

Anterior cruciate ligament regeneration using mesenchymal stem cells and collagen type I scaffold in a rabbit model

David Figueroa · Maximiliano Espinosa · Rafael Calvo ·
Maximiliano Scheu · Alex Vaisman · Marcela Gallegos ·
Paulette Conget

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Abstract

Purpose The objective of this study was to determine whether using mesenchymal stem cells (MSC) seeded in a collagen type I scaffold would be sufficient to regenerate the torn anterior cruciate ligament (ACL).

Methods Anterior cruciate ligament transection was performed on both knees in 10 New Zealand rabbits and then repaired with as follows: suture alone (suture-treated group, $n = 6$), suture associated with collagen type I scaffold (collagen type I scaffold-treated group, $n = 8$) or suture associated with autologous MSC seeded on collagen type I scaffold (MSC/collagen type I scaffold-treated group, $n = 6$). At 12-week post-intervention, the animals

were killed and the ACLs were characterised macroscopically and histologically. Data of the 3 groups were against normal ACL (normal group, $n = 10$).

Results Macroscopic observation found that in MSC/collagen type I scaffold group, 33 % of specimens showed a complete ACL regeneration, with a tissue similar to the normal ACL. Regeneration was not observed in the group treated with suture alone or associated with collagen type I scaffold without cells. In the latter, only a reparative attempt at the ends was observed. Histological analysis of the regenerated ACL showed a tissue with organised collagen and peripheric vessels.

Conclusions These results provide evidence that the use of MSC seeded in a collagen type I scaffold in the treatment of ACL injuries is associated with an enhancement of ligament regeneration. This MSC-based technique is a potentially attractive tool for improving the treatment of ACL ruptures.

D. Figueroa · M. Espinosa (✉) · R. Calvo · M. Scheu ·
A. Vaisman
Orthopedic Surgery Department, Clínica Alemana de Santiago,
Avda Vitacura 5951, Santiago, Chile
e-mail: maxespinosa@gmail.com

D. Figueroa
e-mail: dhfigueroa@gmail.com

R. Calvo
e-mail: rcalvo61@gmail.com

M. Scheu
e-mail: max.scheu@gmail.com

A. Vaisman
e-mail: avaisman@alemana.cl

M. Espinosa · P. Conget
Faculty of Medicine, Clínica Alemana–Universidad del
Desarrollo, Las Condes 12438, Santiago, Chile
e-mail: pconget@udd.cl

M. Gallegos
Pathology Department, Clínica Alemana de Santiago,
Vitacura 5951, Santiago, Chile
e-mail: mgallegos@alemana.cl

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Introduction

Anterior cruciate ligament (ACL) injuries are common, especially in young athletes. The incidence of ACL disruption is estimated to be 0.81 in 1,000 persons [13]. Since the ACL does not usually heal spontaneously [21], surgical intervention is often required to restore knee function and, although controversial, to prevent the development of early osteoarthritis [15]. Reconstruction techniques include autograft or allograft with hamstring tendon or patella tendon. Despite the improvement of surgical interventions

in the last decades, there still remain some limitations as prolonged recovery times, donor site morbidity and pathogen transfer [6, 19].

The emerging field of tissue engineering offers the possibility of creating functional engineered tissues to treat ACL injuries without many of the undesirable side effects associated with current reconstructive options [32]. It is based on the using of appropriate cells, biocompatible scaffolds and growth factors to substitute the natural tissue.

Mesenchymal stem cells (MSC), also referred as multipotent stromal cells, are an attractive tool for ACL tissue engineering since they are relatively easy to isolate and to expand in culture under conditions in which they maintain their potential to differentiate into multiple lineages [4, 5, 20, 26]. In fact, many studies have shown that MSC are one of the most optimal cell sources for ACL regeneration because of their high potential for proliferation and collagen production [10, 14, 18].

On the other hand, the selection of a scaffold to seed the cells is of major importance in the field of tissue engineering. In vitro studies have shown that three-dimensional porous matrices provide a suitable environment for the adhesion, growth and development of the MSC [3, 31]. Furthermore, the ideal ACL replacement scaffold should be biodegradable, porous, biocompatible, exhibit sufficient mechanical strength and promote the formation of ligamentous tissue [32]. Collagen scaffolds are an attractive alternative in ACL regeneration since they possess almost all the features listed above.

The aim of this study was to evaluate, in a rabbit model, the contribution of MSC seeded in a collagen type I scaffold to ligament regeneration in the treatment of complete ACL ruptures.

The hypothesis of the present study was that MSC associated with collagen type I scaffold in ACL ruptures might enhance the possibility of ligament regeneration.

Materials and methods

Study design

See Fig. 1.

Animals

Anterior cruciate ligament ($n = 30$) from 15 New Zealand male rabbits (3 months old, 2.5–3.5 kg) was used in this study. The animals were housed by themselves, at constant temperature and humidity, with a 12:12 h light–dark cycle and with unrestricted access to a standard diet and water, in individual 40 cm × 40 cm × 60 cm cages. The research protocol was reviewed and approved by the Ethics

Committee of the Faculty of Medicine of Clínica Alemana–Universidad del Desarrollo. All the procedures were carried out under aseptic conditions, using intramuscular anaesthesia with ketamine (35 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg). Enrofloxacin (10 mg/kg) and tramadol (4 mg/kg) were administered to all the animals preoperatively and up to 2 days after surgery.

ACL defect interventions

Sharp transections were created in the middle third of ACL of each knee by making a medial longitudinal parapatellar arthrotomy. The knees were divided into four study groups: suture-treated group ($n = 6$), suture plus collagen type I scaffold-treated group ($n = 8$) and suture plus autologous MSC/collagen type I scaffold-treated group ($n = 6$). An unlesioned, untreated group of ACL was used as a control group (normal group, $n = 10$). The number of knees per experimental group was defined in order to obtain an alpha value of 0.05 and a statistical power of 80 %. Collagen type I scaffold used was Duragen™ (Integra LifeSciences Corporation, Plainsboro, NJ, USA). Briefly, the ACLs were transected with scalpel and then sutured with a 6-0 Prolene. In the collagen type I scaffold-treated group, after passing the suture at the ends of transected ACL, the same suture through the collagen scaffold was used to adhere it to the tissue. In the MSC/collagen type I scaffold-treated group, the same procedure was done, but previously, 1×10^6 MSC were seeded into the scaffold. The arthrotomies were then closed by layers with a 4-0 suture in the deep layer and 3-0 suture to the skin. After surgery, the animals were kept in separate cages and allowed to walk freely with full weight bearing and without immobilization. No complications were observed and no animal had to be killed before the end of the study. Twelve weeks after surgery, the rabbits were euthanised using an intravenous overdose of pentobarbital, and the ACLs were dissected and analysed.

MSC isolation, expansion and characterisation

Bone marrow aspirations were carried out under general anaesthesia. Briefly, from both iliac crests, bone marrow was aspirated with a 19-gauge needle that was fastened to a 10-mL syringe containing 1 mL Heparin 250 U (Laboratorio Chile, Chile). After centrifugation, nucleated cells were seeded at a density of $1 \times 10^6/\text{cm}^2$ in α -MEM culture medium supplemented with 10 % foetal bovine serum and 40 mg/mL gentamicin (Sanderson Laboratory, Chile). The next day non-adherent cells were discarded by medium replacement. Thereafter, the medium was changed every 4 days. Cultures were maintained at 37 °C in an atmosphere of air: CO₂ 95:5 %. The cells were further

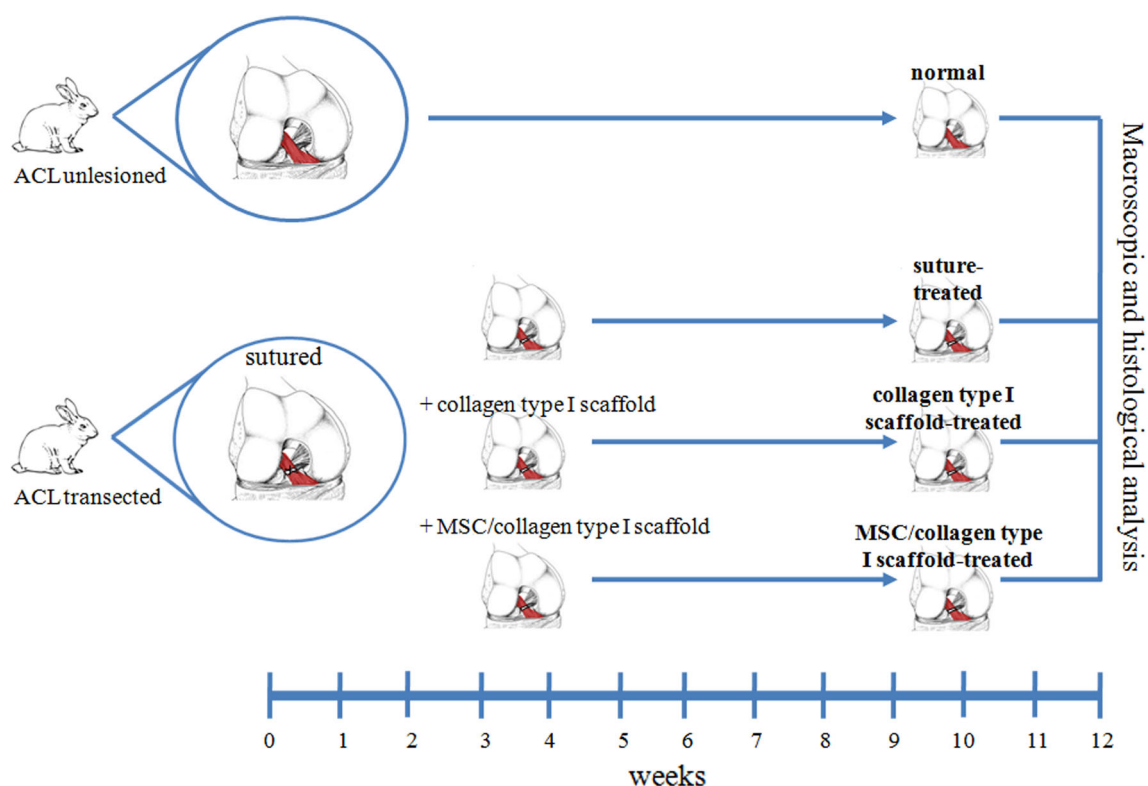


Fig. 1 Study design. Complete sharp transections were established in the anterior cruciate ligament of adult New Zealand male rabbits. The knees were divided into four study groups: suture alone treated group ($n = 6$), suture plus collagen type I scaffold-treated group ($n = 8$)

and suture plus autologous MSC/collagen type I scaffold-treated group ($n = 6$). An unlesioned, untreated group of ACL was used as a control group (normal group, $n = 10$). Twelve weeks after interventions, ligaments were analysed macroscopically and histologically

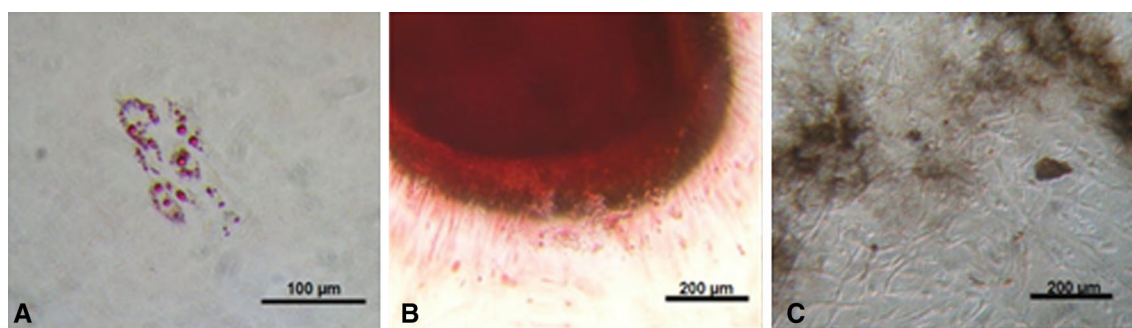


Fig. 2 Mesenchymal stem cells characterisation. **a** Adipogenic, **b** chondrogenic and **c** osteogenic differentiation of mesenchymal stem cells

subcultured by trypsinization and used at passage 3 at 2 weeks after their obtention. Expanded cells were characterised according to their adipogenic, chondrogenic and osteogenic differentiation potential as previously described [5] (Fig. 2).

Macroscopic evaluation

All of the ACLs ($n = 30$) were observed directly, immediately after sacrificing the animals, and the characteristics

of the tissue were recorded describing presence or absence of regenerative tissue and colour/texture of them.

Histological analysis

Anterior cruciate ligaments ($n = 30$) were fixed in formalin, decalcified in nitric acid and embedded in paraffin. Sagittal cross-sections 10 μm thick were cut through the tissue.

Sections were stained with haematoxylin/eosin. The samples were blindly evaluated by a trained pathologist

describing macroscopic union, collagen arrangement and central/peripheral blood vessels.

Statistical analysis

The unit of analysis used in this study was each knee ($n = 30$). The estimated number of ACL required per group based on an average difference between groups of 25 % and a power of 80 % was a minimum of 6.

Results

Macroscopic observations

As expected, the normal group presented a smooth, shiny, white tissue. At 12 weeks after intervention, in the suture-treated group, no tissue was observed and retraction of the ACL remnant ends was present in all samples. In the collagen type I scaffold-treated group, there was no continuity of the ACL, but a redundant fibrous tissue was observed in the proximal and distal ends of the ligament in most

samples. The MSC/collagen type I scaffold-treated group showed a 2 out of 6 knees with macroscopic continuity and tissue regeneration, presenting those samples a tissue with similar features of native ACL (Fig. 3).

Histological analysis

Haematoxylin and eosin staining were used to evaluate the microscopic features of normal and regenerated ligaments. As describe above, no samples of suture treated group or collagen type I scaffold-treated group showed regeneration of ACL. In the latter, a fibrous tissue was observed at the ends of the ACL with no recognisable collagen arrangement and high central and peripheral blood vessel density (Fig. 4). In the MSC/collagen type I scaffold-treated group, 2 out of 6 knees presented macroscopic union of the ACL, with a tissue that showed organised collagen fibres and predominance of peripheral blood vessels. Both tibial and femoral insertions were normal in location and tissue attachment (Fig. 4). As expected, the normal group showed a typical extracellular matrix composition and cell distribution of the ACL (not shown).

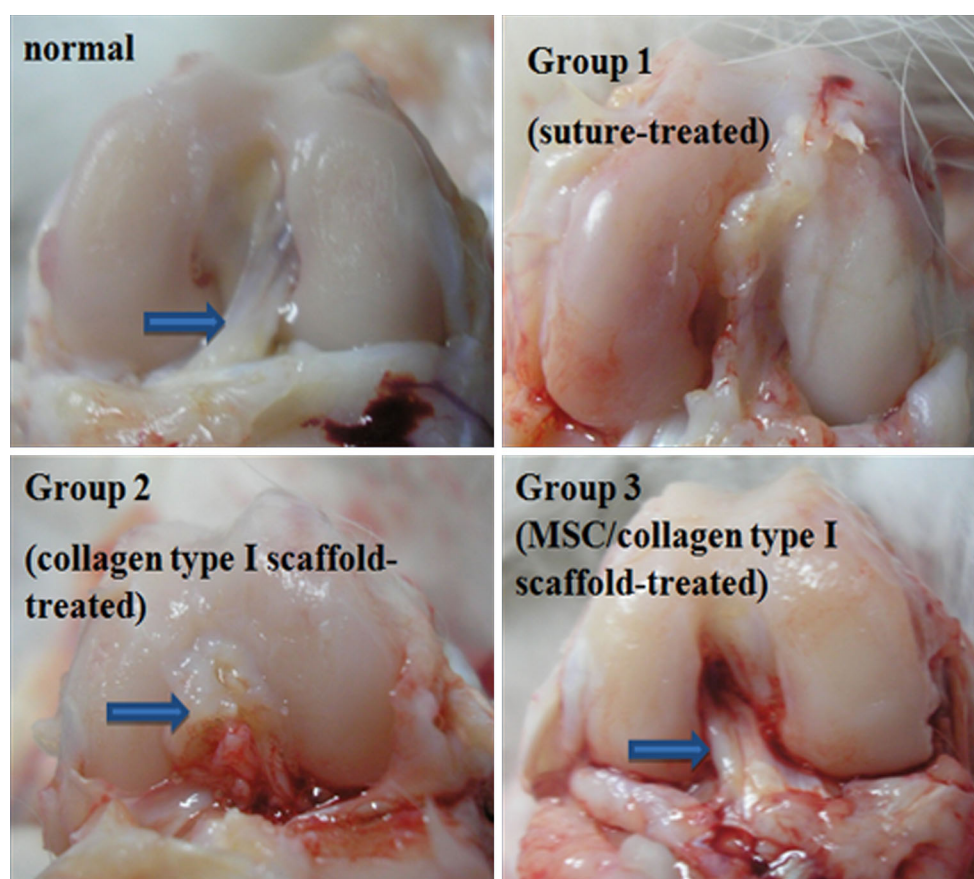


Fig. 3 Macroscopic appearance after anterior cruciate ligament intervention. Grossly evaluation of anterior cruciate ligament presence and features. Normal ($n = 10$), suture-treated ($n = 6$), collagen type I scaffold-treated ($n = 8$) and MSC/collagen type I scaffold-treated ($n = 6$)

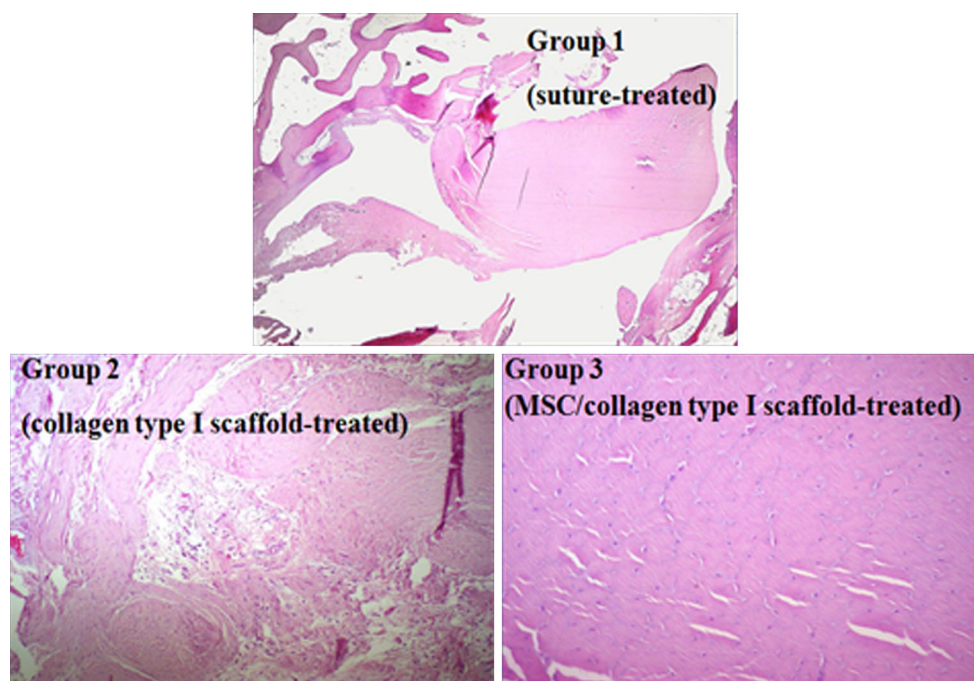


Fig. 4 Qualitative histological analysis after chondral defects intervention. Cartilage histological analysis 12 weeks after interventions. Data shown are representative of 4 sections ($\times 20$) per animal (6–10)

Discussion

The most relevant finding of the present controlled laboratory study was that MSC seeded in a collagen type I scaffold was able to contribute in the ligament regeneration on ACL ruptures in a rabbit model.

Despite the success of ACL reconstruction surgery improving clinical stability, it has not been able to modify consistently the onset of osteoarthritis [30]. This fact, coupled with other problems such as donor site morbidity and loss of proprioceptive fibres, have prompted research in other directions like tissue engineering.

In the past, several studies have shown a decrease in growth factors, cellularity and expression of molecules in ACL repair process, when compared with other ligaments [1, 17, 21, 25, 28]. However, when attempting to improve the biological potential with cytokines and/or growth factors alone, no healing has been obtained [23]. However, their uses as an adjunct to surgery have been evaluated. Darabos et al. [7, 8] observed that in patients with ACL rupture, pro-inflammatory cytokines such as IL-1 β in the synovial fluid was increased, which could affect the bone tunnel remodelling. Moreover, the use of autologous conditioned serum that contains antiinflammatory cytokines, decreased bone tunnel widening when used as adjuvant of an ACL reconstructive surgery.

As it has been suggested previously by Murray et al. [24], the most important issue to achieve healing of a ligament is the union of the ends through a scaffold, since

inside joint does not occur fibrin clot formation [27]. In our results, it seems not enough the single addition of the scaffold. This agrees with the results obtained by Murray [12, 24] in that the addition of platelet factors improves the tensile strength of the repair over the scaffold alone.

Recent literature in large animals has shown successful result of biostimulated ACL repair with collagen scaffold and platelet-rich plasma [16, 29]. More than that, those studies have shown results comparable to those of ACL reconstruction, showing the promising future that repair could have in clinical field. Another recent study has done the same with extracellular matrix scaffold, with promising results [11]. Moreover, the use of scaffolds associated with MSC has showed a ACL regeneration with a tissue with structural and biomechanical properties similar to the native ACL [10, 18]. The results of these studies have made mesenchymal cells an attractive candidate for the treatment of complete ACL injuries. While in this study, only 2 of 6 rabbits in the collagen scaffold/MSCs group achieved macroscopic and histological continuity, our study shows a tendency to heal, not seen among other groups without scaffold or MSCs.

This study has several limitations. No methods were used with bone anchor fixation [22, 29], and animals were able to move on demand inside the cage, which may have compromised healing. The use of a small animal model hinders the reproducibility of the repair and makes difficult accurate placement of scaffold. Studies are needed in larger animals for better approximation of ends, addition of

fixation methods to promote healing and incorporation of scaffolds with increased strength [2, 9, 18]. While in most publications, ACL repair in animals has used PRP as an adjunct to the scaffold, no comparative studies suggest that PRP is superior to other biological stimulators. Finally, from a methodological point of view, it seems important to develop an injury model that is capable of reproducing the ACL injury pattern in a knee trauma, as well as biomechanical analysis of the tissue regenerated, as the latter can significantly affect postoperative outcomes in terms of knee stability.

Mesenchymal stem cells seeded in a collagen type I scaffold appear to have a beneficial role on the treatment of ACL ruptures. More studies in large animal models and clinical trials are necessary to evaluate the real impact of this therapy.

Conclusion

The addition of MSC embedded in type I collagen scaffold with ACL suture may contribute to the healing of ACL in a small animal model.

Conflict of interest The authors declare that they have no conflict of interest.

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