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Vanadate ingestion enhances the organization and collagen fibril diameters of rat healing medial collateral ligaments

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Abstract Although an injured medial collateral ligament (MCL) will naturally heal, the quality of healing tissue is inferior to the uninjured MCL tissue. Previous studies have shown promising results of sodium orthovanadate (vanadate) in enhancing the quality of rat skin wounds. This study therefore investigated whether vanadate enhances the quality of the rat healing MCL in terms of the collagen fibril organization and diameter. Six mature male Sprague–Dawley rats, with weight ranges of 475–505 g and ages of 25 weeks, were used in this study. Three rats in the experimental group received vanadate (0.2 mg/ml) in their saline drinking water (150 mM NaCl), whereas three rats in the

control group were only given saline water. Three weeks after transection, the rat MCLs were harvested for hematoxylin and eosin (H&E) staining and transmission electron microscopy. It was found that vanadate promoted organization of collagen fibrils and significantly increased the diameters of collagen fibrils by 14% in healing MCL ($P < 0.001$). These results indicate that application of vanadate may be a promising tissue engineering approach to enhance the quality of healing tissues such as injured MCLs.

Keywords Orthovanadate · MCL · Organization · Collagen fibrils

Introduction

The incidence of knee ligament injuries has increased in recent years due to the general public's increase in sports activity participation. Injury to the medial collateral ligament (MCL) of the knee is one of the most common knee ligament injuries [16]. Although injured MCLs heal without surgical intervention [22], healing MCLs remain biochemically and histologically abnormal for a long period of time [7, 8, 27]. In addition, the mechanical properties of the healing MCL are inferior to those of the normal MCL [7, 8, 25, 27]. With a rabbit MCL-gap-injury model, even 2 years after the injury, the size and distribution of collagen fibrils in the healing MCL are still far from normal; instead, the healing MCL contains both collagen type I and III and roughly 90% small

fibrils with occasional interspersions of larger collagen fibrils, and orientation of collagen fibrils is disorganized [7]. These studies indicate that treatment of an injured MCL is necessary to restore its normal histological, biochemical, and mechanical properties.

During the last 2 decades, tissue-engineering approaches, such as growth factors, gene delivery, and cell therapy have been applied in an attempt to enhance ligament healing [1, 14, 20, 24]. Growth factors, such as platelet-derived growth factor, basic fibroblast growth factor, and insulin-like growth factor type I, have been shown, with either independent usage or combined usage, to have some positive effects on mechanical properties of healing tissues [1, 14]. However, since large amounts of these growth factors have to be used, the high cost of growth factors prohibits potential clinical

treatment. Gene therapy also shows some promise for ligament healing, but it still remains in preliminary stage of study [12, 26]. Recently, application of sodium orthovanadate (a.k.a. vanadate) has been shown to be a promising tissue engineering approach to enhance tissue healing [4, 5]. The administration of vanadate into rat incision wounds improved the organization of collagen fiber bundles and increased collagen fibril diameter in the healing site. The vanadate treatment also increases the breaking strength of the skin wound by twofold [4]. Although the exact cellular and molecular mechanisms of enhancing tissue healing by vanadate are not clear, the application of vanadate to wound sites may maintain tyrosine phosphorylation, which prevents myofibroblast differentiation in granulation tissue through reducing α -SMA expression and the resulting elimination of myofibroblasts may enhance the rate of collagen fiber bundle maturation within healing tissues [18]. Therefore, the purpose of this study was to test the hypothesis that vanadate treatment enhances the organization and increases collagen fibril diameters of rat healing MCLs.

Materials and methods

Administration of vanadate to rats

Six mature male Sprague–Dawley (SD) rats, with weight ranges of 475–505 g and ages of 25 weeks, were used in this study. The protocol for experiments on the rats was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol # 0208756). These rats were divided into two groups: three in the experimental group, and three in control group. The rats in the experimental group rats were given vanadate (Sigma, St. Louis, MO, USA) in their drinking water, at a concentration of 0.2 mg/ml in 150 mM sodium chloride (NaCl). Rats in the control group received only 150 mM NaCl in their drinking water. The salted water was necessary to make the vanadate compound palatable. Unlike other animals, rats tolerate the long-term ingestion of a NaCl solution instead of water [5].

Surgery for transecting MCLs

After both groups of rats received vanadate-containing saline water and saline water only for 1 week, surgery on rats was performed as follows. Under sterile conditions and anesthesia with xylazine (7 mg/kg) and ketamine (80 mg/kg IM), a small incision (10 mm) was made in their skin on the “femoro-tibial joint” (knee joint) of the right hind limb over the site of MCL. After skin incision, the overlying connective tissue was dissected to expose the knee’s MCL. Then, a 1-mm gap in the mid-substance

was surgically created and the gap was left without suturing to reproduce a similar condition with the traumatic injury. The skin incision was closed using 5–0 Ethibond suture. The left knees of all rats remained intact. After operation, the rats were allowed cage activities and given post-operative ketoprofen (5 mg/kg) for 4 days. The rats in the experimental group continued to drink water containing vanadate with the same concentration, whereas rats in the control group drank water only containing saline (150 mM NaCl). Three weeks after surgery, the rats were sacrificed, and MCLs were harvested for H&E staining and transmission electron microscopy (TEM).

H&E staining and TEM analysis

To minimize possible biases in processing MCL samples, experienced pathologists, who were blind to the treatment protocol, performed H&E staining and TEM analysis. The H&E staining was used to evaluate gross matrix organization. After harvest, the MCLs were immediately fixed in 10% formalin, followed by embedding in paraffin, and then sectioned. Briefly, the procedure for H&E staining is as follows. The MCL sections were de-paraffinized for 10 min in xylene. After a brief wash in distilled water, the sections were stained in hematoxylin for 5 min, followed by washing with running tap water. The MCL sections were then differentiated in 1% acid alcohol for 5 min, dehydrated through 95% alcohol, and allowed to dry. For H&E staining, two cross-sectional regions for each sample were chosen and so a total of six MCL sections for both vanadate-treated and non-treated groups. The stained MCL sections were viewed and photographed at two different magnifications ($\times 10$ and $\times 20$) using a light microscope (Nikon, Model# TE-DH100W) and a digital camera (Diagnostic Instruments Inc, Fredericksburg, VA, USA; Model# 221) attached to the microscope assembly.

Harvested MCLs were also prepared for TEM analysis to examine collagen fibril organization and diameter. Briefly, the MCL specimens were immediately fixed in Karnovsky’s fixative. Then scar tissue was isolated and prepared as follows. The MCL specimens were rinsed several times with 0.1% cacodylate buffer to remove the fixative solution. Following incubation in a 1% aqueous solution of osmium tetroxide, the MCLs were dehydrated by a series of rinses in increasing concentrations of ethanol (50, 70, 95, and 100%). Next, the MCLs were treated for 1 h with a 2:1 mixture of propylene oxide and epon, followed by an additional hour in a 1:2 mixture of propylene oxide and epon. The MCLs were finally embedded in pure epon. Thin cross sections at 1 μ m thickness were obtained using an ultra microtome and stained with toluidine blue. Two cross

sections were obtained from each for each ligament sample and were examined with a transmission electron microscope (Philips, model# EM208-S) and photographed in a random pattern. For each cross-section, 3 TEM photos were taken, so a total of 18 TEM photos were obtained from vanadate-treated and control groups, respectively.

Quantification of collagen fibril diameters

Using a custom-developed image analysis software, the diameters of collagen fibrils were quantified. With Adobe Photoshop CS 8.0 each fibril on the image was manually circled with a 2-pixel wide pencil tool in order to separate the fibrils from the rest of the image. Then the image was saved in TIFF format and processed by Matlab image-processing toolkit. Thresholding, with a very low threshold, was applied to convert the image into a binary image. Using the “imclearborder” function, all the structures that are connected to the image border were cleared out. Because some irregular shaped objects were left on the image, shape index (shape index = $4 \times S / P^2$, where S and P are the area and perimeter of the object, respectively) was used as a criterion to separate the round collagen fibrils from the irregular-shaped objects. The areas of the fibrils were computed automatically and they were converted to diameters by the equation: $S = (1/4) \pi D^2$, where D and S are the diameter and the area of collagen fibril, respectively. A total of 5,225 fibrils in the experimental group and 7,096 fibrils in the control group were analyzed.

Statistical analysis

An unpaired student's t test was used to determine whether the diameter of collagen fibrils from vanadate-treated MCLs was significantly different from that of the injured but not treated MCLs. The difference was considered to be significant if P value was less than 0.05.

Results

Three weeks after surgery, the healing MCLs from rats in both vanadate-treated and non-treated groups were characterized with hyper-cellularity. This was in contrast to the normal MCL with sparse fibroblasts interspersed within collagen matrix. Compared with non-treated rats, however, collagen matrix in the healing MCLs of rats given vanadate treatment appeared to be more organized and cells in healing sites were more elongated, aligning along the axis of MCL and resembling those of the normal MCL (Fig. 1).

Transmission electron microscopic analysis further showed that with vanadate treatment, the collagen fiber bundles in the healing MCLs were easily distinguished and evenly spaced (Fig. 2a), whereas without vanadate treatment, the collagen fibrils were often attached to each other, the spacing among the fibrils was inconsistent, and large voids among the fibrils could be easily observed (Fig. 2b). In addition, it was evident that in vanadate-treated rats, collagen fibrils had smoother contour, instead of an irregular contour as seen in non-treated controls. Moreover, with vanadate treatment, rough endoplasmic reticulum was distinct, which is one of the structural features of fibroblasts actively synthesizing collagen matrix (Fig. 3).

Furthermore, TEM image analysis showed that the diameter of collagen fibrils in the vanadate-treated rats was on average 14% larger than in the control rats that were injured but not treated ($P < 0.001$). Overall, in both vanadate-treated and non-treated groups, the distributions of their collagen fibril diameters were widely spread (Fig. 4).

Discussion

It is known from both laboratory and clinical studies that isolated MCL injuries are capable of healing spontaneously and can result in excellent knee function [8–10]. But the quality of a healing MCL is far from normal even 1 year after the injury. Thus, it is of great importance for the clinicians to improve their armament to treat these kinds of injuries faster and better. Also, as it becomes clear that non-invasive treatment is the chosen method for MCL injuries, any additional help in terms of the conservative treatment would be definitely useful. Therefore, many approaches, such as application of growth factors, have been used, but only with limited success [1, 14, 20]. This study used vanadate to treat healing MCL in rats. Three weeks after MCL injury, the healing MCLs of the rats treated with vanadate had much more elongated cells aligned with the longitudinal axis of MCL compared with those of the control rats without vanadate treatment. In addition, collagen matrix of the healing MCL in the vanadate-treated rats appeared to be more organized than that of the healing MCL without vanadate treatment. Finally, the collagen fibrils of the healing MCL in the vanadate-treated rats are more evenly distributed spatially and larger compared with the control group.

From these results, it was suggested that vanadate treatment could accelerate the repairing process of injured MCLs. These results are also consistent with those of previous studies [4, 5]. They reported that vanadate treatment improved organization of both cells and collagen matrix of granulation tissue in rats with skin

Fig. 1 The effect of ingestion of vanadate on collagen fibril organization. **a** With vanadate treatment, fibroblasts and collagen matrix were more organized in healing MCL. Collagen matrix was uniformly aligned along the longitudinal axis of MCL. The MCL fibroblasts were interspersed within collagen matrix. **b** Without vanadate treatment, fibroblasts and collagen matrix were oriented randomly in healing MCL. (H&E Staining, $\times 20$ light microscope)

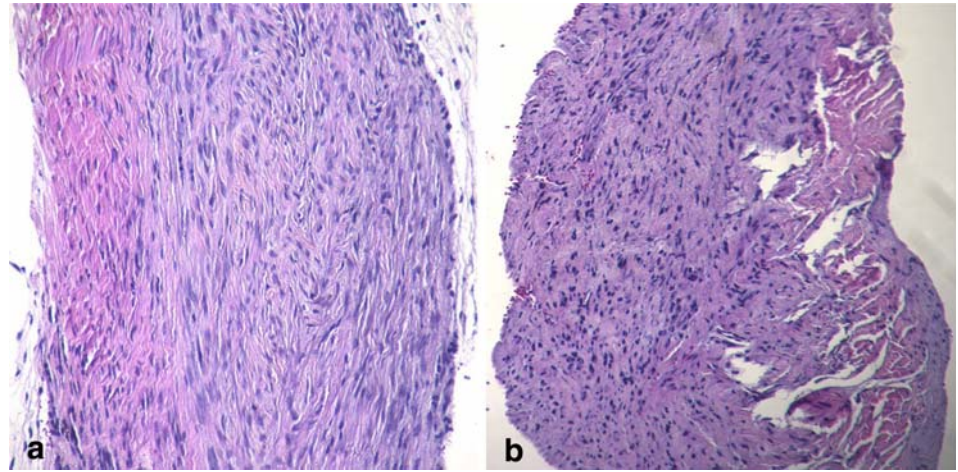


Fig. 2 TEM photographs of rat MCL collagen fibrils. **a** Vanadate-treated healing MCL. **b** No-treated healing MCL. In the vanadate-treated group, there were distinct, uniform, and evenly distributed collagen fibril bundles at the healing ligament. In the non-treated group, however, collagen fibrils were less distinct, with some attached with each other (*arrow*), the distance among fibrils was inconsistent, and there were voids among collagen fibril bundles. (Magnification $\times 60,000$)

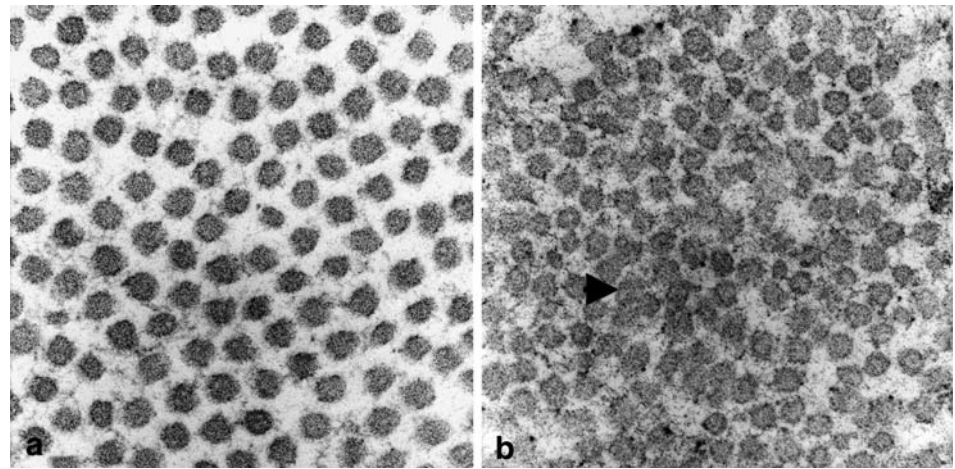
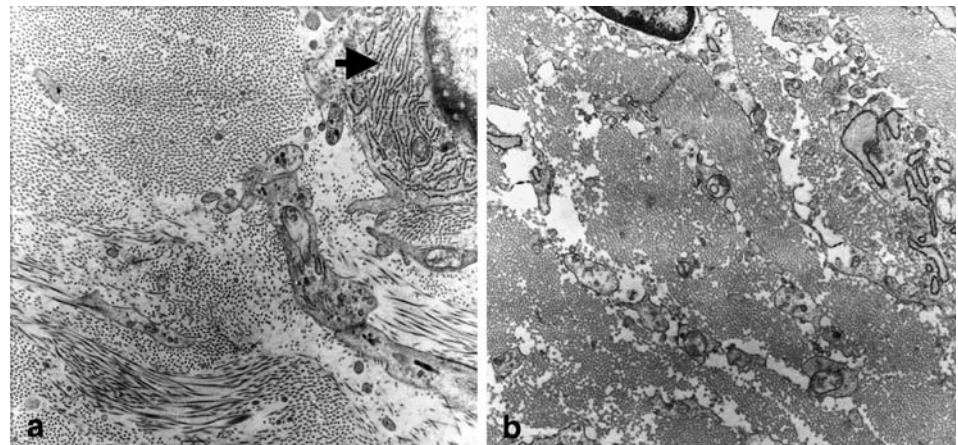


Fig. 3 TEM photographs of rat MCL collagen fibrils. **a** Vanadate-treated healing MCL. **b** No-treated healing MCL. With vanadate treatment, rough endoplasmic reticulum was distinct (*arrow*), which is one of the structural features of fibroblasts actively synthesizing collagen matrix. (Magnification $\times 8,000$)



incision [4]. A recent study also showed that vanadate treatment of healing tendons results in thick, uniformly packed, and more mature collagen fiber bundles [18].

The mechanism by which vanadate enhances healing of the collagen matrix in healing tissue is unclear. It was

suggested that vanadate might induce the more organized collagen fibrils of granulation by preventing the fibroblasts from differentiating into myofibroblasts [5]. Vanadate inhibits the formation of cytoplasmic stress fibers both in vitro and in vivo [5, 15]. In normal MCLs,

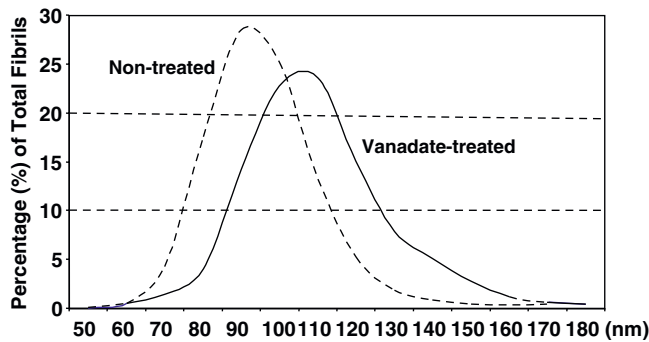


Fig. 4 Distribution of collagen fibril diameter in healing MCL with and without vanadate treatment. There is an increase in the percentage of larger diameters of collagen fibrils in vanadate-treated healing MCL compared to the control. A wide distribution of collagen fibril diameters was evident for both groups

myofibroblasts are generally absent. However, during the wound healing process, the ligament fibroblasts are differentiated into myofibroblasts in granulation tissue [21]. It is thought that the “tractional force” [11] produced by fibroblasts is responsible for organizing collagen fibril during connective tissue healing. However, when fibroblasts become myofibroblasts through differentiation in the granulation tissue, they lose the ability to produce the tractional forces [6], and so lose the ability to reorganize the newly produced collagen fibrils in the granulation tissue. Also, how vanadate in healing tissue increases collagen fibril diameter is not known. Since vanadate inhibits protein tyrosine dephosphorylation, it is possible that enhanced tyrosine phosphorylation may be involved in promoting the maturation of newly formed collagen fibrils [18]. Further studies are required to elucidate the molecular mechanisms of vanadate effects in enhancing tissue healing.

Vanadate was shown to have some toxic effects after chronic use [3, 23]. Systemic administration of vanadate through ingestion may cause some unknown side effects. However, toxic effects of vanadate were observed in a dose-dependent manner, and they were apparent only at markedly high doses. In a recent review, the element

vanadium was reported to have an anti-carcinogenic effect [19]. In order to avoid any significant side or adverse effects through a possible clinical use of this substance, more research in a long-term basis needs to be done in the future.

It is also noted that to relieve pain after surgery, ketoprofen (5 mg/kg) was administered to rats for 4 days. Ketoprofen is a nonsteroidal anti-inflammatory drug (NSAID), which decreases inflammation in injured tissues by inhibiting the production of prostaglandins. NSAIDs, such as piroxicam, has been reported to increase the early tensile strength of the healing MCL in rats [2]. However, the inconsistent results from using NSAIDs were also reported. For example, oral administration of Ibuprofen for 14 days after rabbit MCL injuries did not result in significant changes in the mechanical properties of the healing MCL in ibuprofen-treated rabbits compared to rabbits treated with placebo [17]. Also, ketoprofen at the dose (5 mg/kg) has been shown not to interfere with wound healing after complete corneal de-epithelialization in rabbits [13]. These studies suggest that while short term use of ketoprofen may influence the early healing process of the injured MCL, its effect on collagen organization and collagen fibril diameter at the later healing stage (3 weeks) may be minimal.

In summary, we have investigated the effect of vanadate on the quality of healing MCLs in rats. It was found that vanadate treatment, through ingestion, enhanced collagen matrix organization of the healing MCL and increased collagen fibril diameter at 3 weeks after injury. Future studies, however, are required to examine the long term effect of vanadate on histological, biochemical, and mechanical properties of the healing MCL. In addition, the cellular and molecular mechanisms by which vanadate enhances tissue healing in terms of collagen fibril organization and diameters need to be investigated.

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