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# The Effects of Hyaluronic Acid and Glucosamine on the Migration and Proliferation of Tenocytes

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Tendinopathy or tendon rupture is a common disease. Studies have showed that hyaluronic acid and glucosamine benefit the process of tendon repair. Tendon healing requires the migration of tenocytes to the repair site, followed by the proliferation and synthesis of the extracellular matrix. However, the effects of hyaluronic acid and glucosamine on tenocytes remain to be explored.

Here, we treated tenocytes from rat Achilles tendons with hyaluronic acid or glucosamine. The ability of tenocytes to migrate and proliferate was assessed by transwell filter migration assay and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, respectively. Results from transwell filter migration assays showed that hyaluronic acid has no significant effect on tenocyte migration, whereas glucosamine can inhibit tenocyte migration. The results of MTT assays revealed that tenocyte proliferation decreases after hyaluronic acid or glucosamine treatment.

In conclusion, hyaluronic acid and glucosamine inhibit tenocyte proliferation. Moreover, glucosamine inhibits tenocyte migration. The *in vivo* effect of hyaluronic acid and glucosamine on tendon healing needs to be further assessed. (Tw J Phys Med Rehabil 2013; 41(1): 13 - 19)

**Key Words:** hyaluronic acid, glucosamine, tenocyte, transwell filter migration assay, MTT assay

## INTRODUCTION

A tendon is dense and regular connective tissue that connects muscle to bone. As a connective tissue, tendon is comprised of tenocytes and extracellular matrix, of which type I collagen is most predominant.<sup>[1]</sup> The collagen fibers are arranged in a parallel array and are densely packed. Thus, it can provide strength to withstand tension when we exercise. Additionally, there are many proteogly-

cans, multi-adhesive glycoproteins, and glycosaminoglycans in the extracellular matrix.<sup>[2]</sup> They can both provide mechanical and structural support for tissue, and influence extracellular communication.

Tendon rupture has been associated with increased morbidity, especially in elderly patients.<sup>[3]</sup> The healing of tendons usually takes a long time and is painful for the patient. Currently, the main non-operative treatments of tendinopathy or tendon injury include RICE (rest, ice, compression, and elevation), non-steroidal anti-inflammatory drugs, and

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physical modalities. Sometimes, the pain remains chronic and persistent. Therefore, new therapeutic strategies are necessary to promote tendon healing.

Glucosamine is a common dietary supplement for patients with osteoarthritis.<sup>[4]</sup> It is a hexosamine sugar, which is an important precursor in the biosynthesis of connective components, such as proteoglycans, glycosaminoglycans, glycoproteins and glycolipids.<sup>[5]</sup> There are studies showing that glucosamine is helpful for the prevention of osteoarthritis progression.<sup>[4]</sup> Recent studies using mice as an animal model showed that glucosamine supplements can improve the ability of cartilage healing.<sup>[4,6]</sup> As tendons are similar to cartilage, which contains lots of proteoglycans, glycoproteins, and glycosaminoglycans, it is possible that glucosamine will also promote tendon healing.

Hyaluronic acid is a high-molecular-weight polysaccharide, that is a major component of synovial fluid and cartilage.<sup>[2]</sup> It is commonly used in the treatment of various joint disorders, including osteoarthritis.<sup>[7,8]</sup> Apart from its role in the structural support and lubrication of joints and other tissues,<sup>[9]</sup> hyaluronic acid has been shown to have a profound effect on cell proliferation.<sup>[10,11]</sup> In addition, hyaluronic acid has biological function, providing a hydrated matrix through which cells can migrate, and has been used to improve wound healing.<sup>[12]</sup> It has been reported that exogenously applied hyaluronic acid enhances tendon healing,<sup>[13]</sup> and evidence suggests that hyaluronic acid could prevent the formation of adhesions.<sup>[14,15]</sup> Therefore, we hypothesized that hyaluronic acid can improve tendon healing through modulating the proliferation and migration of tendon fibroblasts.

It is of clinical relevance to provide laboratory-based evidence supporting the use of glucosamine or hyaluronic acid in tendon healing. Thus, the purpose of this study is to investigate the effects of glucosamine and hyaluronic acid on the migration and proliferation of tenocytes.

## MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of Chang Gung University.

### *Primary Culture of Rat Achilles Tenocytes*

Sprague-Dawley rats were chosen as the source of

Achilles tendons.<sup>[16]</sup> The excised tendons were washed in phosphate-buffered saline (PBS). Each tendon was then cut into small pieces of approximately 1.5–2.0 mm<sup>3</sup> (6 pieces in total), and these pieces were individually placed in six-well culture plates. After 5 min of air-drying for better adhesion, 0.5 mL of Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT), with 20% fetal bovine serum (FBS; Cansera, Rexdale, ON Canada), 100 U/mL penicillin, and 100 mg/mL streptomycin was added to each well. The explants were then incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. After migrating out from the explants, the cells started to grow rapidly, and the confluence culture was sub-cultured by trypsin digestion at a 1:3 dilution. Tendon cells between passages 2 and 4, with proper growth rate and normal fibroblast shape were used in the following experiments.

### *Cell Culture Protocol*

Trypsinized rat tenocytes were cultured in 10% DMEM under six different concentrations of glucosamine (control, 0.1 mM, 0.5 mM, 1.0 mM, 2.0 mM and 4.0 mM) and hyaluronic acid (1 mg/mL; control, 0.5%, 1.0%, 5.0%, 7.5%, and 10.0%) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air for 24 h.

### *Transwell Filter Migration Assay*

Transwell filters (Costar, Cambridge, MA) with 8.0 µm pores were used for the migration assay. After 24 h of growth with different concentrations of treatment, tendon cells were seeded at a density of  $1.0 \times 10^5$  cells per filter. The inner chamber was filled with 200 µL serum-free DMEM and the outer chamber was filled with 600 µL DMEM with 10% FBS. Cells were allowed to migrate for 3 h at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air. After that, the cells were stained with Liu's stain and then washed 3 times with PBS. Cells on the upper surface of the filter were removed using a cotton swab. The cells on the lower surface of the filter were counted using four random high-power microscopic fields (HPF; 100×) per filter, and the mean number of migrating cells was calculated for each concentration. The experiments were performed in triplicate.

### *MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide) Assay*

Tendon cells were seeded at the same density in each culture well. After 24 h of growth with different concentrations of treatment, the medium was removed, and 0.1% MTT in DMEM without phenol red was added. Then, the tendon cells were incubated for 1 h, the medium was removed, and 0.5 mL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Optical density (OD) at 570 nm was read using a VICTOR™ X3 spectrophotometer, (PerkinElmer Inc., Waltham, MA) to detect the survival and proliferation of tendon cells. The experiments were performed in triplicate, at minimum.

### Statistical Analysis

All data were expressed as mean  $\pm$  SEM. The analysis was performed with IBM SPSS statistics 20 (IBM, Armonk, NY). Data among the different conditions were compared using the non-parametric Kruskal-Wallis test. The post-hoc test was used for comparison between any 2 conditions. *P* values less than 0.05 were considered significant.

## RESULTS

### The Effect of Hyaluronic Acid on Tenocyte Migration

The results of transwell filter migration assays showed that tenocytes migrate across the upper surface to the lower surface of the inner chamber during the assay. Figure 1 shows the results of Liu's stain of tenocytes that successfully migrated through the transwell filter.

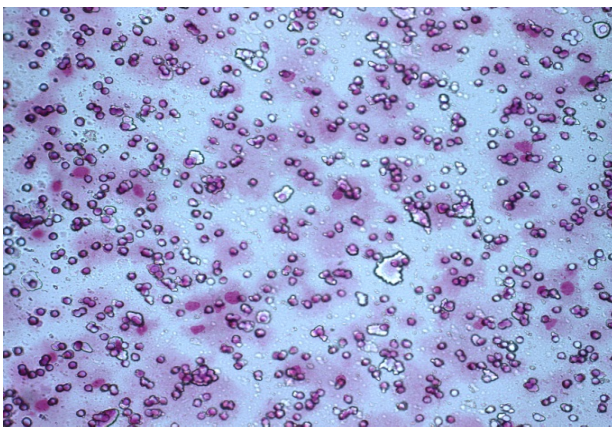


Figure 1. Liu's stain of tenocytes after the transwell filter migration assay (100 $\times$ ). After migration, tenocytes were fixed and observed under a microscope. Round dots in the figure represent migrated tenocytes.

There was no significant change in tenocyte migration ability when tenocytes were treated with hyaluronic acid, as compared to control tenocytes (Figure 2). The average percentage of migrated cells detected on the lower side of the filter was  $100.72 \pm 9.26\%$ ,  $95.24 \pm 7.69\%$ ,  $83.10 \pm 6.98\%$ ,  $89.18 \pm 7.79\%$ , and  $111.16 \pm 4.06\%$  of the control, at concentrations of 0.5%, 1.0%, 5.0%, 7.5% and 10.0% hyaluronic acid, respectively. The result revealed that hyaluronic acid does not significantly affect the migration of tenocytes.

### The Effect of Hyaluronic Acid on Tenocytes Proliferation

It was seen that the OD value of control tenocytes was greater than that of tenocytes treated with hyaluronic acid. The decrease in OD value after hyaluronic acid treatment was dose dependent (Figure 3). The respective OD<sub>570</sub> values of the 0.5%, 1.0%, 5.0%, 7.5%, and 10.0% hyaluronic acid were  $96.16 \pm 0.93\%$ ,  $91.06 \pm 1.28\%$ ,  $86.45 \pm 4.91\%$ ,  $59.10 \pm 7.46\%$ , and  $52.73\% \pm 7.90\%$  of the control. The trend showed that hyaluronic acid significantly inhibits tenocyte proliferation in a dose-dependent manner ( $n = 4$ ;  $p < 0.05$ ).

### The Effect of Glucosamine on Tenocyte Migration

There was a significant change in tenocyte migratory ability when tenocytes were treated with glucosamine, as compared to control tenocytes (Figure 4). The average percentage of migrated cells detected on the lower side of the filter was  $87.60 \pm 3.27\%$ ,  $79.44 \pm 2.40\%$ ,  $68.89 \pm 2.80\%$ ,  $62.75 \pm 1.93\%$  and  $38.29 \pm 1.90\%$  of the control, at concentrations of 0.1 mM, 0.5 mM, 1.0 mM, 2.0 mM and 4.0 mM, respectively. The trend also showed that glucosamine significantly inhibits tenocyte migration in a dose-dependent manner ( $n = 3$ ;  $p < 0.01$ ).

### The Effect of Glucosamine on Tenocyte Proliferation

The respective OD<sub>570</sub> values at concentrations of 0.1 mM, 0.5 mM, 1.0 mM, 2.0 mM and 4.0 mM glucosamine were  $99.60 \pm 1.64\%$ ,  $96.71 \pm 2.35\%$ ,  $96.00 \pm 1.29\%$ ,  $94.64 \pm 3.75\%$ , and  $79.01\% \pm 4.99\%$  of the control, respectively. The trend revealed that glucosamine can inhibit tenocyte proliferation. The inhibitory effect is noted to be statistically significant when tenocytes were treated with glucosamine at levels above 1 mM ( $n = 4$ ;  $p < 0.05$ ).

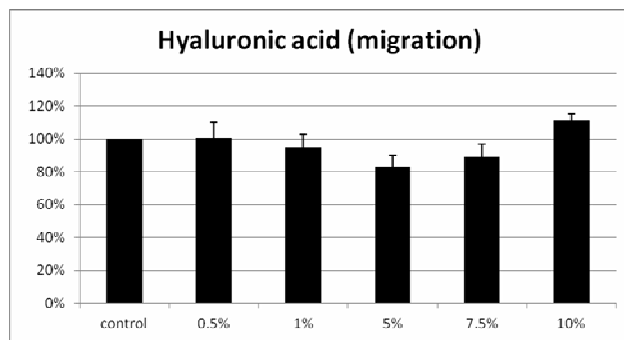


Figure 2. Hyaluronic acid had no significant effect on cell migration of tenocytes (n = 3).

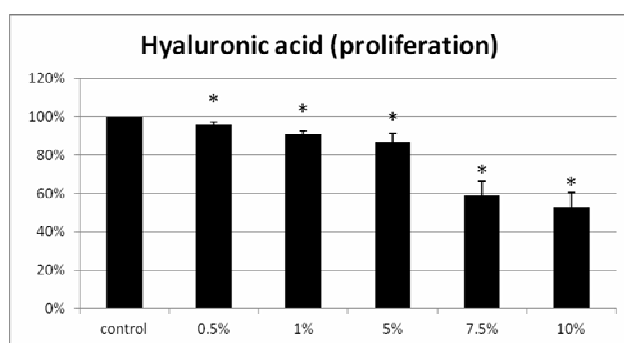


Figure 3. Hyaluronic acid decreased tenocyte proliferation in a dose-dependent manner (n = 4; \* $p < 0.05$ ).

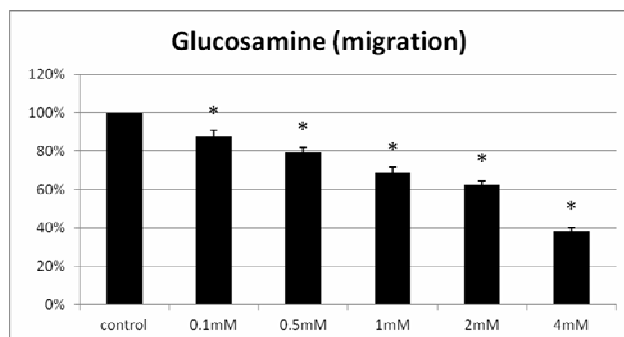


Figure 4. Glucosamine decreased tenocyte migration in a dose-dependent manner (n = 3; \* $p < 0.01$ ).

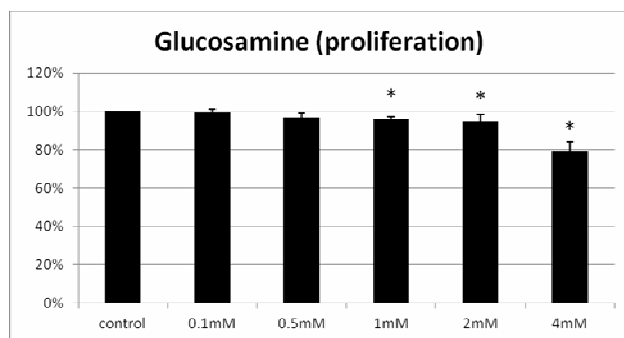


Figure 5. Glucosamine had the tendency to decrease tenocyte proliferation, which was significant at high concentrations (n = 4; \* $p < 0.05$ ).

## DISCUSSION

There are some studies supporting the use of hyaluronic acid or glucosamine for treatment of injured tendons,<sup>[13,17]</sup> although the mechanism of the healing effect has not yet been clarified. Healing of the injured tendon proceeds through 3 overlapping phases: inflammation, regeneration, and remodeling/maturation.<sup>[18,19]</sup> During the regenerative phase, tendon cells from the endotenon and epitenon move to the site of injury.<sup>[15]</sup> These cells are able to establish an extracellular matrix and an internal neovascular network. Before extracellular matrix production begins, a sufficient number of tendon cells must colonize at the repair sites. Thus, tenocyte migration and proliferation are fundamental to the healing process of the injured tendon.<sup>[16]</sup>

It was reported in an animal study that hyaluronic acid can promote the healing of injured tendons.<sup>[20]</sup> This implies that, hyaluronic acid may be able to increase cell proliferation and migration. It has been suggested that hyaluronic acid promotes the proliferation of many cell types, including chondrocytes, hematopoietic cells, and dermal fibroblasts.<sup>[10,11,21]</sup> However, this study demonstrated that the proliferation of tenocytes decreases after hyaluronic acid treatment, and this decrease becomes more significant with higher concentrations of treatment. One explanation of this result was that hyaluronic acid might potentiate an inhibitory effect in early phases of tendon healing. Similar to our observation, hyaluronic acid has been shown to inhibit synovial cell proliferation *in vitro*, in a concentration-dependent manner.<sup>[22]</sup>

The hyaluronic acid mediated expression of collagen in tenocytes has also been explored. Studies showed that hyaluronic acid does not affect the synthesis of proteoglycan, collagen, or non-collagen proteins, as measured by the mean radioactive uptake of <sup>35</sup>S-sulphage, <sup>3</sup>H-hydroxyproline, and <sup>3</sup>H-proline, respectively.<sup>[23,24]</sup> The actual *in vivo* cellular response is an important question that needs to be addressed in a following study.

Maniwa et al examined the cell motility of synovial cells and showed that hyaluronic acid increased the velocity of cell migration *in vitro*.<sup>[25]</sup> Yagi et al reported that hyaluronic acid at various concentrations (0.1–5.0 mg/mL) dose-dependently promoted the cell migration of

tendon fibroblasts derived from rabbit,<sup>[26]</sup> and this effect may be exerted through the CD44 pathway. In contrast to the above observation, there was no effect of hyaluronic acid on tenocyte migration in our study. The reasons for the discrepancy between our results and the above-mentioned studies might be due to different species of animals and tendons from different body parts that were used. In addition, the different molecular weights of hyaluronic acid might have different effects on promoting cell migration. Therefore, the underlying molecular mechanism accounting for hyaluronic acid acting on tenocytes is still unclear. Further molecular studies are needed to clarify this issue.

This study also showed that the proliferation and migration of tenocytes decreased after glucosamine treatment. More glucosamine is seen as more a source or supplement for tendon cells to repair tendon injuries intuitively. Ozer et al showed that glucosamine can help the healing process of impaired tendons in rats, and this result may be due to decreased inflammation and stimulation of collagen synthesis.<sup>[27]</sup> However, our study did not indicate if glucosamine could increase the abilities of tendon cell proliferation and migration. In their study, glucosamine was given orally and the histological results of collagen formation and inflammation were analyzed from 4 to 12 weeks.<sup>[27]</sup> It is possible that longer research duration and different dosages might be used in future studies. Ilic et al investigated the effects of glucosamine on the loss of newly synthesized radiolabeled large and small proteoglycans in bovine tendons, ligaments and joint capsules.<sup>[6]</sup> Their study reported that glucosamine does not have an effect on the catabolism of small or large proteoglycans in tendons. It also showed that the extracellular matrix of tendons is not affected by glucosamine. However, the effect of glucosamine on tenocytes proliferation was not discussed. Lippiello et al found that low dose combinations of glucosamine and chondroitin sulfate effectively stimulate *in vitro* collagen and non-collagenous protein synthesis by ligament cells, tenocytes and, chondrocytes.<sup>[28]</sup> Their results might relate to the combination use of glucosamine and chondroitin sulfate acting as biological response modifiers to upregulate the metabolic activity of tenocytes. Further molecular studies are needed to clarify the exact effect on extracellular matrix of tendons.

## CONCLUSION

Our study showed that glucosamine inhibited the migration and proliferation of tenocytes. Furthermore, hyaluronic acid inhibited tenocyte proliferation at higher concentrations. Further molecular studies are needed to investigate the underlying mechanism. Additionally, the *in vivo* effects of hyaluronic acid and glucosamine on tendon healing would be assessed in animal models.

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## 玻尿酸與葡萄糖胺對肌腱細胞移行與增生的影響

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肌腱斷裂或是病變是運動醫學中常見的疾病。已經發表的研究指出注射玻尿酸(hyaluronic acid)或口服葡萄糖胺(glucosamine)對於肌腱的修復有助益。肌腱的修復需要肌腱細胞(tenocyte)移行(migration)至受損處，而後肌腱細胞會增生(proliferation)與合成細胞外基質來幫助修復。然而玻尿酸或葡萄糖胺對肌腱細胞的影響至今仍然不明。

本實驗使用來自於大鼠阿基里斯腱(Achillis tendon)的肌腱細胞。在肌腱細胞中加入不同濃度的玻尿酸與葡萄糖胺，再使用細胞穿透移行分析法(transwell filter migration assay)來評估肌腱細胞的移行能力；另外使用細胞存活率分析法(3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay)來評估肌腱細胞的增生。細胞穿透移行分析法顯示玻尿酸不會影響肌腱細胞的移行，而葡萄糖胺則會使肌腱細胞的移行率下降( $p < 0.05$ )。細胞存活率分析法顯示玻尿酸濃度增加與葡萄糖胺濃度增加皆使肌腱細胞的增生率下降( $p < 0.05$ )。

本研究結果顯示玻尿酸與葡萄糖胺都會抑制肌腱細胞增生。此外，葡萄糖胺甚至會抑制肌腱細胞移行。然而，玻尿酸與葡萄糖胺在肌腱修復的實際生理效益還需以動物實驗進一步驗證。(台灣復健醫誌 2013；41(1)：13 - 19)

**關鍵詞：**玻尿酸(hyaluronic acid)，葡萄糖胺(glucosamine)，肌腱細胞(tenocyte)，細胞穿透移行分析(transwell filter migration assay)，細胞存活率分析(MTT assay)