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Original Research

A novel glucosamine derivative with low cytotoxicity enhances chondrogenic differentiation of ATDC5

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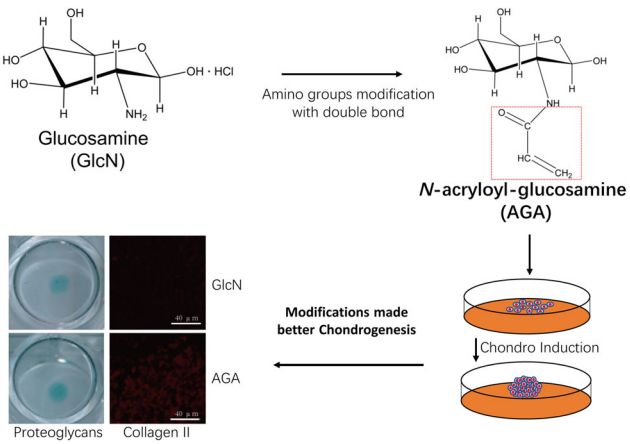
Abstract Glucosamine (GlcN) is a component of native cartilage extracellular matrix and useful in cartilage repair, but it was limited by toxicity in high concentrations. With the aim of altering bioactive properties of GlcN to reduce the toxicity and to facilitate chondrogenesis for hyaline cartilage formation, we introduced an amino-group modification with double bond into GlcN to produce *N*-acryloyl-glucosamine (AGA). The cell ATDC5 was chosen to evaluate its cytotoxicity and chondrogenesis capability. Cell proliferation and cytotoxicity assay showed that AGA had significantly reduced the cytotoxicity compared to GlcN, and promoted ATDC5 proliferation. Alcian blue staining and biochemical analysis indicated that AGA enhanced extracellular matrix deposition. Both the mRNA and protein levels of articular cartilage markers, like Collagen II and Aggrecan were up-regulated, as shown by quantitative real-time PCR and immunofluorescence staining. Moreover, the level of fibrocartilage marker Collagen I and hypertrophic marker Collagen X weren't significantly changed. Overall, these results demonstrated that the AGA achieved the functional double-bond, reduction in toxicity and enhancement in chondrogenesis could be more potential in cartilage repair.

Graphical abstract



1 Introduction

Cartilage tissue experiences high frequency of external stress, strain and loads every day. Because of its avascular characteristics, it has limited regeneration and self-healing ability once injured [1]. The method used to treat cartilage degeneration caused by trauma or diseases such as osteoarthritis is therefore of great significance. Oral administration of glucosamine (GlcN) is a commonly used method for the prevention and treatment of osteoarthritis [2, 3]. GlcN is a critical fundamental building block in the biosynthesis of glycolipids and glycosaminoglycans, that participates in the construction of human tissue and cell

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membranes [4]. The ability of GlcN to enhance the biosynthesis of cartilage-relevant proteoglycans and to prevent the occurrence of inflammation by inhibiting the activity of pro-inflammatory mediators has been proven by many researchers [5–7]. Because of the cytotoxicity of GlcN at high concentrations (10 mM or above) [4], researchers begin to investigate the optimal concentration of GlcN to enhance chondrogenic differentiation. Studies showed that GlcN has the same or even better effects on the reduction of the pain in patients with osteoarthritis compared to low concentrations of anti-inflammatory drugs, and the highest limitation concentration for chondrogenesis induction is 2 mM [8–10]. There are many active functional groups in GlcN, and GlcN modification can produce a variety of derivatives which have special biological activities. Among them, *N*-acetyl-glucosamine (GlcNAc) not only exhibits low cytotoxicity even at high concentrations, but also has an equal or even better effect on chondrogenesis compared to GlcN [11–13]. Thus, appropriate amino-group modification of GlcN is critical for its functional promotion, such as chondrogenesis.

ATDC5 derived from mouse embryonic carcinoma was isolated and expanded in 1990 by Atsumi [12, 14]. This cell line retains chondroprogenitor cell properties, exhibits more efficient chondrogenic differentiation ability and is more stable than other cell lines in vitro, thus it's widely used in the study of chondrogenic differentiation [15–17]. It has been proved that insulin could induce the chondrogenesis of ATDC5 cell in a postconfluent phase [18]. Consequently, it has functioned as an in vitro model to investigate chondrogenesis, as reported in many published studies [19, 20].

In this study, GlcN was modified with acryloyl chloride, which resulted in the addition of a double bond to GlcN to generate *N*-acryloyl-glucosamine (AGA). This molecule exhibited no obvious cytotoxicity to ATDC5 cells determined by water soluble tetrazolium (WST) assay even when added at high concentrations. It also exhibited improved chondrogenic ability when used at the same concentrations as GlcN and GlcNAc, demonstrated by the detection of cartilage-relevant extracellular matrix (ECM) deposition, and the expression of chondrogenesis markers both in mRNA and protein levels. Moreover, the functional double-bond would make AGA be applied in drug deliveries or bioactive graft about cartilage tissue repair.

2 Materials and methods

2.1 Materials

AGA was synthesized as previously described with minor modifications [21]. Briefly, a mixture of methyl alcohol and deionized water (v:v = 1:1) as solvent, GlcN and acryloyl chloride were reacted for 24 h in an ice bath. The product was purified by rotary evaporation, filtration and vacuum drying, resulting in AGA obtained as a white powder. The yield was approximately 38%. The AGA was analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Brucker Avance 600 MHz NMR spectrometer) and Fourier transform infrared spectrophotometry (FT-IR) (Bruker Vector 33 spectrometer).

2.2 Cell culture and differentiation

The ATDC5 cell line was acquired from Abgent (San Diego, USA). Cells were cultured with the medium of 1:1 mixture (v/v) of Dulbecco's modified Eagle medium and Ham's F12 medium (DMEM/F12, GIBCO) supplemented with 10% (v/v) fetal bovine serum (GIBCO). Cells were passaged at 80% confluence using 0.25% trypsin/EDTA and incubating at regular culture environment of 37 °C and 5% CO_2. The medium was changed every other day.

For chondrogenesis, the method of macromass was used. Briefly, cells were resuspended to 2×10^7 cells/ml after digest. Ten microliter cell suspension were dropped into the center of each well of 24-well plates (Corning, USA) and incubated in the incubator for 2–3 h, then, the cultures were maintained with chondrogenesis medium supplemented with 2, 5, 8 mM AGA (AGA 2, AGA 5, AGA 8), 2 mM GlcN (GlcN 2) or 5 mM GlcNAc (GlcNA 5) for 14 days, respectively. The control groups contained the negative control (ATDC5 groups), which culture with normal culture medium, and the positive control (N) with chondrogenesis medium. DMEM high glycose (Hyclone) supplemented with supplemented with 100 µg/ml sodium pyruvate (Gibco), 40 µg/ml L-proline (Sigma), (Sigma, USA), 50 mg/ml ITS-Premix (BD, USA), 50 µg/ml ascorbate-2-phosphate (Sigma), 100 nM dexamethasone, 1% penicillin/streptomycin (Hyclone) was used to induce chondrogenesis [22].

2.3 WST assay

The cytotoxicity of AGA, GlcN or GlcNAc to ATDC5 was characterized by WST assay following the instructions of cell counting kit-8 (CCK-8, Dojindo Molecular Technologies Inc, Kumamoto, Japan). Different concentrations of AGA, GlcN or GlcNAc were tested at the time points of day 1, 3, 5, 7. Briefly, the cells were seeded in 96-well plates at a density of 1000 cells/well and cultured with the medium containing different concentrations of AGA, GlcN or GlcNAc. At the indicating time point, the medium was discarded, and added CCK-8 working solution to incubate for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. To generate an IC_{50} curve, the medium of ATDC5 was added AGA or GlcN, and cultured for 72 h, then the metabolic

activity/toxicity screening was conducted with CCK-8. The OD value of absorbance was measured at 450 and 600 nm.

2.4 Alcian blue staining for ECM

Cells at the day 14 of chondrogenic differentiation were fixed with 4% paraformaldehyde for 12 h at 4 °C and stained with 1% Alcian blue 8GX (Sigma) water solution [17]. The stained cells aggregated in plates were washed at least three times with deionized water to guarantee only specific staining left and scanned using a flatbed optical scanner (HP, USA).

2.5 Biochemical analysis for ECM production

At the day 14 of chondrogenic differentiation, cells were washed with phosphate buffer saline for three times, cells in culture plates were snap-frozen in liquid nitrogen and lyophilized for 48 h. The remaining substance was harvested and digested for 16 h at 60 °C. DNA content was measured using Hoechst 33342 solution (Dojindo) [23]. Calf thymus DNA was used to generate a standard curve. Sulfated glycosaminoglycans (GAG) content was measured by 1,9-dimethylmethylene blue (DMMB) assay with chondroitin sulfate used to generate the standard curve [24].

2.6 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. ReverTra Ace qPCR RT Kit (Toyobo, Japan) was used to generate cDNA. Quantitative real-time PCR reactions were carried out and monitored using SsoAdvanced™ universal SYBR® Green on a CFX-96 Real-time PCR Detection System (BioRad, USA). The gene expression level was defined based on the threshold cycle (Ct), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the measured transcript. The samples were run in triplicate. The relative expression was calculated as $2^{-[(\text{Ct of type II collagen, aggrecan, type X collagen}) - (\text{Ct of GAPDH})]}$. The quantitative real-time PCR primers are listed in Table 1.

2.7 Immunofluorescence staining

The induced cells were fixed in 4% paraformaldehyde for 12 h at 4 °C. The cell aggregate masses were collected carefully from the plate and immersed a graduate concentrations of sucrose solutions to dehydrated for frozen section. The aggregate masses were embedded in frozen section medium (Leica, Germany) and cut into 10-μm thickness sections using a freezing microtome (Leica). For immunofluorescence staining, samples in section slides were permeabilized and blocked with 0.3% Triton X-100 and 10% goat serum for 2 h in room temperature. Samples the incubated with primary antibodies for Collagen II (1:200, ab34712), Aggrecan (1:200, ab36861), Collagen I (1:200, ab90395) and Collagen X (1:200, ab49945) at 4 °C for overnight, these antibodies were purchased from Abcam, washed with PBS for three times and incubated with the secondary antibodies (GIBCO) for 2 h at room temperature. Finally, samples were stained with DAPI for nuclear. Photo images were taken by a fluorescence microscope (Zeiss, Germany).

Table 1 List of primers used in the qRT- PCR analysis of gene expression in ATDC5 cells

Primer ID	Primers (5′-3′)
GAPDH-F	TGTGTCCGTCGTGGATCTGA
GAPDH-R	TTGCTGTTGAAGTCGCAGGAG
Collagen II-F	AGGGCAACAGCAGGTTCACATAC
Collagen II-R	TGTCCACACCAAATTCCTGTTCA
Aggrecan-F	AGTGGATCGGTCTGAATGACAGG
Aggrecan-R	AGAAGTTGTCAGGCTGGTTTGGA
Collagen I-F	ATGCCGCGACCTCAAGATG
Collagen I-R	TGAGGCACAGACGGCTGAGTA
Collagen X-F	CTCCTACCACGTGCATGTGAA
Collagen X-R	ACTCCCTGAAGCCTGATCCA

2.8 Statistical analysis

The results are presented as the mean ± standard deviation (STDEV) for at least three repeated individual experiments for each group using SPSS version 20.0 (SPSS, Chicago, IL, USA). Significant differences were confirmed by analysis of variance (ANOVA single-factor) for independent samples. Statistically significance was considered as $P < 0.05$.

3 Results

3.1 Syntheses and characterization of AGA

AGA was synthesized and confirmed by FT-IR and ^{1}H-NMR with GlcN and GlcNAc. The chemical structure of GlcN was shown in Fig. 1a. Compared to GlcN, AGA had an amide group, compared to GlcNAc, AGA had a double bond. As shown in Fig. 1b, an absorption peak at 1530 cm^{-1} represented the amide bond in both AGA and GlcNAc. A peak at 3072 cm^{-1} represented the double bond in the AGA only. Figure 1c showed the chemical shift of 6.2–6.4 and 5.67–5.68 ppm, indicating the formation of a double bond only in the AGA. Shifts at 5.18 and 4.63–4.64

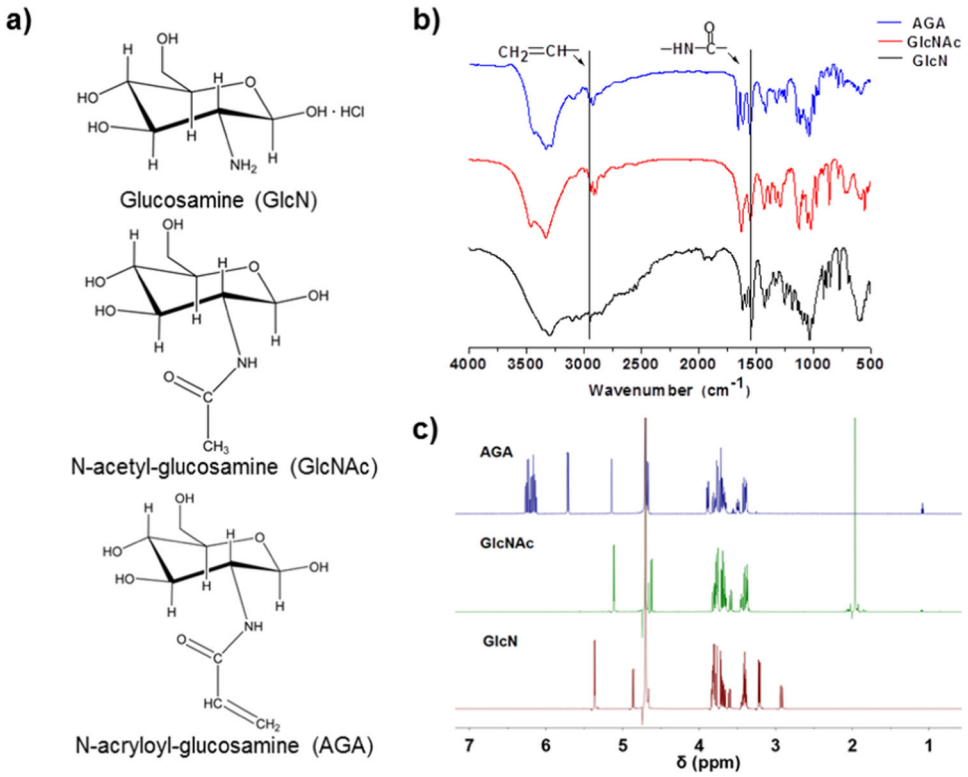

Fig. 1 Chemical structures and characterization of GlcN, GlcNAc and AGA. **a** Chemical structures of GlcN, GlcNAc and AGA. **b** The FT-IR spectrum of GlcN, GlcNAc and AGA. **c** ^{1}H-NMR spectrum of GlcN, GlcNAc and AGA

ppm indicated the formation of amide bonds in the AGA and GlcNAc groups. The result of FT-IR spectrum and ^{1}H-NMR spectrum not only indicated the successful synthesis of AGA, but also confirmed the chemical structure of the three types of small molecules used in subsequent experiments (Fig. 1b, c).

3.2 Cytocompatibility of AGA, GlcN, and GlcNAc

The inhibitory concentration 50% (IC_{50}) is defined as the half maximal inhibitory drugs concentration. It's always used to represent the cytotoxicity index of a drug [25]. Figure 2a showed the IC_{50} value of AGA was about 66 mM, much higher than that of GlcN, which was approximately 13 mM.

To further understand the cytotoxicity of AGA and GlcN, we chose an chondrogenesis effective concentration range (0–10 mM) to treat ATDC5 up to 7 days. Figure 2b showed that AGA didn't affect the cell proliferate in all concentration range, but GlcN had a significance inhibit effect on cell proliferation beyond concentration of 2 mM, which was consistent with the early research [9]. The toxicity reduction in AGA were proved by the assay, which met our aim.

Previous reports have showed that 2 mM GlcN and 5 mM GlcNAc are the best concentration for chondrogenesis, so we respectively treated with ATDC5 with GlcN (2 mM) and GlcNAc (5 mM) to compare the cytotoxicity and chondrogenesis with AGA [12]. Meanwhile, we used 2, 5 and 8 mM AGA to treat ATDC5 for 1 week. Figure 2c showed that ATDC5 proliferated normally in all groups. AGA not only had little cytotoxicity compared to GlcN and GlcNAc in the same concentration, but also promoted ATDC5 proliferation (Fig. 2c).

3.3 ECM accumulation in chondrogenesis with AGA, GlcN and GlcNAc treatment

The deposited cartilaginous extracellular matrix can be visualized by Alcian blue staining [17], because Alcian blue is a cationic dye which can chemically bind to acidic functional groups in GAG. As shown in Fig. 3a, the staining of the N group was stronger than the ATDC5 groups, which illustrated the basic chondrogenesis effect in the study. Compared to the N group, the groups containing GlcN derivatives exhibited stronger Alcian blue staining and the same trend with the early reports [12], demonstrating that three types of small molecules could enhance chondrogenesis indeed. But, it was visually obvious that AGA 2 and AGA 5 groups were corresponded to the highest accumulation of GAG, suggesting AGA had stranger extracellular matrix accumulation in chondrogenesis than GlcN and GlcNAc.

GAG contents were also quantified at Day 14 of the differentiation by DMMB assay [24]. As shown in Fig. 3b, compared to undifferentiated ATDC5 cells (ATDC5 group),

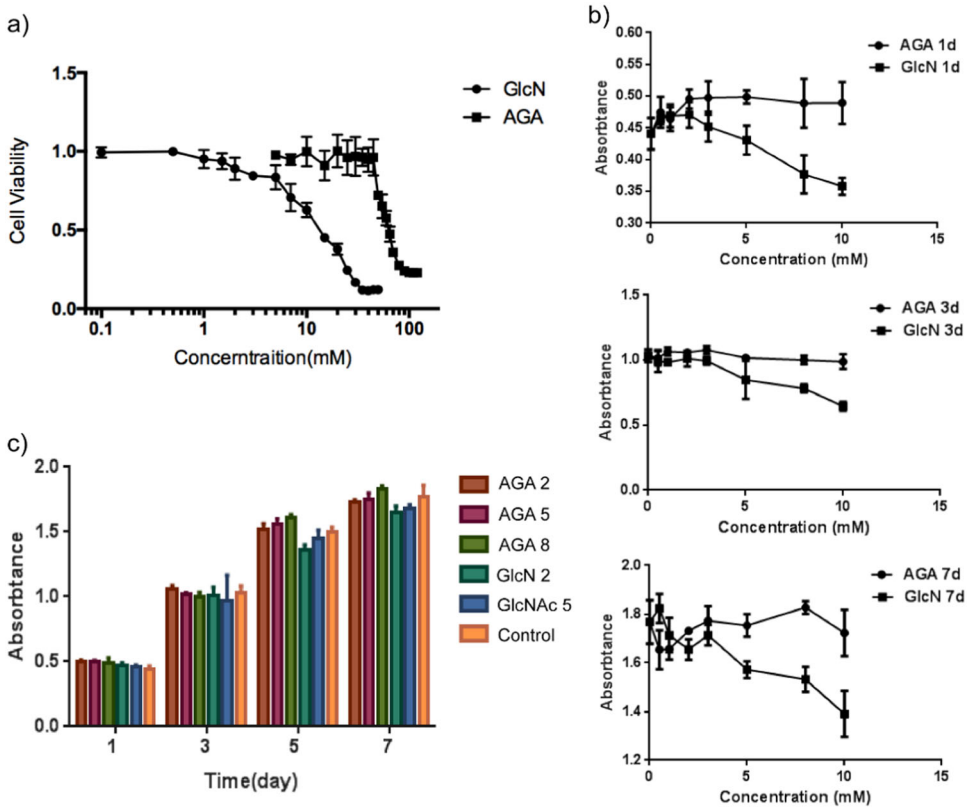

Fig. 2 Cell viability of ATDC5 cells when treated with different concentrations of AGA, GlcN and GlcNAc. **a** IC_{50} curves of AGA and GlcN. Cells were treated with AGA and GlcN for 72 h. **b** Survival curve of ATDC5 cells when treated with different concentrations of GlcN and AGA at day 1, day 3, and day 7. **c** Proliferation histogram of ATDC5 cells when treated with three types of molecules on experimental concentrations for up to 7 days

all groups exhibited a significant increase in GAG content, indicating that the induction method was an effective method for chondrogenic differentiation. The groups treated with small molecules accumulated more GAG than the N group, consistent with the results obtained with Alcian blue staining. Among the experimental groups, all AGA-treated groups accumulated more GAG than the GlcN- and GlcNAc-treated groups, while the AGA 2 and AGA 5 groups significantly promoted GAG secretion and accumulation ($P < 0.05$ compared to the GlcN and GlcNAc treatment groups), further revealing that AGA had stranger ECM accumulation in chondrogenesis than GlcN and GlcNAc.

3.4 mRNA expression of chondrogenesis phenotypes with AGA, GlcN and GlcNAc treatment

Cellular differentiation and the cartilaginous phenotypes were further characterized through analyzing the expression of chondrogenesis markers, which aimed to explore the chondrogenesis ability induced by treatment with AGA, GlcN and GlcNAc. Expression of cartilage-specific genes such as Collagen II, Aggrecan, Collagen I and Collagen X was examined at day 14 of chondrogenic induction using qRT-PCR. As shown in Fig. 4, compared to undifferentiated ATDC5 cells (negative control), the gene expression of the N groups was up-regulated significantly in all four genes, which showed the origin chondrogenesis effects and indicated that the differentiation method we chose was effective. The glucosamine and its derivants showed different regulations in specific genes.

Collagen II is a crucial protein in hyaline cartilage development [26]. Figure 4a showed that AGA 2 and AGA 5 groups both had significantly higher expression of Collagen II compared to all other groups ($P < 0.0001$ compared to the GlcN 2 group). Collagen II gene expression in the 2 mM GlcN-treated group was almost the same as the level in the 8 mM AGA-treated group, indicating that a high concentration of AGA might not enhance chondrogenesis compared to GlcN and 2 and 5 mM AGA treating had better chondrogenic ability. The 5 mM GlcNAc-treated group showed moderate mRNA expression of the Collagen II, which indicated that appropriate modification of GlcN could enhance chondrogenesis when administered at an appropriate concentration. In parallel to collagen II, all the experimental groups were up-regulated compared with the N group in mRNA expression of Aggrecan. Among them, the expression up-regulated significantly after treating with either 2 or 5 mM AGA, with the best enhancement exhibited by the 2 mM AGA-treated group (Fig. 4b). The Aggrecan gene expression level at high concentrations of AGA (8 mM) remained almost the same as that in the 2 mM

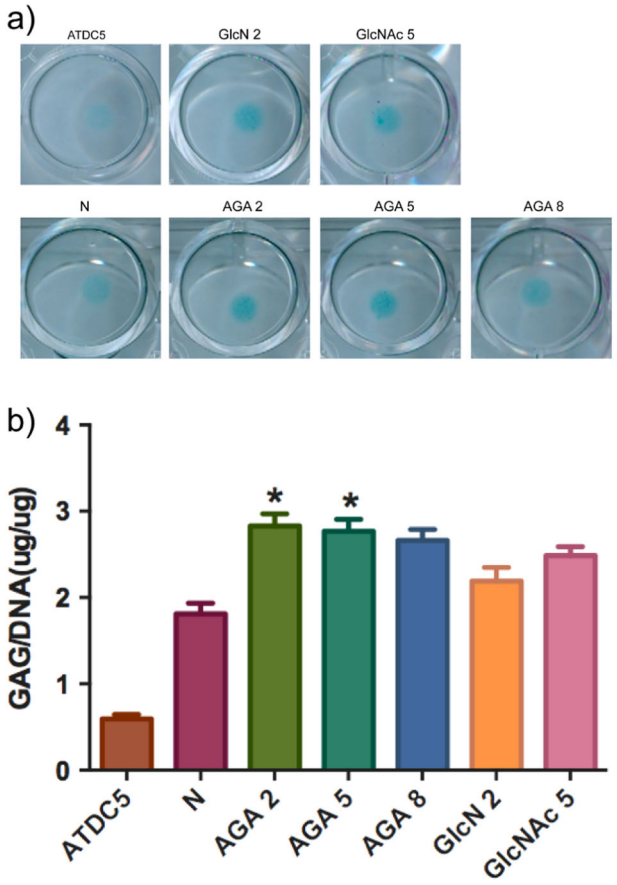

Fig. 3 GAG accumulation with AGA, GlcN and GlcNAc treatment. **a** Alcian blue staining of ATDC5 cells treated with AGA, GlcN and GlcNAc on day 14. **b** Biochemical analysis for GAG production of ATDC5 cells on day 14 with the treatment of AGA, GlcN and GlcNAc ($n = 3$, $^*p < 0.05$ vs GlcN 2 group) (color figure online)

GlcN-treated group. The 5 mM GlcNAc-treated group also exhibited a moderate gene expression level of Aggrecan in all the experimental groups.

In addition, we detected the gene expression level of Collagen I, since Collagen I is one of the markers of dedifferentiation which is found in fibrocartilage [26]. All the experimental groups were slightly up-regulated compared to the N group. Among them, except for 8 mM AGA-treated group, all AGA-treated groups showed higher expression compared to GlcN- and GlcNAc-treated groups (Fig. 4c). However, in the higher expression groups, such as the 2 mM AGA-treated group and 5 mM AGA-treated group, but it's expression hadn't any significant difference. In parallel to Collagen I expression, Collagen X, which is a maker of hypertrophic cartilage and was found in the late time of articular cartilage formation, all the experimental groups were significantly down-regulated compared to the N group, indicating that the glucosamine could inhibit the hypertrophic cartilage trends. During experimental groups, the results showed the same trend as Collagen I expression (Fig. 4d).

3.5 Chondrogenesis protein phenotypes following AGA, GlcN and GlcNAc treatment

Collagen II, Aggrecan, Collagen I and Collagen X protein levels were also assessed by immunofluorescence staining. As shown in Fig. 5, on day 14, positive staining of these proteins was observed in all of experimental groups although the intensity of the positive staining was different among the four proteins under the same experimental conditions. On the whole, the synthesis of cartilage-relevant proteins showed the same trend as the expression of cartilage-relevant genes. 2 mM AGA and 5 mM AGA treated groups had more Collagen II and Aggrecan expression compared to other groups, especially 2 mM GlcN- and 5 mM GlcNAc-treated groups. However, 5 mM GlcNAc did not exhibit an enhanced effect on Collagen II and Aggrecan expression compared to 2 mM unmodified GlcN addition (Fig. 5). This indicated that the change of the amino group to an acetyl group might not have an enhancing effect on chondrogenesis, while the change of amino group to acryloyl group might be beneficial in reducing the chondrogenic differentiation period. It was worth noting that the difference between AGA-treated groups and GlcN- and GlcNAc-treated groups were not obvious on Collagen I and Collagen X expression (Fig. 6a, b). However, compared to the N group, the AGA-treated group showed obvious inhibition of the expression of Collagen X. This indicated that a difference in gene expression might not result in a difference of protein synthesis, this might be caused by mRNA modification. In this experimental section, addition of low concentrations of AGA, especially the addition of 2 mM AGA, exhibited an enhanced effect on Collagen II and Aggrecan expression at the protein level but did not promote the synthesis of Collagen I or Collagen X proteins. These results suggested AGA had better ability for chondrogenesis compared to GlcN and GlcNAc at the same concentration.

4 Discussion

GlcN has been widely studied in chondrogenesis and anti-inflammatory to reduce the pain of osteoarthritis. The aims in this study are to overcome the drawbacks of GlcN, such as toxic at high concentrations, and improve its chondrogenesis ability by modifications in amino-group with a functional double-bond. Glucosamine is found to inhibit cell proliferation by affecting the cellular metabolism system, especially in high concentrations, this adverse effect is attributed by its amino-group, which is proved by comparing the cell viability of three pairs of amino sugars, GlcN, mannosamine, galactosamine and their acetylation derivants included [27]. Other studies also suggest that the

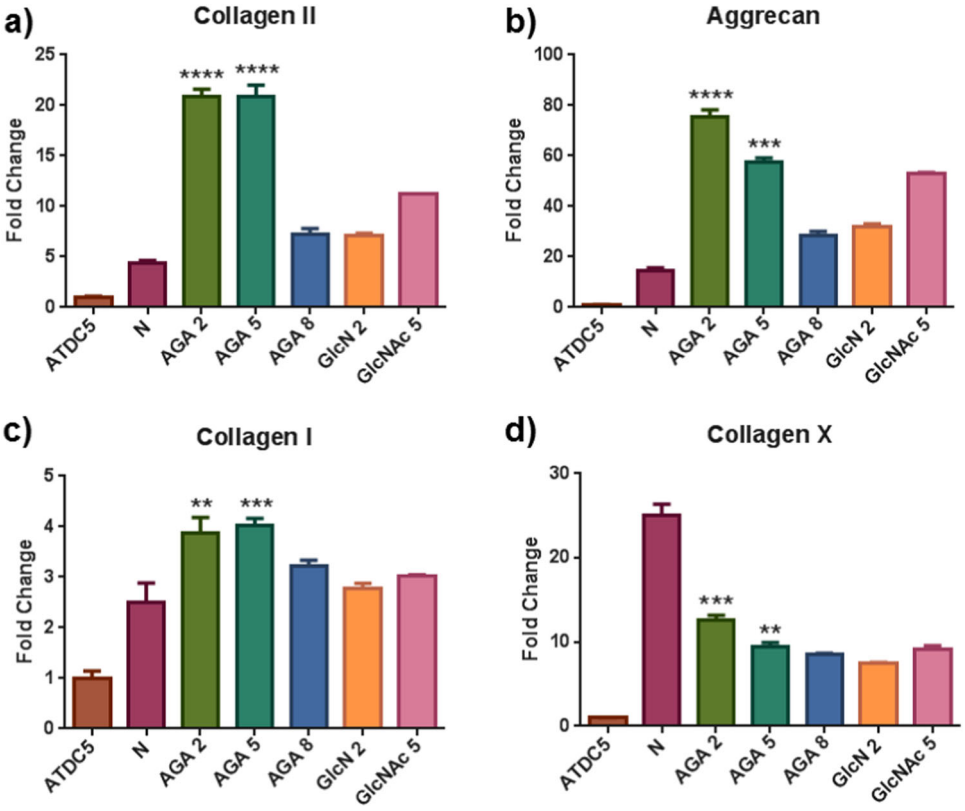

Fig. 4 Gene expression analysis of ATDC5 cells with treatment of AGA, GlcN and GlcNAc on day 14. **a** Collagen II gene expression. **b** Aggrecan gene expression. **c** Collagen I gene expression. **d** Collagen X gene expression. The fold change of each gene was normalized against ATDC5 group and GAPDH was used as a reference gene ($n = 3$, $^{**}p < 0.01$ vs. GlcN 2 group, $^{***}p < 0.001$ vs. GlcN 2 group, $^{****}p < 0.0001$ vs. GlcN 2 group)

hexosamine structure with amino groups would affect the cell attachment, leading cell death [9, 28]. In this study, the modification of introduction the double-bond was also at the position of amino-group in glucosamine and was successfully achieving AGA proved by structure characterizations (Fig. 1). The greater IC_{50} value (66 mM) of AGA indicated its lower cytotoxicity to ATDC5 cells after modification. It was consisted with early studies that the amino-group modification was useful in reducing the cytotoxicity of GlcN (IC_{50}: 13 mM). Apart from this, AGA also increased the cellular proliferation ability compared to GlcN and GlcNAc at certain concentrations (Fig. 2c). Previous research has reported that the butyryl GlcN also showed a significant up-regulation of chondrocytes proliferation and proteoglycans secretion [28]. Although the reason is not clear, it is indicated that functional acylation of GlcN could promote its proliferation.

Good facilitating ECM production ability in chondrogenesis and anti-inflammatory effect keeping well articular chondrocytes phenotype of GlcN is the basic for our functional modification. Although its regulatory mechanism is not totally clear, the earlier studies thought GlcN promotes chondrogenesis not just through one manner. First, As the building-block of proteoglycans, GlcN's primary role is to synthesize GAGs and hyaluronic acid [29]. The exogenously supplied GlcN at proper concentration could directly incorporated into ploysaccharide synthesis [30]. Second, the GlcN could influence the hexamine biosynthesis pathway to modulate the transforming growth factor β1 (TGF-β1) expression, which is very important for chondrogenesis with articular cartilage phenotypes [4, 31]. but in the process of the GlcN with amino groups promoting chondrogenesis would depletion of adenosine triphosphate (ATP) levels, especially in high concentrations, but the GlcNAc hasn't this effect [32–34]. In present studies, the AGA at 2 and 5 mM significantly improved the GAG accumulation compared to the GlcN 2 (Fig. 3), and increased Collagen II and Aggrecan expression both in mRNA and protein levels. we thought it was attributed to AGA not only maintained the incorporated into polysaccharide synthesis, but also provide a better chondrogenesis by stimulating TGF-β1, and didn't depletion of ATP.

Collagen I and Cartilage X indicate the fibrocartilage and hypertrophic cartilage develops, respectively, they are also the markers for the bone formation. Previous study reports that GlcN would reduce the ALP activity in mineralization of ATDC5, but GlcNAc would not [12], indicating that the acetylation would reduce inhibition effect of GlcN on the bone formation. Our study showed that AGA promoted Collagen I and X expression in 2 and 5 mM in mRNA levels compared to GlcN and GlcNAc (Fig. 4). But they

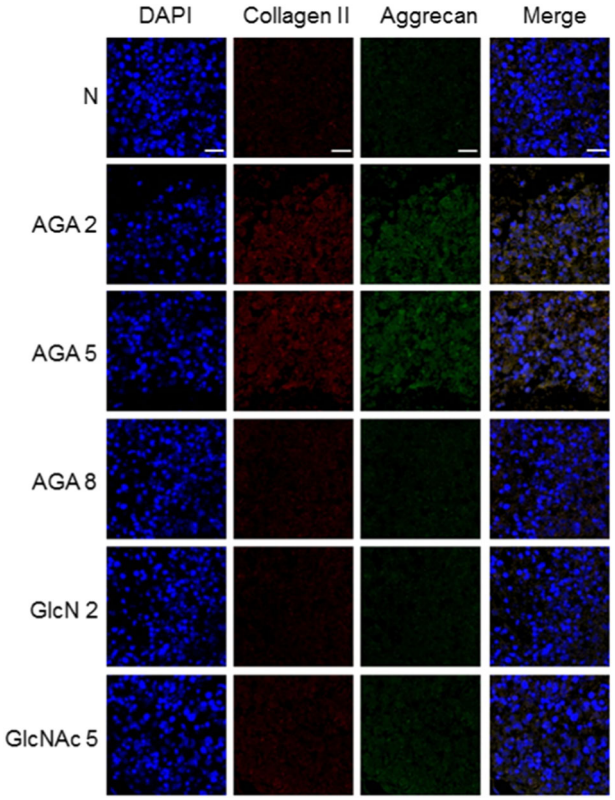

Fig. 5 Immunofluorescence staining for chondrogenic markers collagen II and aggrecan from undifferentiated ATDC5 cells after 14 days of chondro-induction. Collagen II protein was stained with red, aggrecan protein was stained with green and nuclei was stained with blue. Scale bar = 20 μm (color figure online)

hadn't significantly changed their protein levels (Fig. 6), suggesting AGA increased the hyaline cartilage differentiation ability compared to GlcN and GlcNAc

5 Conclusions

In this study, amino-group modification in GlcN achieving AGA was accomplished to overcome GlcN's drawback of toxicity in high concentrations and enhance its advantage of positive effect on chondrogenic differentiation. Several characterizations proved that chemically modifying GlcN with acryloyl chloride not only reduced its cytotoxicity, but also enhanced chondrogenesis in an excellent in vitro model cell line, ATDC5. Compared to GlcN and GlcNAc, AGA has the best effect on chondrogenesis under the concentration of 2 and 5 mM. Taken together, inducting a double-bond in the amino group of GlcN might be application for in drug deliveries and bioactive graft in cartilage repair.

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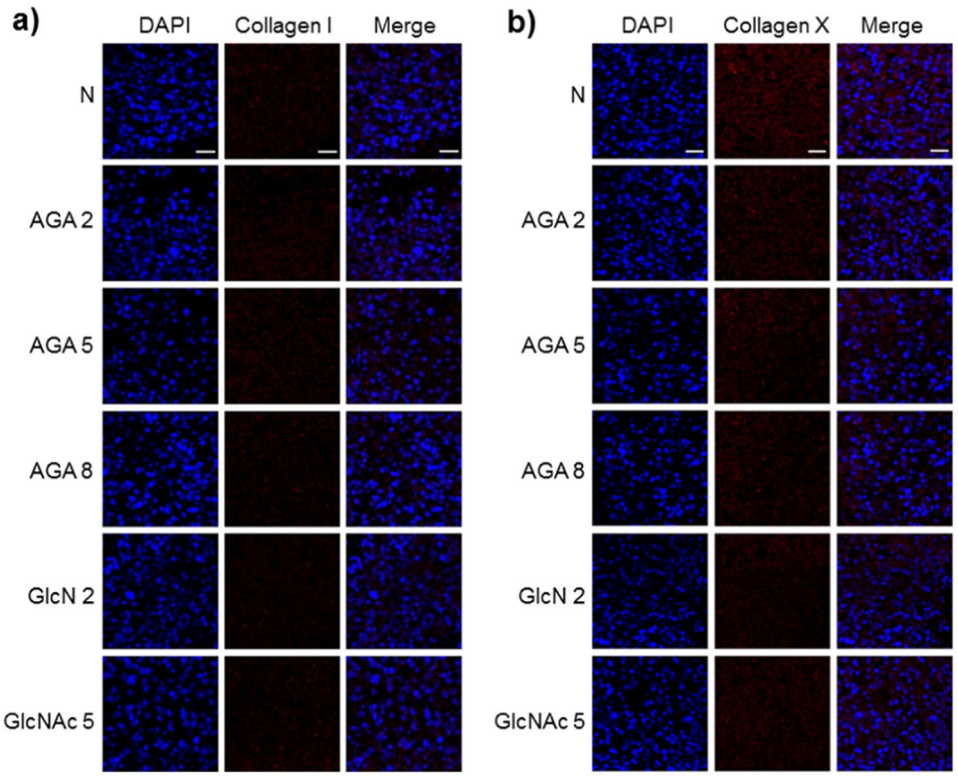

Fig. 6 Immunofluorescence staining for fibrous and hypertrophic cartilage markers collagen I and collagen X after 14 days of chondro-induction. **a** Synthesis of Collagen I protein (nuclei stained with blue and collagen I stained with red) which is an indicator of fibrous cartilage formation. **b** Synthesis of Collagen X protein (nuclei stained with blue and collagen X stained with red) which is an indicator of hypertrophic cartilage formation. Scale bar = 20 μm (color figure online)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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