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CLINIQUE ET BIOLOGIQUE
Transfusion Clinique et Biologique 26 (2019) 3–9

Original article

Intra-articular effects of combined xenogenous serum rich in growth factors (SRGF) and vitamin C on histopathology grading and staging of osteoarthritis in rat model

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Available online 25 August 2018

Abstract

Objective. – Osteoarthritis (OA) is one of the most common degenerative diseases especially in the knee joint. The definitive method for the treatment of this disease is not known. In recent years, use of platelet-derived products has been considered as a new therapeutic approach because of its low cost, easy to use, and minimum side effects. Serum rich of growth factors (SRGF) is one of the biological compounds used to healing and regeneration. Its effects may improve in combination with antioxidants such as vitamin C. This vitamin increases the synthesis of proteoglycans by chondrocytes. The present study investigated effect of xenogenous SRGF in combination with vitamin C on the monosodium iodoacetate-induction osteoarthritis in rats.

Methods. – Animals were randomly categorized into three groups including OA, SRGF, and vitamin C + SRGF. Treatments were performed with 3 time intra-articular injection in weekly intervals. Knee samples were taken after two weeks of the last treatment for histopathologic investigations.

Results. – In the OA group, surface fibrillation and irregularity, multiple clefts, loss of chondrocytes, proteoglycan depletion with Toluidine blue staining were detected. In the treated group with SRGF/vitamin C, the severity of degenerative lesions was decreased. Chondrocytes had proliferated and matrix proteoglycan increased in compared to the SRGF and OA groups. Also, osteoarthritis stage was markedly reduced in this group rather than two other groups.

Conclusion. – The results of this study show the synergic effect of vitamin C and growth factors on accelerating articular repair.

Keywords: Osteoarthritis; SRGF; Monosodium iodoacetate; Vitamin C; Histopathology

Résumé

Objectifs. – L'arthrose (OA) est l'une des maladies dégénératives les plus courantes, en particulier dans l'articulation du genou. La méthode définitive pour le traitement de cette maladie n'est pas connue. Au cours des dernières années, l'utilisation de produits dérivés des plaquettes a été considérée comme une nouvelle approche thérapeutique en raison de son faible coût, sa facilité d'utilisation et ses effets secondaires minimaux. Le sérum riche en facteurs de croissance (SRGF) est l'un des composés biologiques utilisés pour la cicatrisation et la régénération. Ses effets peuvent s'améliorer en combinaison avec des antioxydants tels que la vitamine C. Cette vitamine augmente la synthèse des protéoglycanes par les chondrocytes. La présente étude a étudié l'effet de SRGF xénogène en combinaison avec de la vitamine C sur l'arthrose monosodique d'induction de l'iodoacétate chez les rats.

Méthodes. – Les animaux ont été classés au hasard en trois groupes, y compris l'arthrose, le SRGF et la vitamine C + SRGF. Les traitements ont été réalisés avec une injection intra-articulaire 3 fois par intervalles hebdomadaires. Des échantillons de genou ont été prélevés après deux semaines du dernier traitement pour des examens histopathologiques.

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https://doi.org/10.1016/j.tracli.2018.08.156
1246-7820/

Résultats. – Dans le groupe OA, fibrillation de surface et irrégularité, fentes multiples, perte de chondrocytes, appauvrissement en protéoglycanes avec coloration au bleu de toluidine ont été détectés. Dans le groupe traité avec SRGF/vitamine C, la gravité des lésions dégénératives a diminué. Les chondrocytes avaient proliféré et le protéoglycane matriciel avait augmenté par rapport aux groupes SRGF et OA. De plus, le stade de l'arthrose était nettement réduit dans ce groupe plutôt que dans deux autres groupes.
Conclusion. – Les résultats de cette étude montrent l'effet synergique de la vitamine C et des facteurs de croissance sur l'accélération de la réparation articulaire.

Mots clés : Arthrose ; SRGF ; Iodoacétate monosodique ; Vitamine C ; Histopathologie

1. Introduction

Osteoarthritis, also named degenerative joint disease, is one of the most common skeletal diseases that cause failure of joint motion, severe pain and physical disability in patients. Cartilage destruction results from a number risk factors including aging, obesity, genetic predisposition and mechanical stress [1], reactive oxygen [2] and nutritional status [3]. These risk factors stimulate biosynthesis and degradation of the matrix components. Oxidative stresses contribute in the pathogenesis of OA and cause extensive structural damage, cell death and inflammation [4]. Metabolic reactions in chondrocytes and synoviocytes produce free radicals. Vitamin C (ascorbic acid), vitamin E, thiols (glutathione) and plant polyphenols neutralize free radicals in joints and decrease the oxidative stress related to arthritis progression [5]. Vitamin C (ascorbic acid) is an important water-soluble antioxidant that acts as a powerful scavenger for free radicals. Vitamin C is needed for the growth and repair of tissues [6]. This vitamin stimulates synthesis of type II collagen, the most abundant protein in cartilage, and also increases articular proteoglycan synthesis [7], aggrecan and α-prolyl 4-hydroxylase [8].

Articular cartilage is a specialized avascular tissue that contains embedded chondrocytes in a matrix prominently made of type II collagen and glycosaminoglycans such as hyaluronic acid (HA) and chondroitin sulfate. Articular cartilage has very confined ability for repair because of lack of vessels and nerve supply. Also, unlike the most tissues, the inflammatory processes are not available for its repair, and chondrocytes cannot migrate from an intact healthy site to the site of injury [9]. In recent years, platelet products have been used as a source of growth factors for treatment of damaged tissues in both human and veterinary medicine. Platelets are a potential source of autologous growth factors and proteins involved in tissue regeneration. Provided products from platelets such as plasma rich in growth factors (PRGF) and Serum rich in growth factors (SRGF) are proposed as an effective and safe agent for the healing of different lesions in soft tissues, ligaments, bone and cartilage [10,11]. SRGF is a small volume of enriched serum with released growth factors from the platelets [12]. Growth factors influence chemotaxis, differentiation, proliferation and synthetic activity of different cells such as chondrocytes that produce cartilage [13]. The combination of biomaterials and micronutrients may amplify the effect of each other on tissue regeneration. The present study evaluates possible positive effect of SRGF and vitamin C in decreasing of histopathologic progression of knee osteoarthritis in rat.

2. Materials and methods

2.1. Xenogenous serum rich in growth factors (SRGF) preparation

A total of 10 ml blood from peripheral blood was collected in sodium citrate tubes from the healthy adult human. Then, 2 centrifugations (Sigma 2-16p) including the first at 200 g for 10 minutes to separate erythrocytes and the second at 500 g for 10 minutes to concentrate platelets (PRP) to 1 ml were produced. Ten percent calcium chloride 25 mM was added to the PRP unit and the mixture was incubated at room temperature for 60 min, yielding activated PRP. The activated PRP was centrifuged at 2000 g for 15 min. The supernatants, containing the bioactive platelet factors, were sterilized with filter (0.22 μm) and stored at −80 °C until the examination time be started.

2.2. Induction of osteoarthritis

For induction of osteoarthritis (OA), rats were anesthetized with intramuscular injection of xylazine 2% (5 mg/kg) and ketamine 10% (90 mg/kg) (Alfasan International Group of Companies, Holland). Then, the animals were placed on a heat pad set until deep anesthesia had been achieved. The right knees of animals were shaved and cleaned with povidone-iodine. MIA was prepared freshly before the using. For each knee, 5 mg of monosodium iodoacetate (MIA; Sigma, St. Louis, MO 63103, USA; cat #I2512) was dissolved in the normal saline by vortexing. After shaving and disinfection, a volume of 50 μl of xenogenous SRGF was injected unilaterally into the articular space of the right femorotibial joint through the infrapatellar ligament by a 27-gauge insulin syringe [14]. The left contralateral knee was considered as sham-control group.

2.3. Experimental animals

In the present study, 18 adult male Wistar rats (200-250 g) were used. The animals were housed under a 12-h light-dark cycle with access to the standard food and fresh water *ad libitum*. Animals were randomly divided into 3 groups ($n = 6$ rats in each

group as follow) and all injections in sham-control and treatment groups were performed 3 times at weekly interval:

- group 1 (OA control). Right knee: OA group without treatment + left knee: sham-control of vitamin C (1000 μg);
- group 2 (SRGF treatment). Right knee: OA + treated with SRGF (50 μl) + left knee: sham-control of SRGF (50 μl);
- group 3 (SRGF/vit C treatment). Right knee: OA + treated with combination of SRGF (50 μl) and vitamin C (1000 μg) + left knee: sham-control of combined SRGF with vitamin C (1000 μg).

2.4. Histopathologic assessments

Animals from each group were sacrificed under a high dose of anesthetic ether 2 weeks after last injection of treatments. Femorotibial joints were dissected by removing ligaments and tendons with sharp razor. Samples of the joints were taken and fixed in the 10% neutral buffered formalin for 1 week. The samples were decalcified with formic acid 10%. The calcification took a long time about 3 weeks. After end of decalcification, the samples were cut as frontal plane. Both parts of the joints were embedded in the same paraffin block. Tissue sections in 5 μm thickness were stained with hematoxylin-eosin and toluidine blue for evaluation of degenerative changes and proteoglycan contents. Three sections were cut from each knee with 200 μm step. Histopathologic lesions were graded in accordance with scoring system of Mankin et al. [15]. The scoring of OA histopathlogic stage assessed according to Ostergaard et al. [16]. Grading and scoring of lesions was done in blind counting. The stage is considered as the sum of the total involved surface length with OA to the total surface length (Table 1).

2.5. Statistical analysis

The obtained results were presented as the means ± standard error (SE). Non-parametric analyses with Mann–Whitney test was used to compare of articular scores. P-values ≤ 0.05 were considered as significant difference.

Table 1
Osteoarthritic cartilage histopathology stage assessment.

Stage	% involvement (surface, area, volume)
Stage 0	No OA activity seen
Stage 1	< 10%
Stage 2	10–25%
Stage 3	25–50%
Stage 4	> 50%
Stage	Extent of joint involvement

3. Results

In the all sham-control groups, the structural integrity was preserved and no abnormal microscopic change was found in the articular cartilage. The meniscus was present between femoral condyles and tibia plateau. The articular surfaces were smooth without irregularity and fissures. Cellularity was normal and chondrocytes distributed in their normal orientations. The chondrocytes within the lacunae had light eosinophilic cytoplasm and basophilic nuclei. A clear cartilage capsule surrounded the chondrocytes. Borderline between calcified and non-calcified zone, named tidemark, was intact. Staining with toluidine blue method showed dark blue color of cartilage (Fig. 1). Histopathological grading in according to the Mankin system was in lowest score (0) that shows good quality of cartilage structure. Any inflammatory reaction was observed in the synovium and soft tissues around the knee.

Five weeks after OA induction, the most knee cartilage showed advanced stages of degenerative changes. The joint space between the tibia and femur heads decreased and in some cases was completely disappeared. The articular surface was irregular with multifocal erosions, fibrillation, deep fissures and cysts formation. Chondrocytes were necrotic and lost their cellular details including nucleus and lacunae. There were multiple clefts in the cartilage, which continued until deeper layers of the cartilage in some areas. Disorganization of articular structure was visible in some samples. Hematopoietic tissues declined in the subchondral bone and connective tissue was replaced. Depletion of matrix proteoglycan was demonstrated by toluidine blue staining. No staining was present in completely degraded cartilage. Injured parts of the cartilage were healed with

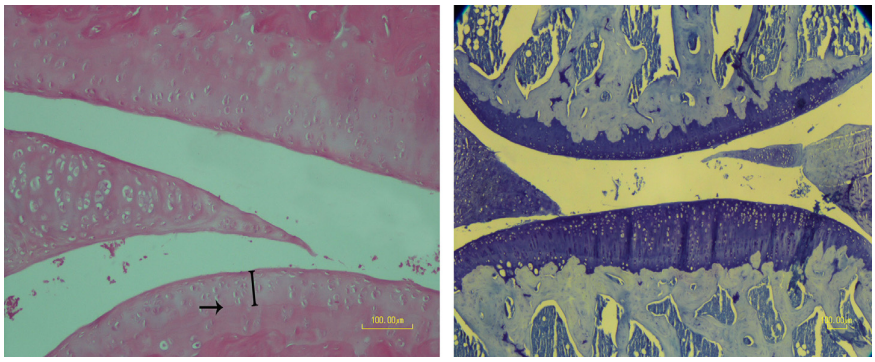

Fig. 1. The sham-control of SRGF joint: a: the smooth articular surface without fissures, intact tidemark (arrow) between noncalcify (I) and calcify zone (HE staining); b: extracellular matrix stained intensively with toluidine blue method.

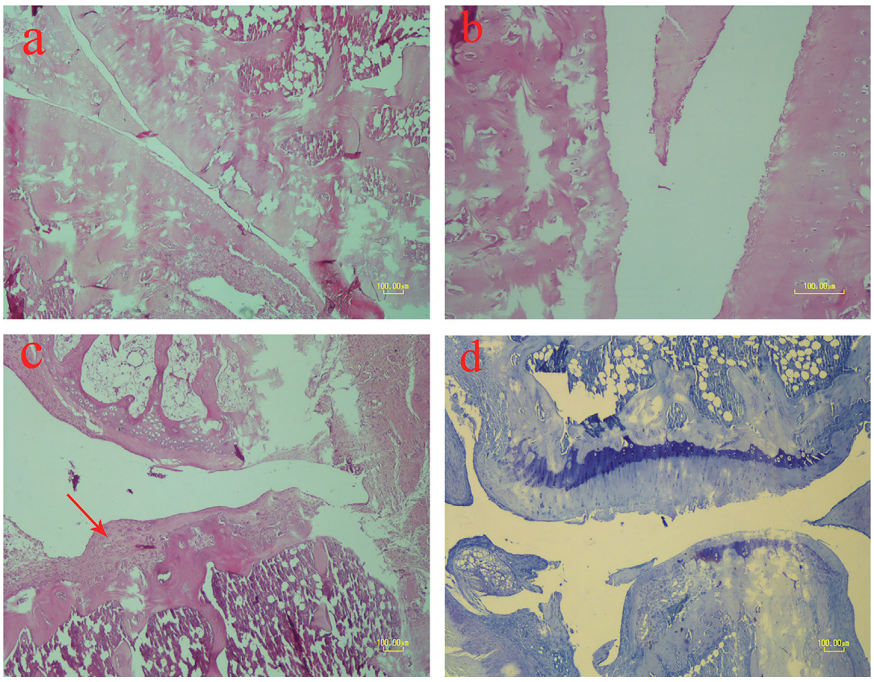

Fig. 2. Osteoarthritis control group: a: decrease articular space and multiple cysts and clefts in the cartilage; b: thinning of cartilage, surface irregularity, presence of several erosions and disappear of tidemark on the left side of joints; c: filling of damaged cartilage with fibrovascular tissues (arrow); d: multiple cracks affected cartilage surface, complete loss of cartilage staining with toluidine blue in damaged areas. Degenerative lesions are in stage 4.

fibrovascular tissue (Fig. 2). The Mankin grading of lesion obtained (10.71 ± 0.918). Extension of lesions on the articular surface (staging) was higher in comparison with other two treatment groups (Mean = 3.28 ± 0.359, more than 50% affected). Soft tissues around the knee joints and synovial membrane exhibited chronic inflammation and increasing fibroplasia (Figs. 3 and 4).

In the treatment group with SRGF/vitamin C, histopathologic grading of lesions showed significant reduction (Mean = $5.83 \pm 0.98 \pm 5$, $P \leq 0.005$) of degenerative changes in knee joint in comparison with OA group and nonsignificant with SRGF group ($P \geq 0.05$) after 5 weeks. Articular erosions, clefts and irregularity decreased considerably and the cartilage surface became smoother. Evidences of healing and regeneration of cartilage was obvious. Chondrocytes proliferated diffusely and also as clusters inside the lacunae. Synthesis of cartilage extracellular matrix was demonstrated with increasing in coloration with toluidine blue especially in proliferated area (Fig. 5). In histopathologic staging, development of degenerative lesions on the cartilage surface of knee joint was appeared as scattered foci and in lower stage (Mean = 1.83 ± 0.040, 10–25%) than OA ($P \leq 0.005$) and SRGF ($P \geq 0.05$) groups (data are shown in Figs. 3 and 4). Severity of chronic inflammation was declined in

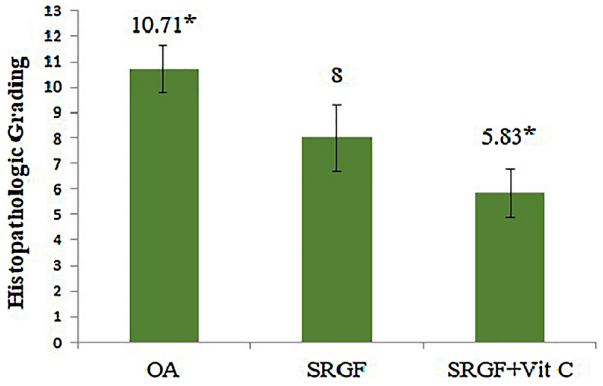

Fig. 3. Effect of SRGF and SRGF/vitamin C on Mankin grading score of osteoarthritis lesions. Data represent mean ± SE (* in the figures shows significantly difference between SRGF + Vit C group in compared to the OA-control group, $P \leq 0.05$).

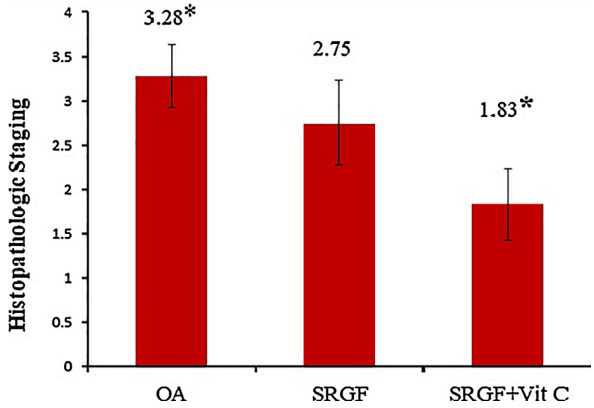

Fig. 4. Effect of SRGF and SRGF/vitamin C on staging score of osteoarthritis lesions. Data represent mean ± SE (* in the figures shows significantly difference between $P \leq 0.05$ in compared to the OA-control group).

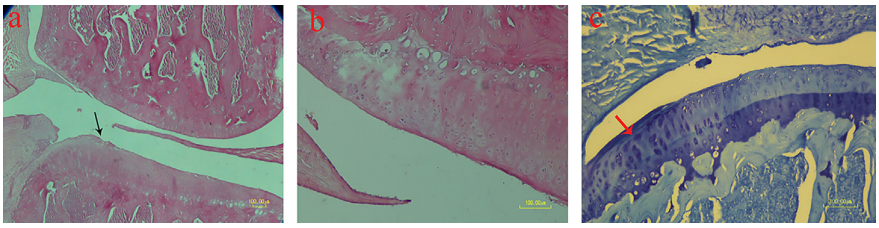

Fig. 5. SRGF/vitamin C treated joint. Considerable reduction of surface irregularity and presence of few fissures (arrow) and degenerative changes (a), proliferation of chondrocytes (b), increasing toluidine blue staining in the areas with chondrocyte proliferation (c) (arrow). The figure shows extension of degenerative lesions on the articular surface is in stage 1.

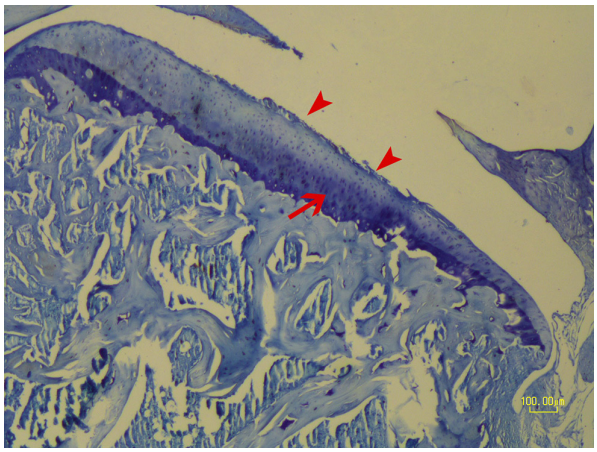

Fig. 6. SRGF treated joint. Toluidine blue staining has increased in the areas with chondrocyte proliferation (arrows). Also, articular surface shows fibrillation and irregularity (arrowheads).

the synovial membrane and around soft tissues rather than that the SRGF and OA groups.

Five weeks post-OA induction, treatment with SRGF decreased grading (Mean = 8 ± 1.29) and staging (Mean = 2.75 ± 0.478, 25–50%) of articular lesions at the joint level (Fig. 6) in compare with the OA group. The better improvement of articular lesions was observed in the SRGF/vitamin C group.

4. Discussion

Osteoarthritis (OA) is a chronic process with gradual and progressive loss of articular cartilage that imposes numerous economic impacts to affected peoples due to work-related disability, cost of long time treatment and need to specific supportive therapy [9]. Current treatments for OA are mainly aimed for decreasing symptoms of the disease and modifying pathologic processes are not considered. Hyaluronic acid, glucosamine sulfate and chondroitin sulfate have been used for relief symptoms, which may be associated with structural changes on OA, but their efficacies are doubtful [17]. In according to limited regeneration ability of cartilage, it is important to find a simple, easy available and inexpensive method that increases chondrogenic differentiation. Nowadays, researches on regenerative medicine and tissue engineering are developing. Regenerative methods can be effective for repair of soft and hard tissues such as bone and cartilage [18,19] based on release of growth factors and bioactive proteins directly at the injured sites for promoting of natural healing cascade. Growth factors and cytokines have essential roles in regulating the mechanisms and pathways that control wound healing and tissue formation following stimulation by thrombin or other materials [20]. These processes may be facilitated by the micronutrients such as (vitamin C or AA), which play as an essential cofactor for extracellular matrix components including type I collagen [21], type II collagen, the alpha subunit of prolyl 4-hydroxylase, lysyl hydroxylase [22], and aggrecan [23]. Additionally, vitamin C serves as a powerful sweeper for reactive oxygen and nitrogen species. It is obvious that a proper supply of ascorbic acid is fundamental for the normal physiology of articular cartilage [24].

In the present study, effect of xenogeneic serum rich in growth factors (SRGF) and vitamin C (1000 μg) was investigated on the femorotibial OA in rat. OA was induced by injection of monosodium iodoacetate (MIA) in the articular space. Intra-articular injection of chemical substances especially MIA is a common method due to its ability to develop rapid OA [14]. The previous researches described that MIA causes clinical, biochemical, and structural changes in the joints similar to human OA [25]. MIA disrupts glycolytic pathway via the inhibition of glyceraldehydes-3 phosphate dehydrogenase of chondrocytes that results in death of chondrocyte [26]. SRGF is a complex of platelets released growth factors (2–3-fold of peripheral blood) without fibrin. It was provided from activation of platelets by $CaCl_2$. After induction of OA, treatment was done with intra-articular injection of xenogeneic SRGF alone and also combined with vitamin C, 3 times weekly. In histopathological investigation, grading and staging of articular damages were done. Grade is an index for the severity or progression of the osteoarthritic process. Stage is based on the extent of the lesions on the joint cartilage surface. MIA led to high grade of articular destruction including surface irregularity, fibrillation, clefts, denudation and narrowing of articular space in OA group. Proteoglycans depletion of cartilage extracellular matrix (ECM) was determined by the toluidine blue staining. ECM is including collagen type II and sulfated proteoglycan that have a crucial role in regulating chondrocyte functions [27]. More than 50% of the cartilage surfaces had damaged. Treatment with SRGF, and significantly its combination with vitamin C decreased severity of cartilage degeneration, increased chondrocytes proliferation and

ECM staining by toluidine blue. Staging of degenerative changes reduced in two treatment groups but was considerable in the treated group with SRGF/vitamin C until 10–20% of surface cartilage.

Scattered studies describe effects of vitamin C on the OA. Consistent with our study, Chiu et al. [28] described vitamin C protects chondrocytes against induced-osteoarthritis through intra-articularly monosodium iodoacetate by several pathways. Treatment with vitamin C had improved these changes. They showed the effects of vitamin C did not strengthen with the increasing dosage. Dose 100 mg/kg was more efficient than 200 or 300 mg/kg. Vitamin C had prevented of OA progress through decrease of apoptosis, expression of pro-inflammatory cytokines and MMPs in addition to its antioxidative properties. Castro et al. [29] evaluated effect of vitamin C, platelet-rich-plasma (PRP) and their combination on differentiation mesenchymal stem cells (MSC) originated from of horse fat. Combining vitamin C and PRP markedly had been effective on cellular differentiation to osteogenic and chondrogenic lineages during 14 days of culture.

Tian and Li [5] investigated application of vitamin C in reducing adverse effects of anesthetics including ropivacaine, bupivacaine and lidocaine on the human chondrocytes viability. Lidocaine had more chondrotoxicity rather than bupivacaine and ropivacaine. Vitamin C effectively improved chondrocyte viability and decreased apoptosis levels. At higher doses, vitamin C had been efficient in reducing the generation of reactive oxygen species and down-regulate the expressions of caspases 3 and 9. Naskar et al. [30] investigated plasma vitamin C and its relation with severity of disease in the patients with knee osteoarthritis. Plasma level of vitamin C was significantly lower in the OA patients in compared with the control population. They suggested that treatment with vitamin C in the early stages of OA may be useful as secondary therapy to prevent the cartilage damage and destruction of the musculoskeletal tissues in osteoarthritis.

Vitamin C influences the progression of OA in animal models. In a study conducted by Schwartz et al. [31], guinea pigs were nourished with low to high doses ascorbate (150 mg/d) before surgical-induced OA in knee. More severe pathologic changes were observed in low dose than animals supplied with high-dose. Also, normal joints showed the higher cartilage retention in high-dose animals. They suggested vitamin C stimulates extra-production of collagen and have a protective effect on the severity of surgically induced knee OA in high doses of ascorbate.

In the present study, reduce injuries in the femorotibilal cartilage treated with SRGF/vitamin C were remarkable. It may be related to protective effects of vitamin C and also new collagen synthesis in the ECM cartilage. To date, only a few studies have used xenogenous platelets and their derivates for the purpose of tissue healing. We concluded that biological therapies such as growth factors have positive role in cartilage reconstruction and increase chondrogenesis and proteoglycan without any signs of inflammation or necrosis. The combination of SRGF/vitamin C can synergistically promote cartilage regeneration with a lower degree of cartilage degeneration.

Ethical approval

This article does not contain any studies with human participants. All animals received human care in compliance with the Guide for Care and use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). The study was approved by the local ethics committee of our veterinary school.

Disclosure of interest

The authors declare that they have no competing interest.

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