From medical strategy to food-borne prophylactic strategy: stabilizing dental collagen with aloin

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Abstract

Objectives: We developed a food-borne prophylactic strategy with aloin to protect dental collagen through enhancing the mechanical strength, thermostability, and the resistance to enzymatic hydrolysis of dental collagen. The mechanism and effect of this food-borne prophylactic strategy were examined.

Methods: Aloin solution (0.1 mg/mL) was prepared. The concentration is equivalent to the natural content of aloin in edible aloe. Attenuated total reflectance flourier transform infrared spectroscopy (ATR-FTIR), Gaussian peak fitting, and X-ray diffraction (XRD) were used to explore the mechanism of interaction between aloin and dental collagen in situ. Mechanical strength and thermostability were separately evaluated via Ultimate tensile strength test (UTS) and thermogravimetric analysis (TGA). Hydroxyproline (HYP), cross-linked carboxy-terminal telopeptide of type I collagen (ICTP), and C-terminal crosslinked telopeptide of type I collagen (CTX) were used as indicators to evaluate the resistance to enzymatic hydrolysis. Wight loss was further tested as macro-parameter to evaluate the effectiveness of aloin on dental collagen stability.

Results: Aloin can interact with dental collagen via hydrogen bonds in humid environment. The interaction shorted the intermolecular distance and enhanced the proportion of stable α-helix chain of dental collagen. Aloin-treated dental collagen exhibited improved mechanical strength, thermostability and enzymatic stability.

Conclusion: Aloin can strongly integrate with dental collagen under physiological conditions via hydrogen bonds and significantly enhance the dental collagen stability.

Clinical Relevance: The use of aloin maybe a daily, nondestructive, and low-cost strategy to protect dental collagen.

1. Introduction

Infectious oral diseases such as caries, pulpitis and periapical periodontitis have become a global public health problem for decades [1–3]. In many low- and middle-income countries, plenty of patients cannot afford the medical expenses for these oral diseases [4,5]. Continuing deterioration of these oral disease further cause systemic infection such as sepsis, multiple organ failure, and even death [6,7]. With the invasion of bacteria, enzyme, and temperature variation, apatite mineral crystals of enamel and dentin collapse incrementally, exposing the organic dental collagen network [8–10]. Denuded dental collagen is much more susceptible to these exogenous infections and their triple helix structure will degrade rapidly. The loosening of collagen structure and the high porosity of collagen network provide more possibilities for the diffusion of these infectious substance. Further, the diffusion of acidic metabolites produced by bacteria makes the infectious area much larger than the zone of bacterial invasion. Hence, it is significant for dentists to extend the scope of surgery to adequately eliminate the infectious tissues. However, the traumatic strategy increases the risk of secondary infection [11]. Furthermore, teeth of people in high-carbonhydrate diet and the patients suffered from temporomandibular disorders, excessive occlusal

forces, and tooth wear are also susceptible to these infectious oral diseases [12,13]. Hence, it is urgent to explore a daily, nondestructive, and low-cost strategy to enhance the dental collagen stability.

In medicine, dental bonding strategy has been proposed to physically isolate the dental collagen from exogenous infectious factors through adhesive resin. Adhesive resin can penetrate into the dentin collagen network and wrap with the collagen fibrils through micro-mechanical interlocking [14]. However, this physical interaction is susceptible to moisture, bacteria, and biomechanics [15–17]. Other studies proposed to rebuild the "inorganic-organic" hybrid structure of dentin through remineralization [18]. Scientists use amorphous mineral precursor nanoparticles like bioactive glass, calcium phosphate, and silica to reproduce the mineralization process [19–21]. However, the period of remineralization is so long to be used for clinical and daily application [22]. The introduction of antibacterial agent such as quaternary ammonium salt, metal ions (Ag^+, Cu^{2+}, Zn^{2+}, etc), and antibiotics can strongly resist the bacterial infection [23,24]. While this strategy is limited by their toxicity and drug resistance. Furthermore, these strategies need to be executed by professional doctors, and it cannot be applied universally by the general population.

Modification of collagen structure based on exogenous agents is a non-invasive strategy to improve the structural stability of dentin collagen. Some synthetic agents, such as glutaraldehyde, dopamine methacrylamide, and isocyanates can chemically interact with amino acid residues of collagen, improving collagen stability [25-27]. However, their toxicity makes them not suitable for daily use and medicine [28,29]. Meanwhile, water molecules can competitively bind with the active group (-OH, $-NH_2$) of dental collagen, restricting the chemical interaction between these agents and collagen [30]. Natural agents including tangeretin, procyanidine, and hesperidin are regarded as candidates to enhance dental collagen structure in humid environment [31]. Compared to artificial agents, they present better biocompatibility, biosecurity and edibility [32]. However, these plant-derived agents need to be purified from the seeds and peels [33]. Aloin, as the main component of aloe, has the potential to be the candidate for food-borne prophylactic strategy because it can be ingested directly [34]. Therefore, present study aims to explore the influence of aloin on dental collagen structure and the effect on enhancing dental collagen stability in situ. The null hypothesis tested is that the application of aloin has no effect on improving dental collagen stability.

2. Materials And Methods

2.1 Reagents and dental collagen preparation

Aloin (CAS: 8015-61-0) and phosphoric acid (PA) were purchased from MedBio (shanghai, China). Sixty non-caries, intact human third molars were collected with the approval by the Institutional Review Board of the last author's institute (#IRB-S2021-655-01). All extracted teeth were used within 1 month after extraction.

Twenty-six dentin slices (10.0 mm diameter, 1.0 mm thick), twenty dentin beams (1.0 mm × 1.0 mm × 6.0 mm), and 310 mg dentin powder were prepared as previously [27]. All specimens were demineralized in 10 wt % PA for 24 h and then rinsed with deionized water for 20 min to prepare dental collagen. Aloin solution (0.1 mg/mL) was prepared through mechanical stirring for 10 min and ultrasonic agitation for 10 min. The tested concentration is based on the natural proportion of aloin in aloe. Aim to evaluate the effect of aloin, half of randomly selected specimens of demineralized dental collagen were used as the control group (DDC group). The other specimens were treated with aloin solution for 2 min and then rinsed with deionized water for 1 min. Aloin treated dental collagen were used as the experimental group (A-DDC group).

2.2 The influence of aloin on dental collagen structure

2.2.1 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and peak fitting

ATR-FTIR (Shimadzu, Tokyo, Japan) was used to explore the interaction between aloin and dental collagen in situ. Demineralized dental collagen slices were used as specimens and placed on the diamond crystal top plate of ATR-FTIR with a gauge force of 100 N. The characteristic peak of amide I, amide II and amide III of DDC and A-DDC group were recorded. The wavelength range was set at $400\sim4000$ cm^{-1} with a resolution of 2 cm^{-1}. Background spectra were collected with no sample on the ATR crystal top plate and this spectrum was subtracted from all absorbance spectra. Gaussian peak fitting was used to quantify the content of each secondary structure of dental collagen (n=3) [35].

2.2.2 X-ray diffraction (XRD)

The crystallinity index of dental collagen specimens was analyzed with XRD (Bruker D8, Germany) using CuKα radiation (40kV, 40mA). Samples were scanned from 0° to 70°.

2.3 Mechanical stability

The ultimate tensile strength (UTS) before and after thermocycling were tested to evaluate the effect of aloin on dental collagen mechanical stability. Twenty dental beams were used in this test. Half of randomly selected specimens were used to evaluate the immediate UTS. Thermocycling was used to evaluate the effect of aloin on the other half of dental collagen stability according to the ISO TR 11,450 standard (1994). All specimens were tested at a crosshead speed of 1 mm/min on a microtensile testing machine (EZ-TEST 500 N, Shimadzu, Japan). Maximum load (N), length (L) and width (W) of the cross sectioned area were measured when the specimens until failure. The UTS (E) of each group of was calculated according to the formula: E= N/(L×W) (n=5).

2.4 Thermostability

Forty-eight mg demineralized dentin powder was divided randomly into two groups. For each group, 8.0 mg of powder was initially used and dried in a vacuum desiccator with silica gel for 24 h before thermogravimetric analysis (TGA, Mettler Toledo, Columbus, OH, USA) from room temperature to 300 °C

at a heating rate of 10 °C/min under N_2 atmosphere (flow rate= 10 mL /min). The five percent mass loss temperature ($T_{95\%}$) was recorded (n=3).

2.5 Enzymatic stability

2.5.1 Release of ICTP and CTX

For each group, 5.0 mg powder were immersed in 2.0 mL artificial saliva at 37 °C to simulate the influence of oral environment for 7 and 14 days. The culture solution was taken and the amount of ICTP and CTX were separately measured using ICTP (Finetest, Wuhan, China) and CTX ELISA kits (IDS, England). The standard curves were plotted according to the requirements of the kit before measurement. OD 450nm was recorded and the concentration of ICTP and CTX were calculated based on standard curve (n=5).

2.5.2 Release of Hydroxyproline (HYP)

The content of HYP in extract is a convincing indicator for collagen degradation. High-performance liquid chromatography (HPLC; Agilent Technologies, Santa Clara, CA, USA) was used to test the release of HYP of each group (n=3). Two groups of demineralized dentin powders (5.0 mg) were immersed in 2.5 mL of collagenase solution (50 μg/ mL, Invitrogen, Carlsbad, CA, USA) for 24 h and 48 h. Detail procedure is the same as we previously published [36].

2.5.3 Weight Loss

Twenty slices of DDC and A-DDC groups were all dried as mentioned in section 2.4 and weighted (m_1). Then the specimens were immersed in collagenase solution (50 μg/ mL) for 7 days and 14 days respectively. Specimens should be washed and dried until the mass unchanged and weighted (m_2). Specimens at the corresponding time point for each group should be washed, dried until the mass unchanged, and then weighted (m_2). The weight loss (W%) was described by the percentage weight loss of individual samples, calculated by the following formula: W%= (m_1-m_2)/m_1×100% (n=5).

2.6 Statistical analysis

After normal distribution and variance evaluation by D'Agostino-Pearson test and Fisher's test, the data of mechanical stability and enzymatic stability were submitted to two-way analysis of variance (ANOVA) and Bonferroni's repeated measure with Graph Pad Prism version 8.0 (La Jolla, CA, USA, RRID:SCR_002798). Student's T-test was used to compare the $T_{95\%}$ and secondary structure parameters in curve fitting. The significance level value was 0.05.

3. Results

3.1 The influence of aloin on dental collagen structure

The schematic diagram of the dental collagen preparation and modification process was shown in Fig. 1. Both of DDC and A-DDC exhibited the typical XRD spectrum of dental collagen (Fig. 2a). The DDC showed XRD peaks at 2θ= 7.51 ° (d= 11.76), 21.01 ° (d= 4.23), and 31.21 ° (d= 2.86). The A-DDC exhibited three characteristic peaks at 2θ= 7.56 ° (d= 11.68), 20.80 ° (d= 4.27), and 31.61 ° (d= 2.83). Nevertheless, an increased intensity of Peak I and a decreased intensity of Peak II and Peak III were detected in A-DDC compared to DDC.

The spectrum of ATR-FTIR of both DDC and A-DDC showed the characteristic amide I, amide II, and amide III peaks of dentin collagen (Fig. 2b). After modification with aloin, the amide I peak of collagen displayed a blue shift from 1639.38 cm^{-1} to 1635.52 cm^{-1}. Meanwhile, the spectrum intensity of A-DDC showed a little decrease compared to DDC, further convincing the interaction between aloin and dental collagen.

The curve fitting procedure allowed determining the secondary structure composition in both groups (Fig. 2c and 2d): α-helix (1650−1660 cm^{-1}), β-sheet (1600−1640 cm^{-1}), β-turn (1660−1700 cm^{-1}), and random coil (1640−1650 cm^{-1}). The intensity of signals decreased after pretreatment with aloin compared to the DDC. The subgroup distribution of the secondary structure was identical but the proportion was different after modification. As shown in Fig. 3a and 3d, the proportion of α-helix increased from 33.40 ± 0.90% to 38.51 ± 1.17% (*p< 0.01*), and the proportion of random coil decreased from 18.70 ± 0.56% to 7.78 ± 0.54% (*p< 0.0001*).

3.2 Mechanical stability

The results of UTS were plotted in Fig. 4a. For immediate UTS, significant difference was noted in A-DDC (24.09 ± 3.47 MPa) compared with DDC (17.16 ± 2.26 MPa) (*p< 0.01*). There were sharp decreases in UTS of both groups with the thermocycling protocol. While A-DDC presented a 33.62% decrease in UTS, the value (15.99 ± 0.39 MPa) remained significantly higher than that of DDC (6.24 ± 1.23 MPa, dropped by 63.64%) (*p< 0.0001*).

3.3 Thermostability

The thermostability of collagen with aloin treatment was evaluated by TGA analysis. As shown in Fig. 4b, the $T_{95\%}$ of the treated collagen was higher than that of the untreated collagen (*p< 0.05*). For DDC, $T_{95\%}$ was 108.60 ± 5.60 °C. After being treated by aloin solution for 2 min, the $T_{95\%}$ rose significantly to 122.70 ± 2.79 °C.

3.4 Enzymatic stability

Release of ICTP and CTX

Fig. 5a summarized the release of the telopeptide ICTP by the endogenous MMPs of dentin. The mean liberation of the more ICTP 20.42 ± 2.48 ng/mg dry dentin for 7 days occurred in DDC, whereas A-DDC

liberated 13.76 ± 2.21 ng/mg dry dentin ($p< 0.001$). The content of ICTP in both groups for 14 days are higher than them for 7 days ($p< 0.05$). A-DDC still showed a significantly lower rate of ICTP telopeptide release for 14 days (19.30 ± 0.89 ng/mg dry mass) than DDC (25.08 ± 2.15 ng/mg dry dentin) ($p< 0.01$).

Fig. 5b revealed the release of the telopeptide CTX produced by cathepsin-K. Similar to ICTP, the demineralized specimens in contact with aloin showed the release of CTX telopeptide both for 7 days (79.24 ± 6.16 pg /mg dry dentin) and 14 days (135.20 ± 5.73 pg/mg dry dentin) significantly lower than that in DDC (129.10 ± 5.30 and 161.40 ± 5.41 pg/mg dry dentin, respectively) ($p< 0.0001$).

Release of HYP

The resistance to collagenase digestion was also determined by measuring free HYP. As shown in Fig. 5c, no significant difference was observed for 24 h between A-DDC (0.08 ± 0.01 mg/g dentin) and DDC (0.09 ± 0.01 mg/g dentin) ($p= 0.17$). After 48 h, significantly lower release of hydroxyproline occurred in A-DDC (0.10 ± 0.00 mg/g dentin) as compared to DDC (0.21 ± 0.01 mg/g dentin) ($p< 0.0001$).

Weight Loss

Fig. 5d showed the weight loss of demineralized dentin for 7 and 14 days. The A-DDC showed a W% with $4.95 \pm 0.72\%$ for 7 days which was obviously lower than that in DDC ($7.15 \pm 0.86\%$, $p< 0.01$). The W% in both groups increased with time and it was significantly lower in A-DDC ($8.10 \pm 0.77\%$) compared to DDC ($10.84 \pm 0.60\%$) for 14 days ($p< 0.001$).

4. Discussion

In the present work, the authors have developed a new non-invasive and dietary strategy for enhancing dental collagen stability with aloin. Results indicate that this strategy can significantly improve the mechanical strength, thermostability and the capacity on resisting enzymatic hydrolysis of dental collagen. Hence, the null hypotheses should be rejected.

Phenolic compounds such as catechol, polyphenols, and procyanidine have been proposed as collagen modifiers [37–39]. Previous researches have proposed they can interact with collagen via covalent bonds, hydrogen bonds and van der Waals forces [40]. While, in this article, the blue shift of amide I from 1639.38 cm^{-1} to 1635.52 cm^{-1} suggests that aloin can interact with dental collagen via hydrogen bonds. The mechanism of interaction is attributed to the hydrogen bonds between the phenolic hydroxyl groups of the natural compounds and amino or amide groups of dentin collagen.

The increased area of the characteristic peak located in 1600 cm^{-1} ~ 1700 cm^{-1} interval of the ATR-FTIR spectra and the increase in intensity of Peak I in XRD suggest that the dental collagen of A-DDC group exhibits an enhanced proportion of α-helix. As the most stable secondary structure in collagen type I, an increase in content of α-helix demonstrates the enhancement of dental collagen stability [41]. Meanwhile, the proportion of relatively unstable structures like β-sheet, and especially random coil shows a

significantly decrease based on the results of curve fitting [42]. The XRD spectrum exhibits a similar tendency that the intensity of Peak II decrease, demonstrating that the dental collagen of A-DDC compared to DDC containing less content of random coil. These phenomenon evidences that the interaction between aloin and collagen improve the structural stability. Meanwhile, the decreased d value of Peak I of A-DDC in XRD suggests that the dental collagen treated with aloin exhibit a smaller interplanar spacing. This phenomenon illustrates that the hydrogen bonds between dental collagen and aloin can reduce the intermolecular distance of dental collagen fibrils, further improving the stability of collagen structures.

Intraoral temperature, bite force and enzyme play significant roles in dentin fate determination [43]. Hence, we chose UTS, TGA, and enzymatic stability tests to assess the effectiveness of aloin on dentin collagen. The increase of UTS and $T_{95\%}$ of A-DDC group are adjunctive evidences for the interaction between dental collagen and aloin. Hydrogen bonds between aloin and collagen increases the collagen density per unit volume [44]. The enhanced binding force in the form of closer intermolecular distances is manifested macroscopically as an increase in UTS. In addition, the UTS of the specimens in A-DDC is significantly higher than that in DDC group after rinsing with deionized water, laterally indicating the interactions remain stable in the humid oral environment.

In terms of thermostability of collagen, the modification may stabilize the structure of the thermally labile domains by forming more hydrogen bonds [45]. A shorter axial distance among amino acid residues indirectly confirms an improved hydrogen bonding content, according to the d value of Peak III in XRD reduced from 2.86 to 2.83. Furthermore, aloin can elevate the proportion of stable secondary structure to increase the stability of thermally-labile domains with dentin collagen. Besides, the temperatures of weight loss are also related to the molecular weight of the reaction system. The graft of aloin can significantly increase the molecular weight of dental collagen and the higher molecular weight also explain why dentin collagen modified with aloin exhibited increased thermostability [46].

The findings prove that aloin has abilities to resist collagenase as measured by the release of ICTP, CTX and HYP, as well as confirmed by the weight loss. The released HYP is due to the action of bacterial-collagenase and other dentine-bound proteases and give indication of tissue collagen concentration [47]. The release of ICTP and CTX fragments represents specific degradation products resulting from MMPs and cathepsin K-mediated activities [48]. Weight loss is the macroscopic manifestation of enzymatic degradation [49]. Effective inhibition of collagenase by aloin occurs in our work is due to several reasons. Firstly, the stabilized collagen matrix acts as a mechanical barrier, preventing unwinding of the triple helix, which is necessary for exposure of the catalytic site of collagenase to cleave. Secondly, aloin promotes conformational changes of collagen and alter the configuration of the active site of collagenase, making protease unable to identify the complementary peptide sequence for collagen. Furthermore, aloin may exert additional effect directly on collagenase via chelation and hydrophobic interactions like other plant-derived polyphenols, reducing collagenase's molecular mobility and altering the 3-D structure of their catalytic or allosteric domains.

While, previous researches have proven that phenols can interact with intraoral microbiota to degrade their activity, enhancing the antibacterial capacity of dental collagen [50]. Whether aloin has this effect are in investigation. Also, studies should be designed to quantify the in−situ reaction kinetics to further explore the daily intake of aloin.

5. Conclusion

A food-borne prophylactic strategy using aloin to enhance the dental collagen stability has been developed. Aloin can interact with dental collagen through hydrogen bonds. Using of this strategy can improve the mechanical strength, thermostability and the enzymatic stability of dental collagen. This daily, nondestructive, and low-cost strategy has the potential to significantly enhance the dental collagen stability, decreasing the risk of tooth infection.

Declarations

Acknowledgements

Author contributions

C.Z. Jia, H. Li and Z.L. Yang performed the methodology, investigation, validation, formal analysis and writing − original draft. R.C. Xu contributed to the conceptualization of intellectual content, writing − review & editing and supervision. L.J. Wang and H.B. Li are devoted to writing − review & editing, supervision, funding acquisition. All the authors reviewed and approved the final form of the manuscript.

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Data Availability

All data generated or analyzed from this study are included in this published article.

Ethical approval This study was performed in accordance with the Declaration of Helsinki and the study protocol was reviewed and approved by Ethics Committee of Chinese PLA General Hospital (#IRB-S2021-655-01).

Consent for publication Not applicable.

Conflict of Interest The authors declare no conflicts of interest

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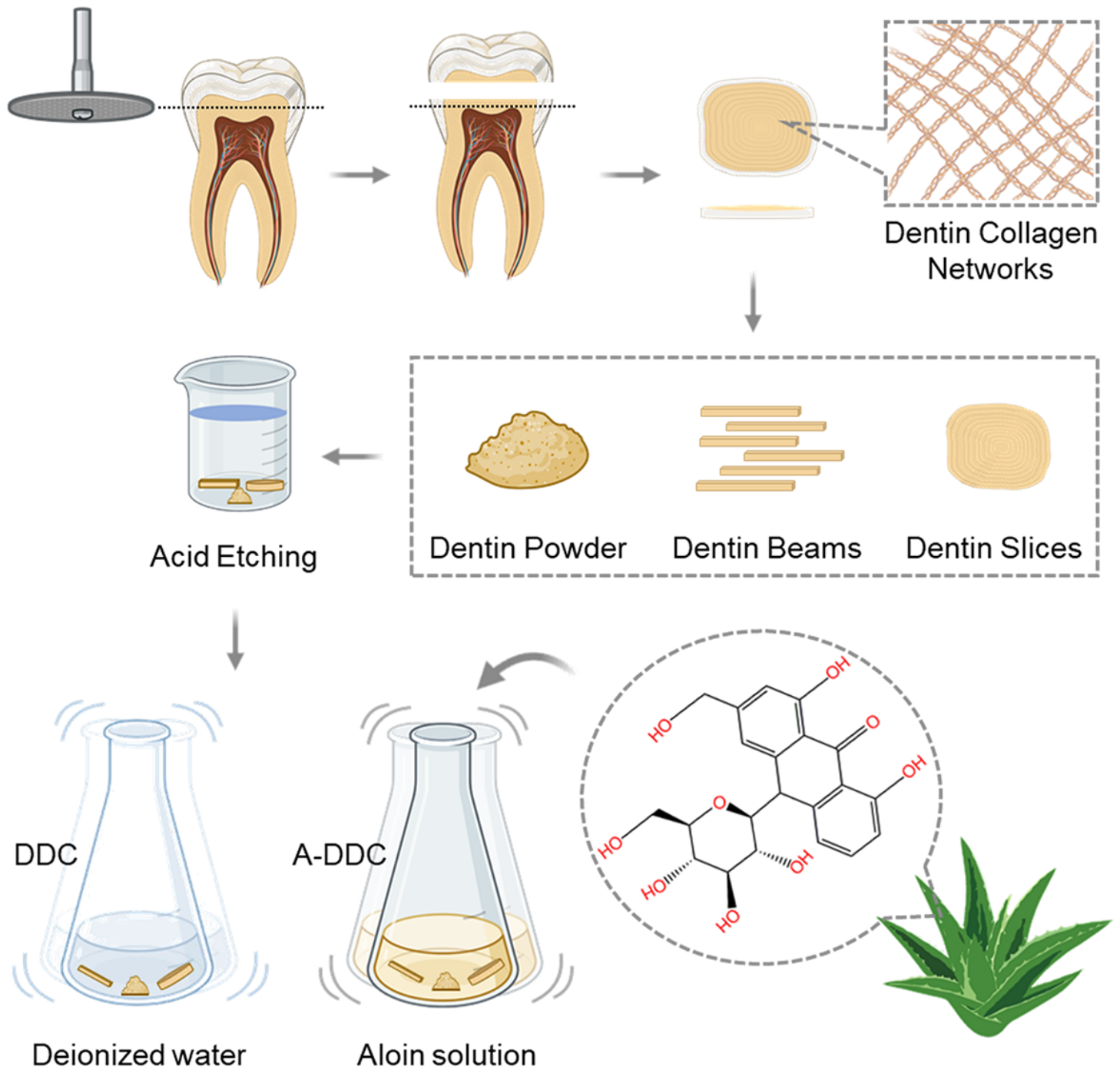
Figures

Figure 1

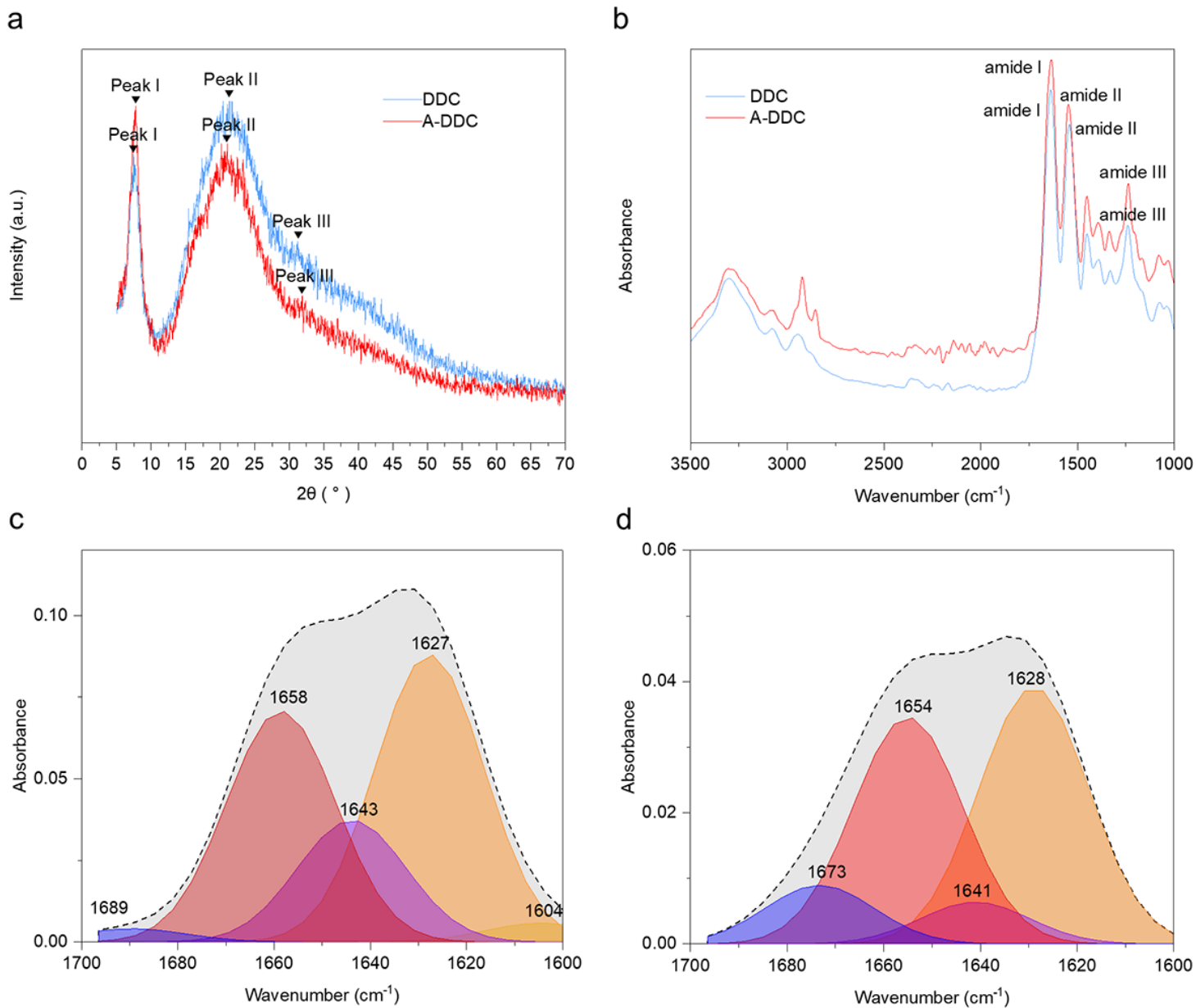Schematic diagram of the preparation of DDC and A-DDC

Figure 2

Interaction between aloin and demineralized dentinal collagen matrix (a) XRD pattern and (b) infrared spectra of DDC and A-DDC. Gaussian deconvolution of the characteristic peak located in the 1600 cm^{-1} to 1700 cm^{-1} of DDC (c) and A-DDC group (d), respectively
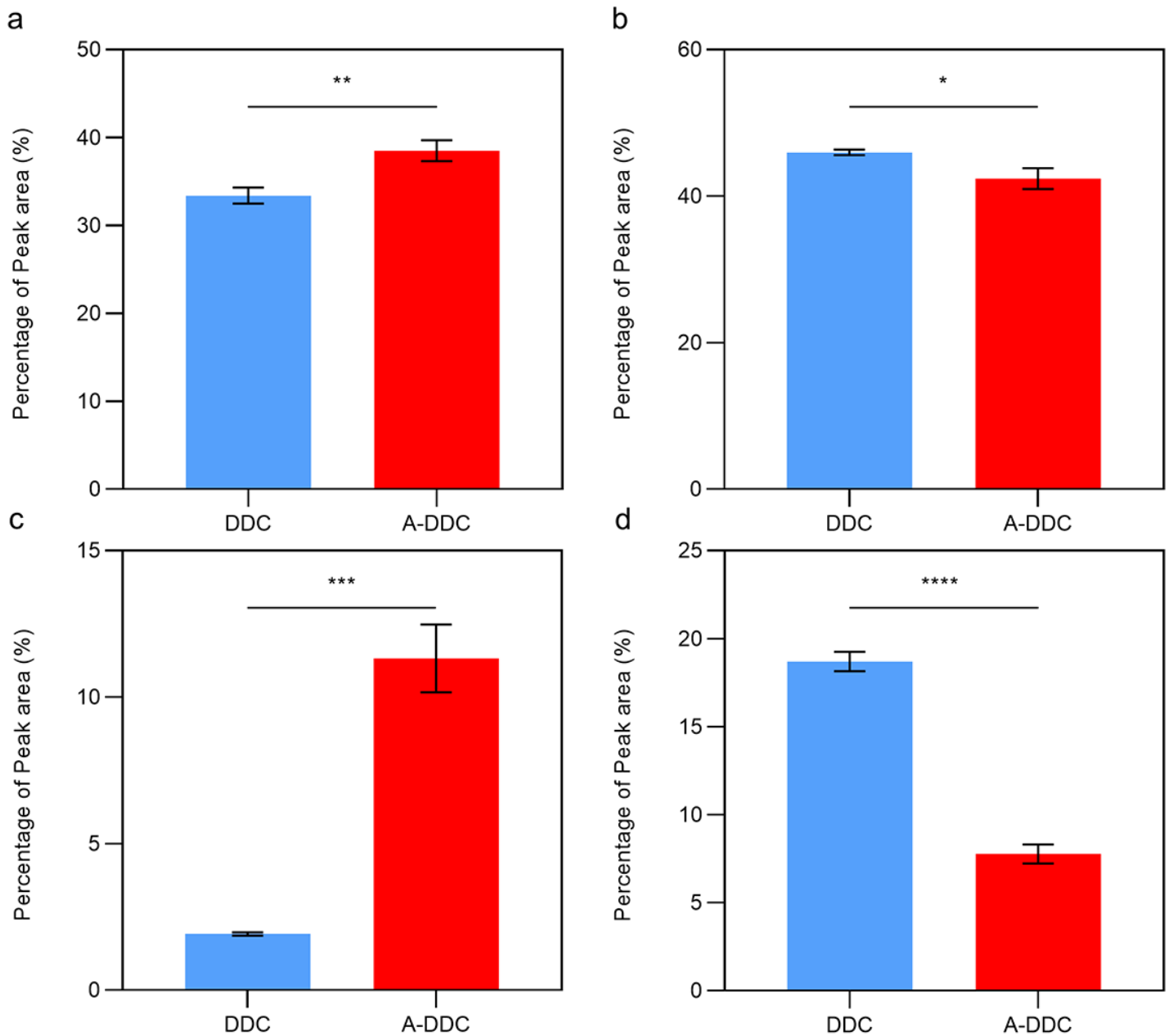
Figure 3

The percentage of secondary structure of DDC and A-DDC (a) the content of α-helix, (b) the content of β-sheet, (c) the content of β-turn, (d) the content of random coil. (Data are presented as the mean ± SD; *p< 0.05; ** p< 0.01; *** p< 0.001; **** p< 0.0001)

Figure 4

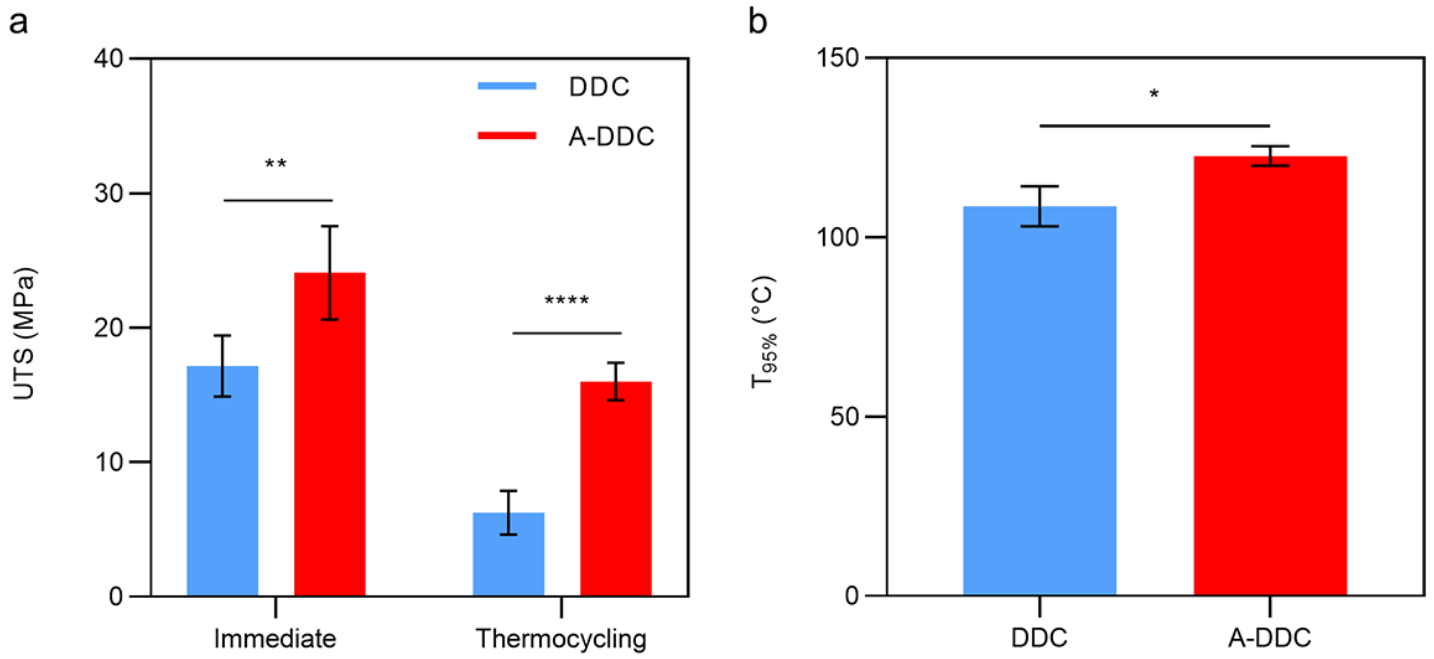The mechanical strength (a) and thermostability (b) of each group (Data are presented as the mean ± SD; *$p< 0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$)
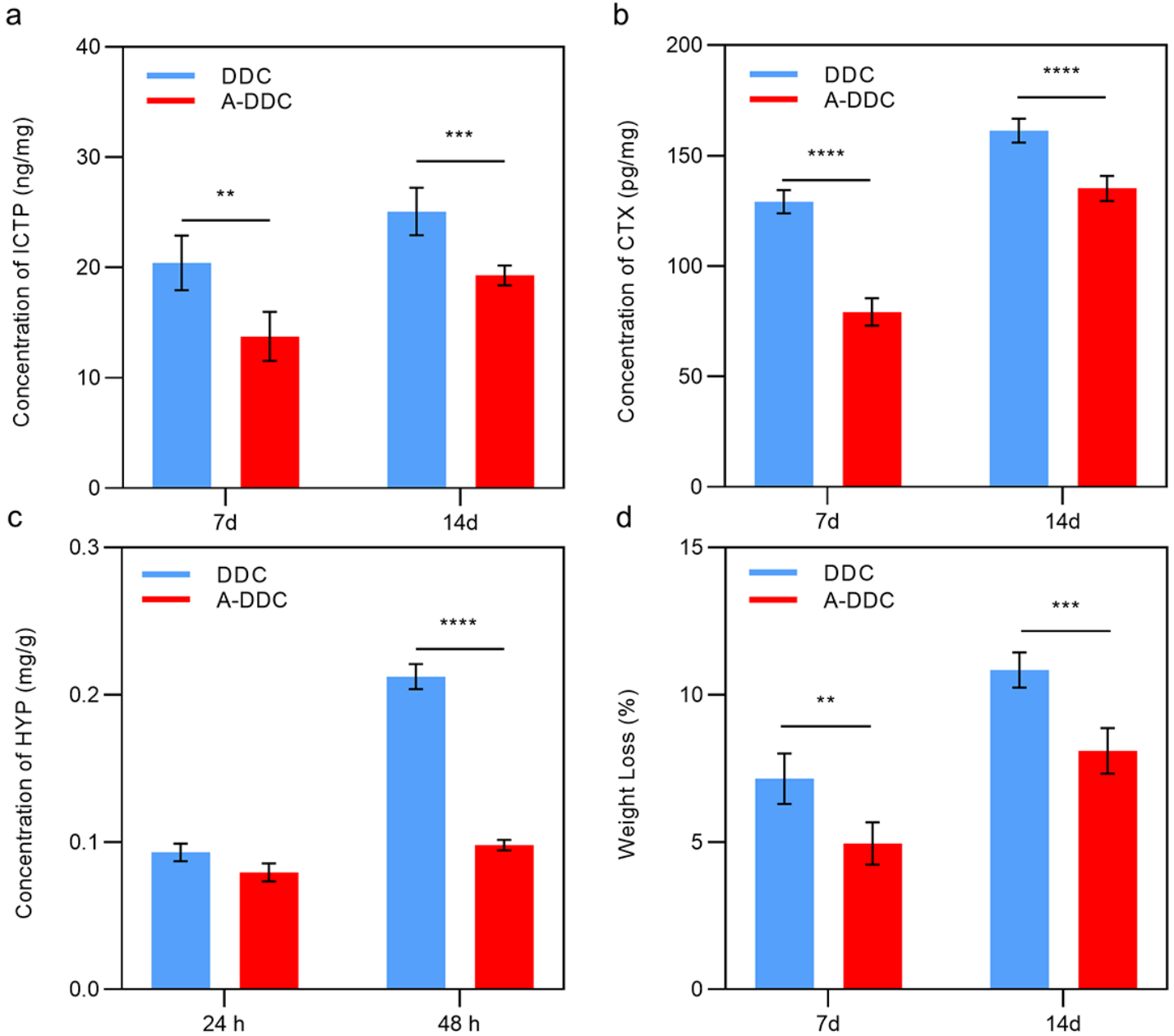
Figure 5

The enzymatic stability of demineralized dental collagen before and after treatment with aloin (a) the concentration of ICTP, (b) the concentration of CTX, (c) the concentration of HYP, (d) the wight loss for each group (Data are presented as the mean ± SD; *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)