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An in vitro study on the effects of various concentrations of low and high molecular weight hyaluronic acid on human chondrocyte cell metabolism

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1. Introduction

Hyaluronic Acid (HA) also known as hyaluronan is a mucopolysaccharide with alternate N-Acetyl-D-glucosamine and D-glucuronic acid that is found in the synovial fluid of joints.[1] It is a responsible for the viscosity of synovial fluid as it binds to water.[2]. In normal the human joint it is present at a molecular mass of 6–7 x 10^6 Daltons[3] There is a constant secretion of hyaluronic acid by the synovium in the knee joint.[4] It regulates and maintains the joints osmotic pressure as demonstrated by Day et al.[5] HA has been postulated to have feature known as shear dependent viscosity where when shear forces in the joint increase, the viscosity of HA increases forming a thick gel like structure across the joint surface in order to counteract the forces applied to the articular cartilag3.[6] Hyaluronon molecules interact with $CD44^+$ receptors on articular chondrocytes to promote collagen synthesis.[7] HA has been shown to have a role in angiogenesis, where high molecular HA inhibits angiogenesis and low molecular weight induce it in the joint.[8] This may be the reason for reduced vascularity in the articular joints. HA has also been shown to remove free radicals from the joint but the biological significance of this has not been proven as of yet.[9]

Commercially various forms of HA are available from a spectrum of drug manufacturers. In the past it was extracted from rooster combs but manufacture has now moved on to recombinant systems using Streptococcus zooepidemicus and Bacillus subtilus.[10] High and low molecular weight HA are the two forms of the molecule sold by manufacturers (high molecular weight being 6×10^6 Daltons and low molecular being 2×10^6 Daltons).

Hyaluronic acid intra articular injections have documented use in treating osteoarthritis in equine joints in a study done by Balaz et al. in the 1970s.[11] They found that horses had reduced symptomology and did have improved performance with the HA injections. As a part of this study owl monkeys were also subjected to bilateral knee injuries, one knee was kept as a control and injected with saline while the other received serial HA injections. Results showed the joint that received HA was smoother and had better cartilage healing.[11] In vitro studies have shown promise in aiding in chondrocyte metabolism, showing stimulation and increased glycosaminoglycan and DNA synthesis especially in low doses of hyaluronic acid in bovine chondrocytes.[12]

In clinical studies Trigkilidas et al. showed that there was a modest effect of the drug in mild to moderate OA knee showing most effectiveness at 6–8 weeks, at 6 months the effect of the drug was questionable.[13] A Meta analysis of double-blinded, sham-controlled trials with a minimum of sixty patients showed no differences of HA treatment over placebo.[14] A conflict of interest was found in an updated systematic review stating that there was a significant association of conflict of interest in the study and a favourable outcome in HA injections. Studies with no industry affiliations showed no more effectiveness of HA than a placebo injection.[15] Thus there is inadequate evidence and controversial data as to whether HA injections are beneficial treatment in early knee osteoarthrosis.

The purpose of this study was to assess the effects of low and high molecular weight hyaluronic acid on human chondrocytes in vitro and determine whether they potentiate chondrocyte cell metabolism.

2. Material and methods

2.1. Study type- comparative study

2.1.1. Tissue culture and harvest

All procedures were approved under the institutional ethical

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committee and with patient consent. Three osteochondral plugs were harvested during total knee arthroplasty from patients suffering from osteoarthritis scheduled for surgery under sterile conditions.

Chondrocytes were isolated and cultured to passage one and three samples from passage one cultured to passage two.

Passage one and two flasks were seeded with 0.1 mg/ml, 1 mg/ml & 2 mg/ml of High (Hylast One) and low (Synject) molecular weight hyaluronic acid all against control.[12] Cells were then harvested on the 14th day of culture and assessed for cell viability, cell count, CD 44^{+} expression.

2.1.2. Cell viability

20 μl Samples of cell suspension was removed on the 14th day culture and dispensed into a 1.4 ml test tube. A hemocytometer was wiped with 70% isopropyl alcohol and allowed to dry. 20 μl of 0.4% Trypan blue was added to the same test tube and the mixture gently mixed by dispensing with a micropipette. 10 μl of the cell suspension Trypan blue mixture is then transferred to the counting chamber under a cover slip. Stained cell (non viable) and non stained cells (viable) are then counted in 4 squares of the hemocytometer areas at 100× magnification. The percentage of the viable cells is then calculated by dividing the viable cells by total cells and multiplied by one hundred.

Table 1a
Concentration of HMW in cell count.

	cultural stage	Mean	sd	F	P value
Control	P1	4.5733	2.93737	.0.081*	.968
1 μM/ml Pred	P1	4.3700	2.61152		
10 μM/ml Pred	P1	3.9433	2.97147		
100 μM/ml Pred	P1	3.6300	2.61064		
Control	P2	6.2800	1.22784	1.47*	.293
1 μM/ml Pred	P2	5.4433	.59501		
10 μM/ml Pred	P2	4.9500	1.12601		
100 μM/ml Pred	P2	4.5600	1.16202		

*not significant (p; >0.05).

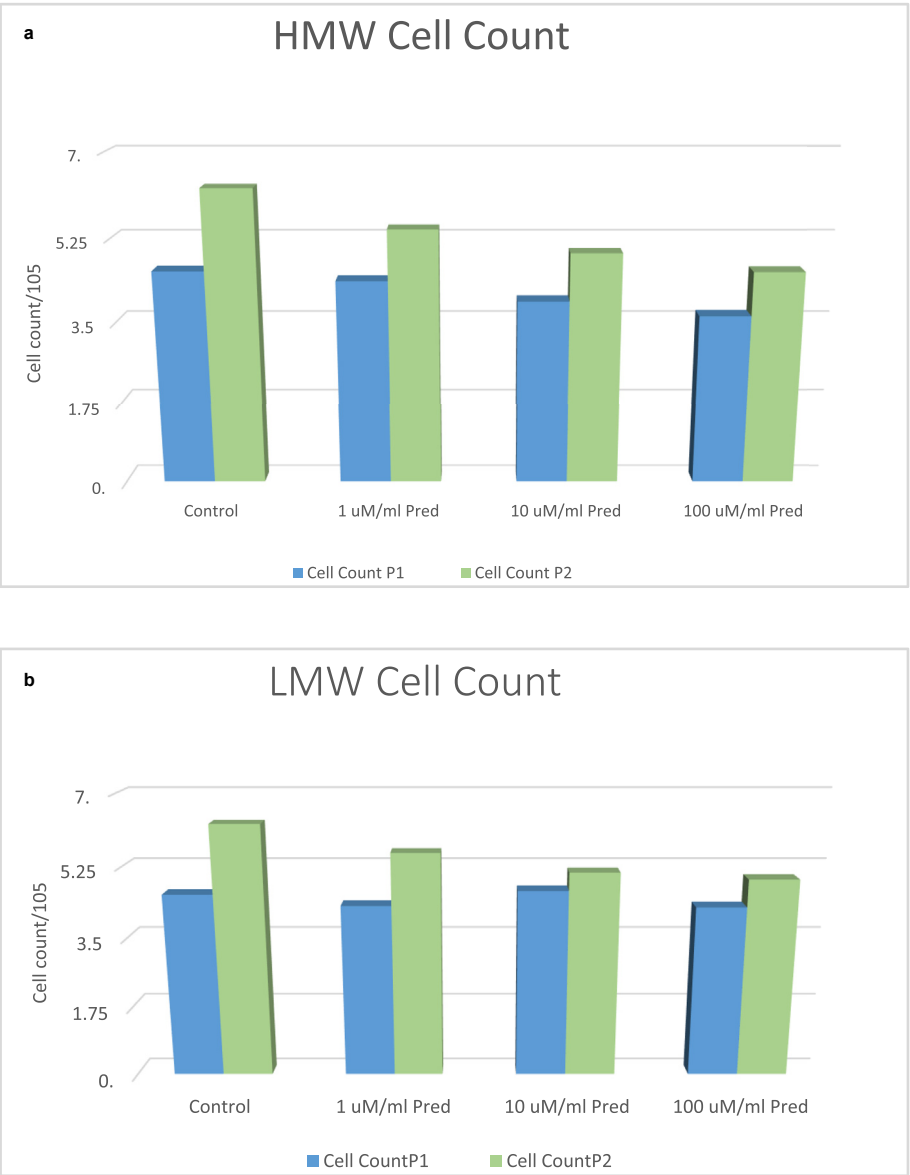

Fig. 1. Graph showing the effect of HMW & LMW HA on cell count.

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Table 1b
Concentration of LMW in cell count.

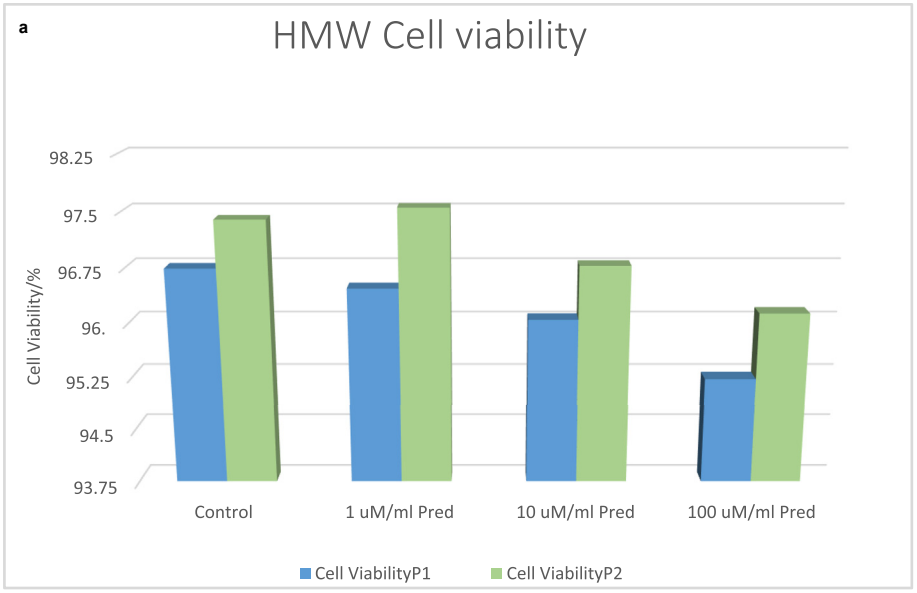
	cultural stage	Mean	sd	F	P value
Control	P1	4.5733	2.93737	.0.015*	.997
1 μM/ml Pred	P1	4.2967	2.61152		
10 μM/ml Pred	P1	4.6633	2.97147		
100 μM/ml Pred	P1	4.2633	2.61064		
Control	P2	6.2800	1.22784	2.12*	.176
1 μM/ml Pred	P2	5.5867	.41885		
10 μM/ml Pred	P2	5.1067	.44456		
100 μM/ml Pred	P2	4.9433	.39107		

*not significant (p; >0.05).

Table 2a
Concentration of HMW in cell viability.

	cultural stage	Mean	sd	F	P value
Control	P1	96.7400	1.64654	.284*	.836
1 μM/ml Pred	P1	96.4700	2.06589		
10 μM/ml Pred	P1	96.0433	2.39529		
100 μM/ml Pred	P1	95.2167	2.44672		
Control	P2	97.3933	1.03549	1.600*	.264
1 μM/ml Pred	P2	97.5533	.23180		
10 μM/ml Pred	P2	96.7767	.50143		
100 μM/ml Pred	P2	96.1300	1.33368		

*not significant (p; >0.05).



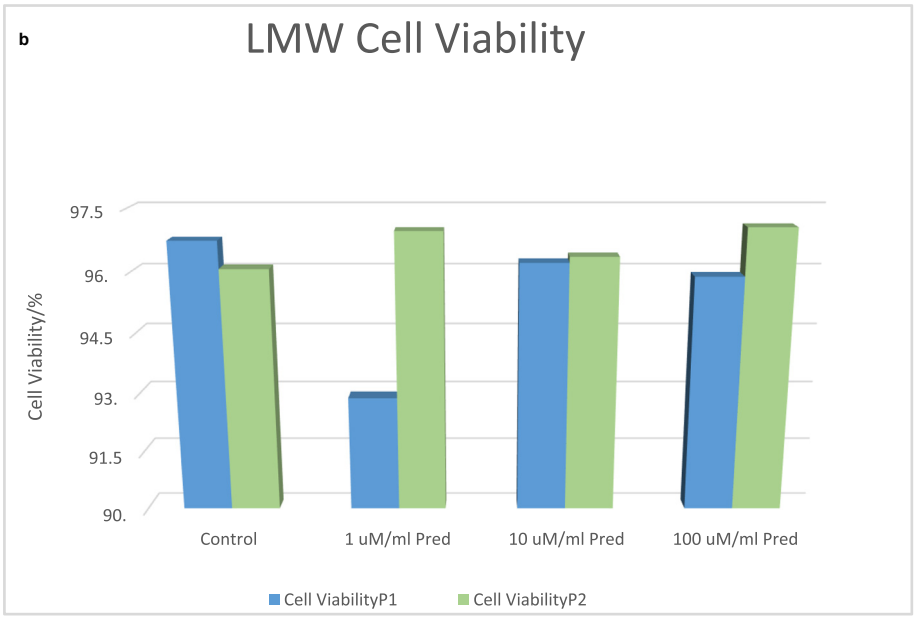


Fig. 2. Graph showing the effect of HMW & LMW HA on cell viability.

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Table 2b
Concentration of LMW in cell viability.

	cultural stage	Mean	sd	F	P value
Control	P1	96.7400	1.64654	.858*	.501
1 μM/ml Pred	P1	92.8700	5.11205		
10 μM/ml Pred	P1	96.2100	2.74255		
100 μM/ml Pred	P1	95.8767	2.44396		
Control	P2	96.0600	1.67896	.363*	.781
1 μM/ml Pred	P2	96.9700	.77949		
10 μM/ml Pred	P2	96.3533	1.94747		
100 μM/ml Pred	P2	97.0600	.70292		

*not significant (p; >0.05).

2.1.3. Cell count

Aperture of coulter counter is first flushed. 200 μl of the culture sample is removed from the cell suspension. It is then dispensed in 9.8 ml of cell counting buffer solution. The sample is then poured into an accuvette sample container and then placed on the coulter counter.

The aperture is then immersed into the cell suspension and the counter is then initiated reading taken and recorded.

2.1.4. CD 44^{+} expression

2.1.4.1. Flowcytometry

2.1.4.1.1. Specimen preparation. 100 μl of well mixed specimen brought to room temperature is added to tube. 10 μl of antibody solution is added to the mixture. The sample is then incubated for 30 min in a dark room at room temperature. The cells were then washed twice with BD FACS Flow Solution. The sample was then centrifuged at 1500 rpm for 5 min. The supernatant is then discarded and the pellet suspended in 500 μl of BD FACS Flow solution. The sample is then run through the flow cytometry within one hour and readings recorded. CD44^{+} expression is an indirect method of measuring collagen II synthesis in chondrocytes.[16]

3. Results

3.1. Cell count

Both High molecular weight (HMW) and Low molecular weight

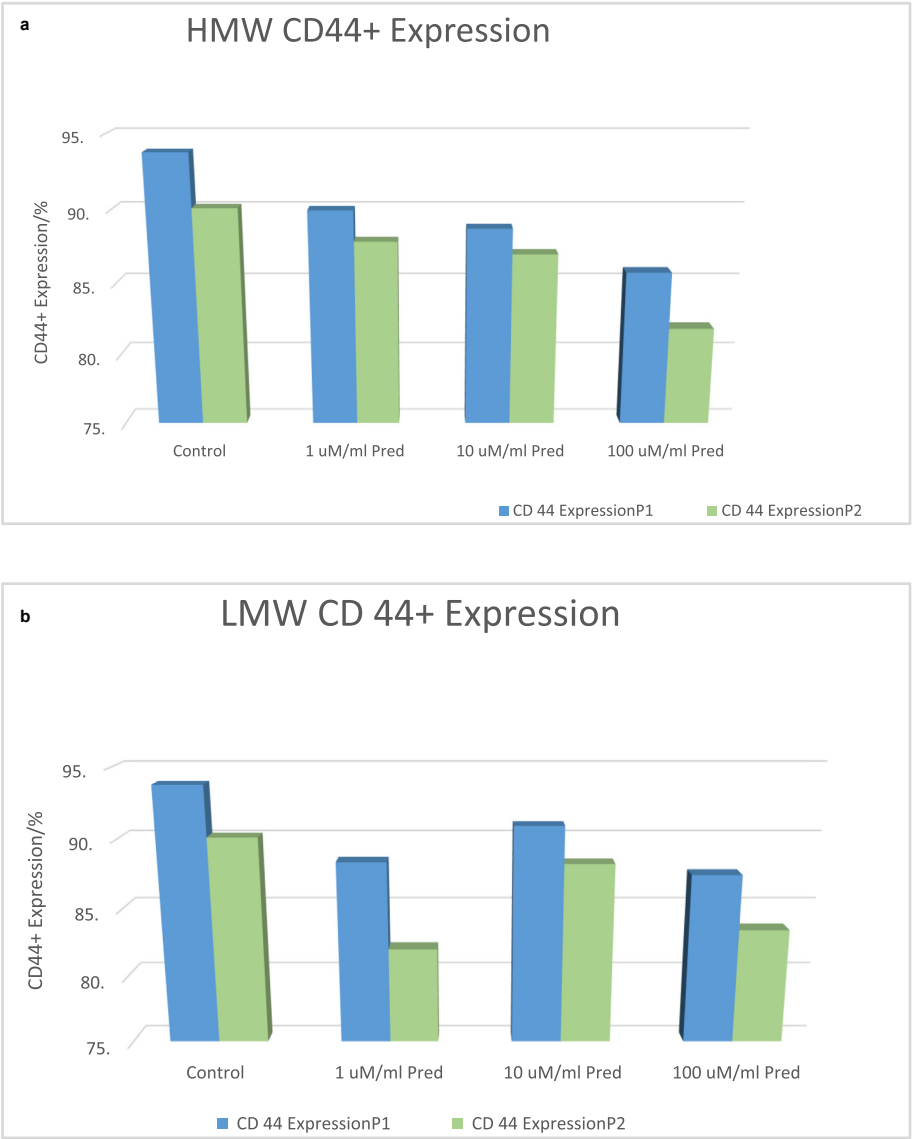

Fig. 3. Graph showing the effect of HMW & LMW HA on CD44 expression.

Table 3a
Concentration of HMW in CD 44 expression.

	cultural stage	Mean	sd	F	P value
Control	P1	93.7933	3.14662	4.457**	.040
1 μM/ml Pred	P1	89.9533	1.93146		
10 μM/ml Pred	P1	88.7333	2.04131		
100 μM/ml Pred	P1	85.7300	3.49309		
Control	P2	90.1000	4.70698	.875*	.493
1 μM/ml Pred	P2	87.8433	5.66224		
10 μM/ml Pred	P2	86.9900	5.08605		
100 μM/ml Pred	P2	81.8267	9.36276		

*not significant (p; >0.05) ** significant (p; <0.05).

Table 3b
Concentration of LMW in CD 44 expression.

	cultural stage	Mean	sd	F	P value
Control	P1	93.7933	3.14662	2.65*	.120
1 μM/ml Pred	P1	88.3433	3.69007		
10 μM/ml Pred	P1	90.9267	1.66692		
100 μM/ml Pred	P1	87.4333	3.25997		
Control	P2	90.1000	4.70698	.783*	.536
1 μM/ml Pred	P2	81.9800	11.1440		
10 μM/ml Pred	P2	88.2267	3.56350		
100 μM/ml Pred	P2	83.4067	8.30456		

*not significant (p; >0.05).

(LMW) HA passages showed a steady decrease in cell count as the concentration of HA increased (Fig. 1a and b). Although the decrease was not statistically significant in both group passages (Tables 1a and 1b).

3.2. Cell viability

Passage 1 in both HMW and LMW showed a decrease in cell viability as the concentration of HA increased but the decrease was not significant. However, in passage 2 both HMW HA and LMW HA demonstrated a slight increase in viability at a concentration of 1 μM/ml although not statistically significant (Fig. 2a and b). At concentrations higher than 1 μM/ml the viability decreased in both groups although not statistically significant (Tables 2a and 2b).

3.3. $CD44^+$ expression

In both passages the HMWHA group showed a decrease in $CD44^+$ expression and it was statistically significant in HMW HA passage 1 ($p = 0.04$) (Fig. 3a). The LMW HA group showed a decrease in both passages with decreased expression at 1 μM/ml, then a slight increase at 10 μM/ml followed by a decrease again at 100 μM/ml (Fig. 3b) though these changes were not statistically significant (Tables 3a and 3b).

4. Discussion

Our study shows that both HMW and LMW HA have no significant effect on articular human chondrocytes in vitro. There was no potentiation of chondrocyte activity in terms of cell viability, cell count and CD 44^+ expression. The results showed decreases in viability, count and CD 44^+ expression though only statistically significant in HMW HA passage one. As our results do not show a uniform statistically significant decrease in all groups it cannot be said that either forms of HA cause toxicity to the chondrocytes. If HA was to increase collagen II synthesis in chondrocytes the $CD44^+$ expression would have increased in our study groups.

Literature has shown that lower concentrations of HA have a stimulatory effect on chondrocyte metabolism in bovine chondrocytes[12], but our study did not support such findings. Clinical studies have shown ambiguous results[13–15] with some studies showing benefit. This may be attributed to the physical properties of HA causing an overall improvement in joint health and lubrication. Our study design does not take into account the physical properties of HA where clinical studies allow for the biological and physical property effects to be studied.

From our study results indicate that HA does not have a significant biological role in promoting cartilage health or growth. It does not potentiate chondrocyte metabolism nor is it chondrotoxic. Intra articular HA injections to promote cartilage growth and joint health may not be an effective treatment option.

Limitations we faced in our study were mainly to do with sample size. Also determining the concentration of HA reached within the knee joint during an intra articular injection in a clinical setting, would be beneficial to help decide what concentrations of HA to study. This would involve studying average joint surface area in a defined population and estimation of synovial fluid volume.

5. Conclusion

Both low and high molecular weight hyaluronic acid did not have a beneficial biological effect on human articular chondrocytes. Both neither potentiated chondrocyte metabolism nor did they cause significant chondrotoxicity.

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