

# Enhancement of Anterior cruciate ligament injury repairing using connective tissue growth factor in a rabbit model

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**Abstract:** The study was to evaluate the contribution of connective tissue growth factor (CTGF) to the regeneration of the torn anterior cruciate ligament (ACL) in a rabbit. ACL transection surgeries were performed on both knees of male New Zealand rabbits. Then injury reparation was done as follows: 0.5ml fibrin glue (FG) alone (FG-treated group, n=24 knees) and 0.5ml FG dissolve with 15ng CTGF (CTGF/FG-treated group, n=24 knees). At 2 or 6 weeks after surgery, the ACLs were characterized histologically (n=6 knees) and biomechanically (n=6 knees). The healing effect of the CTGF/FG-treated group was obviously better than that of the FG-treated group, with an increased amount of collagen fibers and fibroblasts in the ligament tissue. After 2 or 6 weeks of healing, CTGF/FG-treated group exhibited significantly higher maximum loads of  $8.50\pm 0.58\text{N}$  and  $16.35\pm 1.16\text{N}$ , compared with the control group ( $7.52\pm 0.80\text{N}$  and  $13.60\pm 1.35\text{N}$ ). And the stiffness of CTGF/FG-treated group at 2 or 6 weeks post-intervention ( $5.59\pm 1.24\text{N/mm}$  and  $11.64\pm 2.21\text{N/mm}$ ) was remarkably higher than that the control group ( $3.74\pm 0.89\text{N/mm}$  and  $6.83\pm 2.51\text{N/mm}$ ). CTGF could serve as a potentially attractive tool for improving ACL injury treatment by promoting the regeneration of related cells.

**Keywords:** Anterior cruciate ligament injury, connective tissue growth factor, histological observations, biomechanical outcomes.

## INTRODUCTION

Anterior cruciate ligament (ACL) injury is a clinically common arthropathy, which often strikes the young players. The incidence of ACL disruption is estimated at 0.81 per thousand (Frobell *et al.*, 2007). And nowadays, the improved living standards have spurred people to participate in sporting events with greater enthusiasm, leading the morbidity of ACL injury increased year by year. Different from the other ligaments and tendons, ACL's self-healing ability is quite limited, due to the lack of cytokines, growth factors and tissue repair cells (Murray *et al.*, 2000). What's more, ACL injury often lead to poor stability and reinjury of knee joint, which can result in secondary meniscus injury and osteoarthritis (Hayami *et al.*, 2006). Therefore, conservative treatment and surgical intervention of knee anterior cruciate ligament are often necessary for the restoration of knee function and prevention of the development of early osteoarthritis. However, curative effects seem not optimistic because of the inefficiency of the long-term, donor site morbidity and pathogen transfer (Crawford *et al.*, 2005; Mastrokalos *et al.*, 2005). Good strategic news is that tissue engineering which aimed to "replicate tissue and organ", provides another innovative choice for the repair and regeneration of tissue defect repairation (Nukavarapu *et al.*, 2008; Digiovanni, *et al.*, 2012). Replacing natural tissue with appropriate cells,

biocompatible scaffolds and growth factors, tissue engineering is able to treat ACL injuries without many adverse side effects (Figueroa *et al.*, 2014; McCarty *et al.*, 2012; Yates *et al.*, 2012).

Connective tissue growth factor (CTGF) is a member of the C-terminal cysteine-rich protein (CCN) family (Wang *et al.*, 2009; Leask A and Abraham DJ, 2006; Perbal B, 2001), and has been shown to be associated with several biological functions such as fibrosis, angiogenesis, cell growth regulation and tissue modeling (Friedrichsen *et al.*, 2003; Bradham *et al.*, 1991). God *et al.* observed that CTGF over-expressed in injured cartilage, and thought it was a one of the key growth factors which regulated the growth, proliferation and differentiation of cartilage cells (Goel *et al.*, 2003). And mice of CTGF expression defection died soon after birth because of the abnormal proliferation of cartilage cells and cartilage matrix synthesis disorder (Ivkovic *et al.*, 2003). A research reported that CTGF could enhance the repair of meniscal tearing damage in the avascular zone by significantly promoting extracellular matrix and heightening the expression of VEGF activity (Wei *et al.*, 2013). However, whether CTGF is helpful in the repair of ACL injury has remained undetermined. Therefore, in this study, ACL injury rabbit model was established to explore the effect and possible mechanism of CTGF in the treatment of ACL injury.

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## MATERIALS AND METHODS

### *Animals and Reagent*

New Zealand male rabbits (3 months old, 3.0-3.5 kg) were kindly provided by Shanghai Slac laboratory animal Co. Ltd. (Shanghai, China). The rabbits were placed in individual cages at the temperature of 20-26°C and the humidity of 40-70%, with a light/dark cycle of 12h/12h and free access to a standard diet and water. All animal work was approved by Animal Experimental Ethics Committee of the third people's Hospital of Qingdao.

CTGF (HPLC $\geq$ 98%) and FG used in the study were respectively purchased from Shanghai Kanglang Biotech CO. LTD and Shanghai Pine &Power Biotech, CO. LTD (Shanghai, China), and stored away from light at 4°C.

### *Animal Groups*

The healing effect of CTGF in the knee joints was studied using the rabbit ACL injury model. New Zealand male rabbits (N=24 rabbits, n=48 knees) of 3-months age underwent ACL injury surgeries on both sides of their hind limb. The knees were randomly divided into two groups (n=24 per group): FG-treated group and CTGF/FG-treated group. At 2 or 6 weeks after surgery, six rabbits of each group were killed directly through air embolism, and the ACLs were dissected and analyzed.

### *ACL defect interventions*

Undergoing a 7-day adaptation and an intraligamentary injection using penicillin, all animals were performed with the partial ACL injury surgeries. After the ear-vein injection anesthetization with 3% pentobarbital sodium (30mg/1000 g.bw), rabbits were holden on the operating table. The patella was displaced laterally and a ~3cm long incision inside of the patellar ligament was performed through the skin and joint capsule. The subpatellar fat pad was displaced and ACL exposed. For partial ACL injury surgeries, ACL were created sharp transections in the middle half of each knee, and the other half was still connected with the femur and tibia. According the experiment design, 0.5ml FG was used in the femoral bone tunnel of the FG-treated group. In the CTGF/FG-treated group, the same procedure was carried out, but before that, 15ng CTGF was dissolved into the FG. Then, after stanching bleeding and rinsing with saline for the joint capsule, the arthrotomies were performed through closure stratification with a deep 4-0 suture and skin 3-0 suture.

After surgery, rabbits were administered with penicillin (80000 IU) by intraperitonea injection for three days to prevent the infection of knee-joint, and stitches were removed seven days post treatment. Animals were allowed to walk freely with full weight bearing and without immobilization, and were observed for the condition of spirits, health, feeding.

### *Histological analysis*

At 2 or 6 weeks after surgery, six knees of each group were used for histological examination. Centered on the femoral tunnel, bone blocks of 16 mm in diameter were sectioned from the knees joint specimens. Then, the tissues were fixed in 10% neutral-buffered formalin and decalcified in 10% formic acid and formaldehyde for almost a month. Processed routinely by embedding in paraffin, 10  $\mu$ m thickness of sagittal cross-sections were cut through the tissue, which were then mounted on glass slides and stained with hematoxylin and eosin, respectively. A light microscopy BX51 (Olympus, Japan) was used to examine the collagenous permutations and central/peripheral blood vessels.

### *Biomechanical test*

The biomechanical testing was performed in constant environment with the temperature and humidity respectively keeping at 22°C and 70%. At 2 or 6 weeks post treatment, the rest of knees joint specimens (n=6 per group) were sectioned with the tendon grafts, posterior cruciate ligament (PCL) complete, and with all soft tissues external to the joint capsule removed. The specimens were detected on the instrument of biomechanics (CSS-44020), and the joints were wrapped in towels saturated with physiologic saline to prevent dehydration.

## STATISTIC ANALYSIS

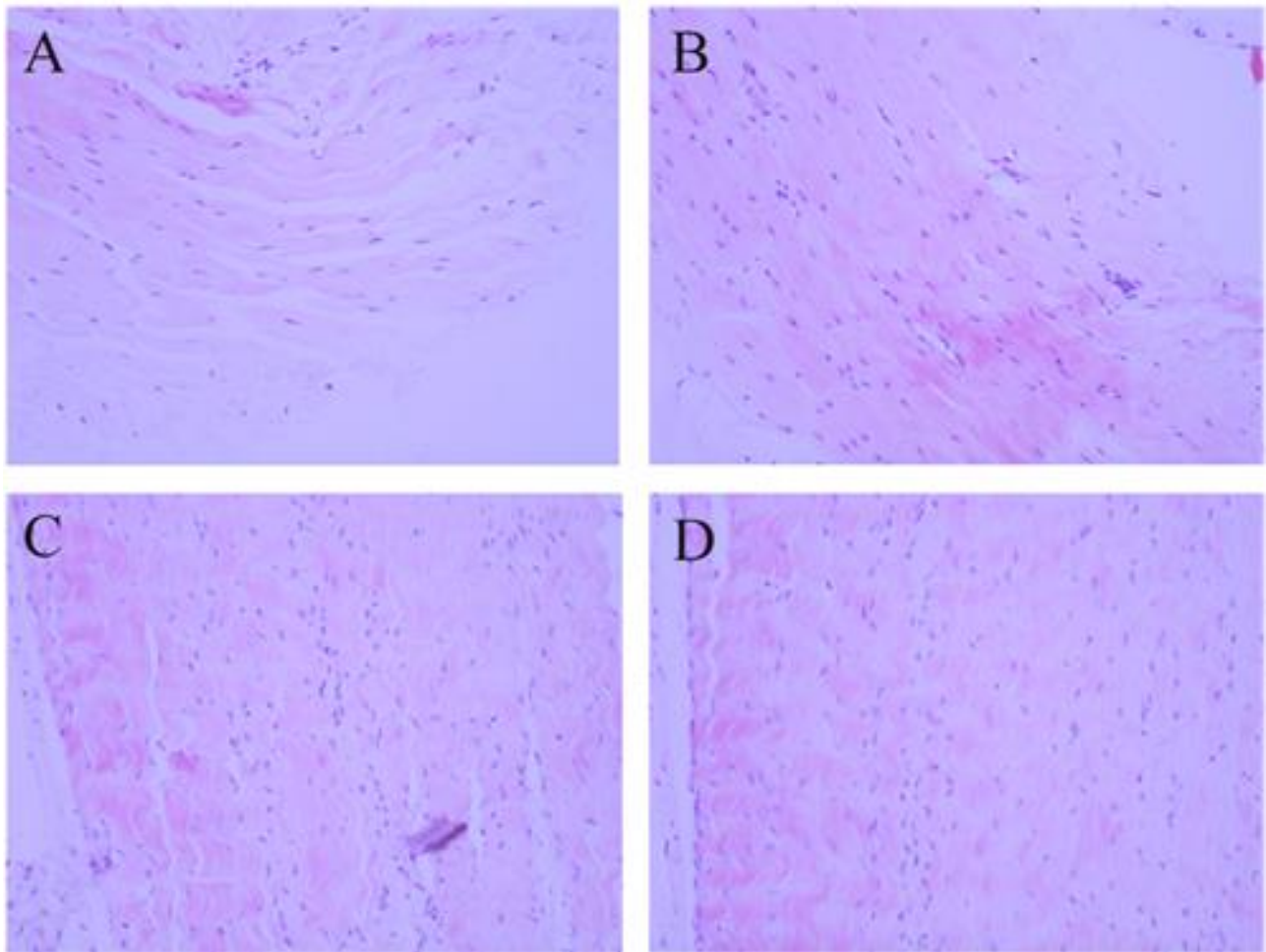
Data statistic analysis was performed by SPSS 16.0. Biomechanics changes between the two groups at the same time point were compared by Student's *t* test, and  $p < 0.01$  was considered statistically significant.

## RESULTS

### *Histological analysis*

Haematoxylin and eosin staining was used to evaluate the microscopic characteristics of the regenerated ligaments in FG-treated group and CTGF/FG-treated group. The morphological results of the two study groups at 2 weeks after surgery were shown in fig. 1 (A, B). According to the results, in the CTGF/FG-treated group, the ligament healing began with the formation of numerous disorganized fibroblast-like cells. As expected, there was no obvious healing tendency for FG-treated group, although loose fibrovascular tissue and a few of fibrocartilage-like cells were observed.

The histological changes of the regenerated ligaments at 6 weeks were shown in fig. 1 (C, D). We found that the ligament interface of the CTGF/FG-treated group became indistinct, with a large amount of aligned collagenous fibers and fibroblasts distributing around. While in the FG-treated group, the ligament began to heal indirectly and loosely. Fibroblast-like cells and a few of



**Fig. 1:** Haematoxylin and eosin (H & E) staining of the ligament tissue in rabbits. (A) Pathological changes of FG group at 2-week post surgery; (B) Pathological changes of CTGF/FG group at 2-week post surgery; (C) Pathological changes of FG group at 6-week post surgery; (D) Pathological changes of CTGF/FG group at 6-week post surgery

disorganized collagenous fibers were also found increased in the ligament interface.

#### **Biomechanical outcomes**

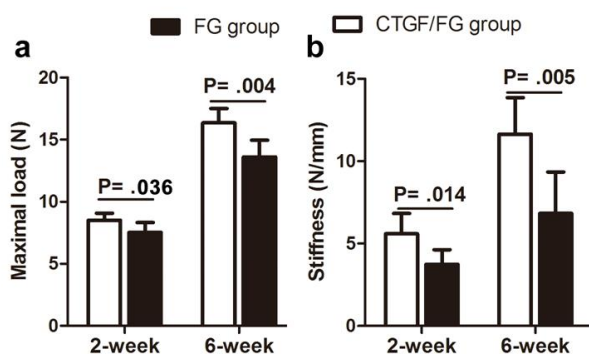
The maximum load and stiffness of specimens at the two time points were calculated to represent the biomechanical properties. As demonstrated in fig. 2 (a,b), after 2 weeks of healing, the CTGF/FG-treated group had a significantly higher maximum load ( $8.50 \pm 0.58\text{N}$  vs  $7.52 \pm 0.80\text{N}$ ;  $p=0.036$ ) and stiffness ( $5.59 \pm 1.24\text{N/mm}$  vs  $3.74 \pm 0.89\text{N/mm}$ ;  $p=0.014$ ) than the G-treated group. When coming at 6 weeks post surgery, as expected, the CTGF/FG-treated group also had a greater maximum load ( $16.35 \pm 1.16\text{N}$  vs  $13.60 \pm 1.35\text{N}$ ;  $p=0.004$ ) and stiffness ( $11.64 \pm 2.21\text{N/mm}$  vs  $6.83 \pm 2.51\text{N/mm}$ ;  $p=0.005$ ) compared with the G-treated group fig. 2(a,b). These findings demonstrated that, at the same time point, the ligament traction and self-healing strength of the CTGF/FG-treated group were always stronger than those of the G-treated group.

#### **DISCUSSION**

Despite the achievement of ACL surgical interventions in the last decades, there still remain plenty of controversies about the loss of proprioceptive fibers, inefficiency of the long-term and donor site morbidity (Crawford *et al.*, 2005; Mastrokalos *et al.*, 2005; von Porat *et al.*, 2004). Thankfully, bone tissue engineering emerges at the right moment and brings new hopes for ACL injury treatment.

Recently, several studies have shown that growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) (Vavken *et al.*, 2010, Schwarting *et al.*, 2015) were involved in the ACL repair process. In fact, a large number of experiments approved that the healing performance of cytokines and/or growth factors, in an ACL reconstructive surgery, used as the adjuvant was much better than that used independently (Darabos *et al.*,

2009; 2011; Murray *et al.*, 2009). The study (Dong *et al.*, 2012) reconstructed the ACL in New Zealand white rabbits using the gastrocnemius tendons wrapped by bMSCs+Lv-BMP-2 virus, and observed that bMSCs+Lv-BMP-2 group exhibited better biomechanical and histological properties on tendon-bone healing, compared with the bMSCs+Lv-Control group. It also (Cheng *et al.*, 2009) found that FGF-2 and TGF- $\beta$ 1 enhanced the proliferation of human ligament-derived stem cells (LSCs), and increased the production of collagenous and noncollagenous extra cellular matrix protein. However, cytokines used in the current study are not so practical as for the short half-life, high cost and ambiguity of efficacy and safety. Therefore, development for more ideal growth factors of treating ACL injury is imperative.



**Fig. 2:** Mechanical examination of graft-bone healing in a rabbit model at various time points after surgery. (a) Maximal load; (b) Stiffness.

CTGF has a wealth of sources, expressing widely in a variety of tissues and organs. Besides, it was shown to be involved in the repair process of meniscal tearing damage and cartilage defect. As a popular scaffold material in the tissue engineering, FG has been successfully transplanted in vitro reconstruction in the field of chondropathy, dermatology and cardiology, because of the good histocompatibility, biodegradability, plasticity and superior mechanical properties (Pankajakshan *et al.*, 2009). In the present study, CTGF combined with FG were used to evaluate the effect on ACL injury treatment. Rabbit was chosen to establish the ACL injury model and do related research since the anatomical features and biomechanical characteristics of ACL in rabbit is very similar to that of the human. The most relevant finding of the present controlled laboratory study was that CTGF combined with FG contributed to the healing process of the ACL injury, biomechanically and histologically.

Tendon-bone healing after ACL injury interventions generally should undergo a series of course, among which vascular regeneration is particularly important (Ritchie *et al.*, 1996). It determines the progress of tendon-bone healing and further influences the stability of ACL reconstruction. The lag of vascular regeneration can slow

down tissue recovery and lead to deterioration and microstructure damage of graft. Several cytokines had been reported to play revascularization promotion effect, in the tendon graft reconstruction of ACL, by combining directly with vascular endothelial cell receptor or by combining with auxiliary cell receptor in paracrine way (Futamura *et al.*, 1998; Rahim *et al.*, 2014). CTGF was first found (Bradham *et al.*, 1991) as a kind of novel mitogen in the human vascular endothelial cells, indicating CTGF may participate in angiogenesis. Using knockout mice, it (Hall-Glenn *et al.*, 2012) proved CTGF was an essential mediator of vascular remodeling by regulating endothelial-pericyte interactions, and verified its contribution on inducing the production of endothelial basement membrane components. Another study also revealed CTGF's role as one of mediators in angiogenesis via the VEGF-A/CTGF/formyl peptide receptor-like1 (FPRL1) axis (Lee *et al.*, 2015). And, angiogenesis and metastasis can be effectively inhibited by down-regulating CTGF mediated PI3K/AKT/ERK signaling (Chang *et al.*, 2016). Findings of the present study were in accordance with the previous study: more peripheral vessels were formed in the CTGF treatment group compared with the control group. It (Miyashita *et al.*, 2016) observed the importance of CTGF in rheumatoid arthritis(RA), and found that CTGF treatment of human umbilical vein endothelial cells (HUVECs) could accelerate the angiogenesis of RA synoviocytes. This suggested that CTGF may exhibit healing effect by promoting the vascular regeneration.

Cells proliferation and regeneration also plays a key role in the healing process post ACL injury. As an important structure in the knee-joint, ACL is mainly organized by fibroblasts, collagen fibers and extra cellular matrix. CTGF, as a matricellular protein, is known to stimulate fibroblast proliferation, promote collagen deposition and up-regulate extra cellular matrix production (Li *et al.*, 2016; Yuda *et al.*, 2015). Yuda *et al* believed CTGF was potentially used as the therapeutic agent for periodontal ligament (PDL) regeneration, because it promoted the growth and migration of human PDL stem cells and up-regulated the expression of type I collagen and fibronectin. Our result showed that CTGF/FG-treated group promoted the formation of cartilage cells, collagen fibers and mesenchymal cells between the tendon and bone, indicating the healing effect of CTGF on ACL injury may depend on its ability to promote the regeneration of related cells.

At the same time, in the present study, biomechanical indicators such as the maximum load and stiffness were also significantly increased by CTGF treatment. This result agreed with Zhu ZQ's observation that CTGF injection not only promoted the early bone tendon junction healing, but also increased the biomechanical strength of bone tendon junction (Zhu *et al.*, 2011). In



return, mechanical stretch was reported to play a vital effect on maintaining cell-specific features by induction of CTGF (Chaqour and Goppelt-Struebe, 2006). We can infer that CTGF may play a key role in strengthening ACL's resistance to mechanical stretch or pressure, which will be of significance for the treatment of ACL injury.

Considering the above three aspects of CTGF in the healing process of the ACL injury, we assumed that they were interacted with each other. By promoting the angiogenesis, CTGF can improve nutrition and metabolism of the surrounding condition, which will lead to the cytological changes and then biological performances enhancement of the ACL tissues.

## CONCLUSION

In summary, the present study demonstrated that CTGF combined with FG has a beneficial effect on ACL injury treatment in a rabbit model, biomechanically and histologically.

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