2 mRNA expressions.

RNA isolation. Dog LMSCs and Ad-MSCs used for control purposes were removed from 3 separate T25 culture dishes by a scraper, washed with PBS, and centrifuged, then RNA was isolated with RNeasy Mini Kit (Qiagen, Cat No./ID: 74104).

Control of isolated RNAs. The quality and quantity of isolated RNAs were determined by a spectrophotometer (NanoDrop ND-1000). RNAs were measured and diluted to 50 ng/5μL.

Conversion of isolated RNA to cDNA. RNA samples were obtained using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat. No. 04 379 012 001) and the Thermal Cycler (Applied
Biosystems); 65 °C 10 min denaturation, 25 °C 10 min, 55 °C 30 min, 85 °C 5 min reaction program was converted to cDNA.

Among the obtained cDNA samples, B-Actin (internal control), p63, ABCG2 primers Fast Start Essential DNA Probes Mastermixes (Roche, Cat No: 06402682001) and Rotor-Gene Q 2plex Platform (Qiagen) and 10 min at 95 °C, 30 sec at 95 °C (35 cycles), 30 sec at 60 °C (35 cycles), 30 sec at 72 °C RT-qPCR was performed using (35 cycle) thermal cycler program. Data were analyzed in Q-Rex Software.

Preparation of contact lenses. Monthly lenses were used (Air Optix Aqua Night and Day, Alcon, USA), which are commercially available and produced for humans, with 24% water content, are transparent, and can be used for 30 days day and night. Lotrafilcon A lenses have a diameter of 13.8 mm and a base curve of 8.6 mm.

Prior to the transfer of LMSCs to contact lenses, the lenses were removed from their packages in sterile conditions in a laminar cabinet, and incubated in dog serum for half an hour. At this time, 10µl of LMSCs prepared for transplantation were taken, cells stained with 10µl Trypan Blue were counted using the Countess automated cell counting device (Invitrogen), and viability was determined.

At least 2×10⁶ LMSCs were prepared by using the existing trough structure of the contact lenses coated with serum in a Petri dish, and were placed in this trough area with the help of a micropipette. In order not to dry the lenses, the surfaces contacting the Petri dish were filled with medium. LMSCs planted on the lenses were incubated at 37 °C, 5% CO₂ humidified atmosphere for 7 days, and the medium was changed every 2-3 days. On the day of transplantation, the prepared contact lenses were checked for the general condition of the LMSCs and the presence of infection, with an inverted microscope (Leica DFC 295), and those in good condition were selected for transplantation (Fig. 2).

The contact lenses were placed in the eyes of the dogs without the need for general or topical anesthesia, and the eyes were protected against external trauma by an Elizabeth collar. On the 5th day after transplantation, the general condition of the eye was checked and the contact lenses were removed.

10 patients in the LMSC group received LMSC transplantation by contact lenses once and no drug was used; in the control group, 10 patients received topical Cs A (Depores, 0.05% Cyclosporine, Deva İlaç, Turkey), artificial tears (Tears Naturale Free, Alcon, USA), and antibiotic eye drops (Tobrex, Alcon, USA) in patients with purulent discharge, three times a day for 4 weeks.

Statistical analysis. Descriptive statistics of the variables included in the study were shown as "Arithmetic Mean ± Standard Error". The Schirmer tear test and tear osmolarity measurement results were analyzed over time and between groups. In the model, time, group, and group-time interaction terms were used. Data were analyzed using SPSS 14.01 (SPSS Inc, USA). The P<0.05 criterion was used for all statistical evaluations.

Fig. 2. View of LMSCs transferred to contact lenses on an inverted microscope. (a) Day 3 of LMSCs in incubation (×20), (b) Day 7 of LMSCs in incubation (×20), (c) Contact lens with LMSCs just before transplantation.
Results

The mean Schirmer tear test values of the LMSC group before treatment were 5 ± 1.09 mm/1 min; and of the control group 5.4 ± 0.91 mm/1 min. The mean Schirmer tear test values increased to 8 ± 0.97 mm/1 min and 8.2 ± 1.5 mm/1 min after 2 weeks of treatment, relatively. The mean Schirmer tear test values were found to be 8.7 ± 0.93 mm/1 min and 8.7 ± 1.41 mm/1 min after 4 weeks of treatment, relatively. There was no statistically significant difference between the groups in terms of Schirmer tear test values (P = 0.879). The increase between 2 and 4 weeks was not statistically significant.

The mean tear osmolarity values of the LMSC group before treatment was 305.5 ± 7.49 mOsm/L; the control group was 306 ± 11.07 mOsm/L. The mean tear osmolarity values were 326.4 ± 7.91 mOsm/L and 314.5 ± 11.25 mOsm/L, respectively, 2 weeks after treatment. The mean tear osmolarity values were 314.4 ± 9.65 mOsm/L and 305.6 ± 14.01 mOsm/L, respectively, 4 weeks after initiation of treatment. There was no statistically significant difference between the groups in terms of tear osmolarity values (P = 0.622).

When the average TBUT values of the groups were examined, the values of the pre-treatment LMSC group were 23.7 ± 11.02 sec, and of the control group 46.3 ± 16.19 sec. Two weeks after initiation of treatment, the mean TBUT values were 21.8 ± 10.96 sec and 40.01 ± 14.12 sec, respectively. The values were measured for 20.5 ± 11.09 sec and 32.9 ± 11.58 sec, respectively, 4 weeks after initiation of treatment. There was no statistically significant difference between the groups in terms of TBUT values before treatment (P = 0.323).

Goblet cells in samples obtained by impression cytology were counted before treatment and 4 weeks after treatment. Accordingly, the mean number of goblet cells in the LMSC group before treatment was 9.7 ± 0.76 and in the control group 9.8 ± 0.93. Four weeks after treatment the mean number of goblet cells was found to be 12.6 ± 0.76 and 10.6 ± 0.58, respectively. There was no statistically significant difference between the pre-treatment groups in terms of goblet cell count (P = 0.33).

Rose Bengal staining was performed in all cases and any staining was accepted as a positive result. In the LMSC group 8 dogs before treatment, 7 dogs 2 weeks after treatment and 5 dogs 4 weeks after treatment were evaluated as positive. In the control group, the results of 7 patients who were evaluated as positive before the treatment did not change during the treatment, and were evaluated as positive again at the 4th week.

Expressions of p63 and ABCG2 were investigated by immunostaining, to determine whether the limbal stem cells retained their phenotypes. A total of 100 cells were counted at ×20 magnification on three different slides, with p63 (+) in 35 (35%), while ABCG2 was detected in 15 cells (15%).

When the results of RT-qPCR were analyzed in the Q-Rex Software program, B-Actin gene expression levels in LMSC and Ad-MSC (3, 3, respectively) were found to be the same, as expected, due to it being the housekeeping gene. p63 gene expression levels were higher in LMSCs compared to Ad-MSCs (2, -3, respectively) and this difference was statistically significant (P<0.05). When the ABCG2 gene expression levels were compared, it was seen that ABCG2 gene expression was higher in LMSCs compared to Ad-MSCs (1, -3, respectively), and this was statistically significant (P<0.05).

Discussion

Inflammation plays an important role in the pathogenesis of dry eye (LEONARDI et al., 2017). Decreased tear volume and increased osmolarity trigger inflammation on the ocular surface. Increased secretion of inflammatory cytokines causes damage to the ocular surface (COASSIN et al., 2005). In the conventional treatment of dry eye, the aim is to lubricate the ocular surface by tear replacement, or to preserve the patient's existing tears without correcting the underlying disease. However, these treatments do not directly target ocular surface inflammation. Therefore, anti-inflammatory drugs are needed. Cs A, an immunomodulatory drug, is routinely used in the treatment of KCS (GUADA et al., 2016).
The most important clinical indication of Cs A is the prevention of organ rejection after transplantation (DEMIRYAY et al., 2011). Cs A inhibits the activity of T-cells and stops inflammatory cytokine release (GULBAG and CELEBI, 2017). In addition to post-transplant immunosuppression, Cs A is widely used in some autoimmune diseases and in the treatment of dry eye syndrome (KULUALP and KILIC, 2012). Cs A was first tested on dogs in 1989, and was found to increase tear production in patients with KCS, and to provide significant regression in chronic corneal neovascularization and granulation. In addition, it has been reported to prevent apoptosis by increasing the density of conjunctival goblet cells (IZCI, 1995).

It is reported that at least 2-3 weeks of Cs A treatment is needed to see clinical improvement in dogs with KCS. This time frame coincides with immunosuppression, and confirms the hypothesis that Cs A suppresses activated T-lymphocytes and local immunity in lacrimal glands in animals with KCS. Studies related to topical Cs A use in dogs with KCS have reported that the degree of clinical improvement varies according to the degree of lacrimal disorder (IZCI et al., 2002).

In a study that evaluated Schirmer tear test results and tear gland histological findings during the treatment of KCS with 2% Cs A, and after discontinuation of drug use, the patients were divided into 3 groups. The first group received %2 Cs A for 30 days, the second group for 45 days, and the third group for 60 days. After Cs A treatment and when the treatment was temporarily suspended and started again, improvements in Schirmer tear test values and clinical results were reported. It has been reported that this improvement is due to reduced damage in the tear gland by themununosuppressive mechanism, and the presence of the lacrimostimulant mechanism (EDASO and KEBEDE, 2018).

In this study, Cs A was administered to the control group for 4 weeks. Compared with pretreatment, the increase in the Schirmer tear test value at weeks 2 and 4 was significant. However, there was no significant increase between the 2nd and 4th weeks. There was no significant difference between TBUT values before and after treatment. A significant increase in tear osmolarity values was found at the 2nd week compared to pretreatment. However, in the 4th week, there was a significant decrease compared to the 2nd week. Goblet cells were found to increase in number at week 4 compared to pretreatment, and this result was consistent with the previous studies. This is thought to be due to the control of the inflammatory process involved in the pathogenesis of KCS by Cs A, via inhibition of the epithelial apoptosis and cytokine produced by T-lymphocytes, and the goblet cells performing their functions once more.

In recent years, the effectiveness of stem cells has been investigated in many diseases in the field of veterinary medicine (PELLEGRINI et al., 2001). However, the number of studies on the use of stem cells for the treatment of dry eye in dogs is limited. In a relevant study, $5 \times 10^6$ allogeneic adipose-derived MSCs (Ad-MSC) were transplanted around the lacrimal glands in dogs that did not respond to conventional KCS treatment (such as tacrolimus, Cs A), and there was a significant increase in the Schirmer tear test value in the patients after the transplantation, as well as a reduction in conjunctival hyperemia and ocular discharge. In conclusion, allogeneic Ad-MSC transplantation in dogs with KCS was shown to increase tear production (VILLATORO et al., 2015).

In another study in which allogeneic Ad-MSC transplantation was performed in dogs with KCS, allogeneic Ad-MSCs were transplanted around the lacrimal glands at a dose of $1 \times 10^6$ and as a result, it was reported that in moderate KCS the patients’ Schirmer tear test values returned to the normal range, and in advanced KCS the patients’ Schirmer tear test values increased and ocular surface improvement was found (BITTENCOURT et al., 2016).

In the present study, fetal limbus-derived MSCs were used as stem cell sources in dogs, and their effects on healing were investigated. The reason for choosing LMSCs as the cell source is that these cells are native cells of the eye. Therefore, when used in corneal injuries, it is thought that they can provide better recovery than MSCs with other tissue or organ origin (e.g. adipose, bone marrow).
Although specific markers have not been identified, studies have reported that ABCG2 and p63 are useful markers in the characterization of corneal epithelial stem cells, and in the regenerative treatment of the cornea (WATANABE et al., 2004; RAMA et al., 2010). In human eye diseases, corneal epithelial cell layers cultured from autologous limbal epithelium have been used in corneal diseases, and layers containing p63 positive cells have been reported to be more successful after transplantation (NAM et al., 2013). Studies have shown that corneal epithelial layers cultured on canine amniotic membrane provide promising results in severe corneal injuries, and ABCG2 and p63 positive cells are present in the basal part of these layers (GUL SANCAK et al., 2014; MORITA et al., 2015). In the present study, the expression of ABCG2 and p63 gene, as stem cell and proliferation markers in limbal stem cells was also demonstrated by immunohistochemistry and the RT-qPCR method. It can be said that the use of LMSCs has a positive effect on healing in dry eye syndrome that causes corneal epithelial damage.

In a study in which a high dose of MSC was administered systemically, inflammatory damage in the cornea was reported to be reduced by TNF-α-stimulated gene/protein 6 secretion, and very few MSCs could be located in the region. As the cornea is an avascular structure, it was shown that systemic administration of stem cells is not an effective method (WILLIAMS and TIGHE, 2018).

The necessity of local transplantation of MSCs has brought up the use of carriers. Studies have shown that corneal epithelial layers cultured on canine amniotic membranes give good results in corneal injuries (MORITA et al., 2015). Although an amniotic membrane is a widely used carrier in limbal stem cell failure, it has some disadvantages, such as transport difficulties, poor optical permeability, phenotypic variation and disease transport potential (YANG et al., 2006; LEVIS and DANIELS, 2009).

In order to transport limbal stem cells without these disadvantages, contact lenses, which do not show immunogenic properties and provide phenotypic and transfer stability, were used in the present study. Contact lenses have protective effects on the corneal surface and the ability to transfer biological agents easily to the corneal surface (DIGIROLAMO et al., 2009; GUPTA and AQIL, 2012). With this method, the aim was to transfer LMSCs to cornea more easily and effectively, and to avoid the disadvantages arising from existing carriers.

In the present study, $2 \times 10^6$ allogeneic LMSCs, grown on contact lenses, were transplanted to the LMSC group only once. There was no complication related to the use of contact lenses in any of the dogs. The increase in the Schirmer tear test value was significant at 2nd and 4th weeks compared to pretreatment. Although this increment continued between the 2nd and 4th weeks, it was not statistically significant. A significant increase in tear osmolarity values at the 2nd week was observed compared to pretreatment, and in the 4th week there was a significant decrease compared to the 2nd week. When TBUT values were compared, there was no significant difference between the groups before and after treatment. The number of goblet cells increased significantly. The possible mechanism here was thought to be related to the immunomodulatory and anti-inflammatory effects of LMSCs as a result of the stimulation of proinflammatory cytokines (such as TNF and IL-6), and secretion of immunomodulatory factors (such as TGF-β). As a result of this effect, the vicious cycle of proinflammatory cytokines and lacrimal lymphocyte proliferation may be broken, and tear production may be reactivated to initiate repair of the ocular surface.

Although the findings obtained by Rose Bengal staining in the present study suggested that LMSC transplantation might be more beneficial in repairing corneal epithelial damage and increasing mucin production compared to Cs A treatment, there was no significant change in TBUT findings that would support this opinion.

No significant difference was found between the LMSC and control group when the Schirmer tear test, TBUT, tear osmolarity values and Goblet cell numbers at 2nd and 4th weeks were compared to pretreatment, indicating that both treatment options contributed to clinical improvement at a similar level. On the other hand, in order to obtain these
similar healing findings, the control group had to be given medication 3 times a day for 4 weeks, in contrast to the transplantation in the LMSC group, with a single application without requiring any other drug or supportive treatment.

Based on our results, single-time transplantation of LMSCs, produced on contact lenses, is a simple, safe and effective treatment method in the treatment of KCS in dogs. When compared with the conventional treatment, there was no need for lifelong drug use, which reduces the cost of treatment, and the clinical improvement improved the quality of life of the patients. However, since the number of cases in the present study was limited, it is necessary to support these results by working on larger groups, and to follow up the clinical results of the patients for a longer period of time.

As a result, we conclude that transplantation of LMSCs by contact lenses for the treatment of KCS is a safe, effective and relatively simple method in dogs, which improves ocular clinical symptoms and tear production.

Conflict of interest
The authors have no conflict of interest to disclose.

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SAŽETAK
Keratokonjunktivitis sika (KCS), poznat i kao sindrom suhog oka, česta je očna bolest u pasa uzrokovana upalom susne žlijezde i posljedično smanjenom proizvodnjom suza. Kako bi se preveniralo dugoročno uzimanje lijekova za sindrom suhog oka, pokušale su se razviti alternativne terapije. Mezenhimske matične stanice (MSCs) su, s obzirom na njihove imunoregulacijske sposobnosti, kod čovjeka i životinjskih modela pokazale učinkovitost u liječenju mnogih imunosno posredovanih bolesti. Cilj ovog istraživanja bio je transplantirati mezenhimske matične stanice izdvojene iz

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ekstremiteta (LMSCs) na površinu oka, odnosno na kontaktne leće, te procijeniti njihov terapijski učinak na temelju kliničkih nalaza. Životinje su slučajnim odabirom podijeljene u pokusne i kontrolnu skupinu. Skupina LMSC (n = 10) primila je LMSCs (najmanje 2x10^6 stanica) uzgojenih na kontaktnim lećama. Konvencionalna pokusna skupina (n = 10) primila je umjetne suze, topički 0,05 % Cs A i antibiotik u obliku očnih kapi 3 puta dnevno tijekom 4 tjedna. U svih su pacijenata učinjeni Schirmer-ov test, test vremena pucanja suznog filma, impresijska citologija, Rose-Bengalovo bojenje i osmolarnost suza. Rezultati su statistički obrađeni dva i četiri tjedna poslije tretmana. U obje je pokusne skupine uočeno znakovito poboljšanje u usporedbi s nalazima prije tretmana, no razlika među skupinama nije bilo statistički znakovita. Zaključno, terapija KCS-a primjenom LMSC-a proizvedenima na kontaktnim lećama ima obećavajuće učinke, prvenstveno zbog jednostavnosti primjene, neimunogenih svojstava i jednokratne aplikacije.

Ključne riječi: pas; kontaktne leće; mezenhimske matične stanice izdvojene iz ekstremiteta; KCS; transplantacija