Influence of D-Amino Acids in Beer on Formation of Uric Acid

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SUMMARY

Excessive intake of beer could increase serum uric acid levels, leading to high-risk gout, which was previously attributed to rich purines in beer. Recent reports that purine-rich vegetables and bean products did not cause the higher uric acid would not support the view. Why excessive intake of beer could increase a high risk of gout has been unclear. Other factors affecting the accumulation of uric acid in the blood were explored. Beer contains a relatively high levels of D-amino acids due to the racemization of L-amino acids induced by food processing. D-amino acid was catalyzed by D-amino acid oxidase (DAAO) to produce H₂O₂, which is further oxidized in the presence of Fe²⁺ to produce...
hydroxyl radicals, resulting in DNA damage and formation of a large amount of purine bases, which are oxidized to uric acid by a series of enzymes. Some food ingredients, such as vitamins and I− ions, promote D-amino acids to form uric acid. D-amino acids in beer are one of key roles inducing an increase in uric acid. The biological response of D-amino acids could illustrate gout events in beer drinkers.

Key words: uric acid, beer, D-amino acid, hydroxyl radical, DNA damage

INTRODUCTION

As we know well, the high level of serum uric acid (H2U) level in vivo would cause hyperuricemia (HUA), which is closely associated with the development and progression of gout, cardiovascular diseases, renal disease and tumor lysis syndrome (1-3). The higher serum H2U levels are also an independent predictors of these diseases (4). There is a great correlation between H2U and daily diets (5,6). Recently, there have been reports that H2U levels are related to beer or beer and seafood intake (7,8), and it had been suggested that the richness of purines in the beer will result in the increase in H2U in blood (5-8). But, it was reported that purine-rich vegetables and bean products did not lead to higher H2U (9,10). Therefore, why beer drinkers get a higher risk of HUA should be explored.

Beer, as liquid bread, contains many amino acids in addition to the rich purines. Each amino acid has two isomers (L- and D- amino acid) (11). It is reported that relatively high D-amino acid (DAA) content in beer was observed (12). Normally, L-amino acids (LAA) are the most crucial structural components in protein, and only rare DAAs are required for some normal biological functions (e.g. Ser) (13,14). The DAAs are maintained at relatively lower concentrations (DAA/LAA<1.77 %, w/w) in healthy biological tissues (13,15-17). Thus, DAAs in beer may be one of factors that cause physiologic dysfunction or some related diseases (such as HUA).

It was reported that the DAA is catalyzed by D-amino acid oxidase (DAAO) to form NH₄⁺, α-keto acid and H₂O₂ (18). H₂O₂ further reacts with Fe²⁺ or Cu⁺ and is easily converted into highly reactive hydroxyl radical (·OH) (19). Furthermore, vitamin B₂ and iodide ion can improve the catalytic activity
of DAAO on the hydrolysis of DAA. The reducing agent helps to reduce Fe$^{3+}$ to Fe$^{2+}$ or Cu$^{2+}$ to Cu$^{+}$, which is beneficial to the formation of active ·OH. In fact, vitamins B$_2$, C and E in beer usually are as high as 0.38, 30 and 3.0 mg/L, respectively (20,21), and iodide ion (0.1 mmol/kg), Fe$^{3+}$ (364~1388 μg/g) and Cu$^{2+}$ (17.1~143.9 μg/g) are usually rich in seafood (22-25). This may provide all possible evidence for the simultaneous consumption of beer and seafood to promote the formation of ·OH. In addition, nucleic acids (DNA and RNA) are catalyzed to H2U through a series of enzymes, which, according to metabolic mechanisms, maintain the balance of H2U in blood under normal conditions. Yet, nucleic acids are damaged in the presence of ·OH to form nucleotides and nucleosides, which are then converted to free purine bases that are necessary for H2U production (26). These data will provide a basis for links between DAA and H2U.

In view of previous arguments, rich purines were regarded as the most important factor in the formation of excess H2U. However, the possible linkage between DAA and H2U was ignored. In this report, the direct linkage between DAA in beer and H2U was based on molecular levels, and each metabolic process of DAA in beer was investigated during the conversion of DAA and H2U. A mechanism for the formation of free purines by nucleic acids damage pathway produced by DAA was proposed, and the key role of DAA in beer in inducing the excessive H2U was found.

MATERIALS AND METHODS

Chemicals and material

All chemicals used are of analytical grade (>99.0 %). L- and D-Ala were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L/D-isomers of Cys, Asn, Phe, Leu, His, Thr, Pro, and Ser were from J&K Scientific Ltd. (Zhejiang, China). Hydroxybenzenesulfonic acid sodium salt (DHBS), adenosine deaminase (ADA; EC 3.5.4.4), phenyl isothiocyanate (PITC) and 4-aminoantipyline (4-AAP, 98 %) were also from J&K Scientific Ltd. (Zhejiang, China). D-amino acid oxidase (DAAO, EC 1.4.3.3), purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) and horseradish peroxidase (POD; EC 1.11.1.7) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Calf thymus DNA, riboflavin, xanthine oxidase (XOD; EC 1.2.3.2), vitamin C, vitamin E and catalase (CAT)
were obtained from Solarbio Co., Ltd. (Beijing, China). PBS and TAE buffer were from Sangon Biotech Co., Ltd. (Shanghai, China). Methanol (chromatographic purity) was purchased from Siyou Co., Ltd. (Beijing, China). Agarose was obtained from Tiangen Co., Ltd. (Beijing, China). Triethylamine (TEA) was purchased from Shanghai Titan Co., Ltd. (Shanghai, China). Acetonitrile (ACN) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetic acid was obtained from Beijing Chemical Works (Beijing, China). Yanjing beer (Yanjing Brewery Co., Ltd., Beijing, China), Tsingtao beer (Tsingtao Brewery Co., Ltd., Qingdao, China) and Harbin beer (Harbin Brewery Co., Ltd., Harbin, China) were purchased from supermarket (Beijing, China). We assured that experimental test was conducted within the warranty period. Ultrapure water was prepared using double distilled water (18 MΩ cm).

**Assay of released H$_2$O$_2$ from the metabolism of DAA**

The effects of different concentrations of L- and D-Ala on the formation of H$_2$O$_2$ catalyzed by DAAO were studied. The L- and D-Ala solution at concentrations of 10, 1.0, 0.1, 0.01 and 0.001 mM were prepared with PBS buffer (pH=7.4, 0.01 M), respectively. Then the chromogenic agent containing 228 U/L DAAO, 0.4 mM 4-AAP, 2.0 mM DHBS and 1500 U/L POD was added into each of the above 3.0 mL solutions, and incubated for 30 min at 37 °C in digital constant temperature water bath pot (HH-4; Xinbao Instrument Factory, Guangzhou, China). DAA was oxidized by DAAO to generate H$_2$O$_2$, which was quantified by enzymatic coupling method and UV-Vis spectroscopy (27) using UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

**Analysis of DNA damage by DAA**

The effect of D-amino acid on the DNA was studied in PBS (0.01 M, pH=7.4). DNA damage was detected using DNA band diffusion assay on agarose gel (1 %). The samples containing 0.1 mM Fe$^{2+}$, 0.75 U/mL DAAO ,50 μg/mL DNA and different concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.6 mM) of D-Ala were parallelly incubated for 30 min at 37 °C, and the degree of DNA damage was detected by agarose gel (1 %) (JY600E; Beijing Junyi Electrophoresis Co., Ltd., Beijing, China). The reaction
mixtures were terminated by the addition of 2 μL of loading buffer. And a 10 μL of aliquot of the above mixtures was performed by gel electrophoresis for 1.5 h at 120 V in the presence of a 1× TAE buffer using 1% agarose gel.

**Measurements of H2U formation induced by the presence of D-amino acid**

The effect of DAA concentration on the formation of H2U was investigated. Samples containing DNA (50 μg/mL), Fe²⁺ (0.10 mM) and different concentrations of D-Ala (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mM) and DAAO (0, 0.05, 0.075, 0.10, 0.13, 0.13 U/mL) were prepared to simulate the formation of uric acid. The samples were incubated for 30 min at 37 °C, then CAT was added at 37 °C and kept at constant temperature for 30 min to remove H₂O₂ residues. Afterwards the samples were heated for 15 min at 70 °C to inactive CAT. To catalyze adenine and adenosine produced after DNA breaking into H₂U and H₂O₂, PNPase (9.4 U/L), ADA (10.0 U/L) and XOD (5.0 U/L) were added and incubated for 30 min at 37 °C. All samples were incubated for 30 min at 37 °C in 2.7-mL reaction mixtures containing 1500 U/L POD, 0.4 mM 4-AAP and 2.0 mM DHBS. The absorbance of H₂U was indirectly detected at 512 nm by UV-Vis spectroscopy (UV-2550; Shimadzu Corporation).

**The relationship between DAA in beer and the formation of H2U**

Beer samples of 50 μL were dried in vacuum drying oven (DZF-6050; Beijing Land and Technology Co., Ltd., Beijing, China). The obtained dry powders were mixed with 0.75 U/mL DAAO and dissolved in 100 μL water, and then incubated for 30 min at 37 °C, followed by drying in vacuum drying oven. The D- and L- amino acids were determined based on the method of Cohen et al. (28). The derivatized amino acids were determined by HPLC (LC-20AT; Shimadzu Corporation, Kyoto, Japan), equipped with Eclipse XDB-C18 column (250 mm × 9.4 mm i.d., particle size 5 μM). The gradient elution was performed at 0–10 min mobile phase A 90 % (0.14 mol/L CH₃COONa, 0.5 mol/L TEA, pH=6.40±0.05), 10–11 min mobile phase B 12.5 % (100 % ACN, pH=7.5, and ultrapure water, 60:40 by volume) and 11-30 min mobile phase A 90 %. Injection volume was 5 μL, the column was maintained at 25 °C, detection wavelength was 254 nm. The flow rate of the mobile phase was 0.8 mL/min, the control
samples were prepared by dissolving dry powders in 100 μL water without DAAO. The total DAA content in beers was calculated by comparing the peak areas of standard amino acid and DAA in beers at same retention time. Each test was done in triplicate and the content of DAA in beers was expressed as mean value±standard deviation.

The samples of 0.50 and 1.0 mL of Yanjing beer were pipetted into individual tubes and dried in vacuum drying oven (DZF-6050; Beijing Land and Technology Co., Ltd.) at 45 °C to remove the ethanol. The dry powders were dissolved in 1.0 mL water, and then the PNPase, ADA and XOD were added to the two samples to remove the adenine and adenosine, thereby eliminating their interference. The interferences were detected by enzymatic coupling method and UV-Vis (27) spectroscopy (UV-2550; Shimadzu Corporation). CAT was added to the above mentioned samples at 37 °C and kept at constant temperature for 30 min to remove H₂O₂, and then the obtained solution was maintained for 15 min at 70 °C to inactive enzyme CAT, followed by being dried again at 45 °C. Control samples containing 50 μg/mL DNA without adenine and adenosine and test group containing 0.75 U/mL DAAO, 0.10 mM Fe²⁺ and 50 μg/mL DNA were prepared. These samples were incubated for 30 min at 37 °C, and then CAT was added at 37 °C and kept at constant temperature for 30 min to remove H₂O₂ residues. Afterwards the samples were heated at 70 °C for 15 min to inactive CAT, and then PNPase (final concentration 9.4 U/L), ADA (final concentration 10.0 U/L) and XOD (final concentration 5.0 U/L) were added and the samples were incubated for 30 min at 37 °C. All samples were kept for 30 min at 37 °C in 2.7-mL reaction mixtures containing 1500 U/L POD, 0.4 mM 4-AAP and 2.0 mM DHBS. The absorbance was read at 512 nm using UV-Vis spectrophotometer (UV-2550; Shimadzu Corporation).

The assay of the role of vitamins and I⁻ ions on the formation of uric acid

The effect of vitamin B₂ on the activity of DAAO was studied. The D-Ala (final concentration 2.8 mM) and DAAO (final concentration 0.1 U/mL) were mixed with different concentrations (0, 0.50, 0.75, 1.0 mg/L) of vitamin B₂ in 3.0 mL of PBS buffer. The samples were incubated for 1 h at 37 °C, and then the absorbance of the pyruvic acid was measured at 260 nm by UV-Vis spectrophotometer (UV-2550;
Shimadzu Corporation) to evaluate the effect of vitamin B$_2$ on the activity of DAAO.

The effects of vitamin C and vitamin E on the formation of H2U were studied. The sample containing 0.1 mM Fe$^{3+}$, 0.75 mg/L vitamin E or vitamin C, 50 μg/mL DNA, 0.1 mM DAA and 0.75 U/mL DAAO were incubated for 40 min at 37 °C, followed by gel electrophoresis (JY600E; Beijing Junyi Electrophoresis Co., Ltd.) for 1.5 h at 120 V on 1 % agarose gel.

The effects of I$^{-}$ and Fe$^{3+}$ ions on the formation of H2U were estimated by the determination of ·OH. The highly reactive ·OH reacts with Phe and produces α-, m-, and p-tyr (29,30). The mixture (pH=7.4) containing 10 mM Phe, 0.1 mM Fe$^{3+}$ (EDTA-Fe$^{3+}$), 0.1 mM DAA and 0.75 U/mL DAAO was incubated for 40 min at 37 °C. The concentration of ·OH ions in the mixture was determined by HPLC (LC-20AT; Shimadzu Corporation, with C18 column 9.4 mm×250 mm), using isocratic elution consisting of 250 mM KH$_2$PO$_4$/H$_3$PO$_4$ (pH=3.01) with 5 % methanol (by volume) at the flow rate of 0.8 mL/min. The absorbance of the samples was evaluated at 274 nm by UV-2550 spectrophotometer (Shimadzu Corporation).

DNA samples (50 μg/mL) were treated with I$^{-}$ (0.10 mM), EDTA-Fe$^{3+}$ (0.10 mM), D-Ala (0, 20, 40, 60, 80, 100 mM) and DAAO (pH=7.4, 0.75 U/mL. The degree of DNA damage was determined using DNA band diffusion assay on agarose gel (1 %).

The effect of the racemization of LAA on the formation of H2U was studied. As we all know, the seafood contains abundance of metal ions (e.g. Fe$^{3+}$ and Cu$^{2+}$) (11). LAA can be racemized by heating or adding metal ions (17). The sample containing 0.03 mmol/L L-Ala, Cu$^{2+}$ and water added to give a total 1 mL reaction volume (pH=7.0) was prepared and incubated for 3 h at 100 °C. The ratios of Cu$^{2+}$ to L-Ala were 0, 1:8, 1:4 and 1:2. The racemization of L-Ala were detected by circular dichroism spectrophotometer (Jasco-815; Jasco Corporation, Tokyo, Japan) in the 190-240 nm wavelength region at room temperature.

RESULTS AND DISCUSSION

The effect of DAA in aqueous solution on the formation of uric acid

As previously reported, H$_2$O$_2$ was one of metabolites of D-isomeric amino acids in the presence of
D-amino acid oxidase (DAAO), due to stereo-specificity of the deamination of amino acids (18). DAA could be oxidized and deaminated to produce H₂O₂, which reacted with 4-AAP and DHBS to produce red quinoneimine dye in the presence of POD. The H₂O₂ as a product of DAA degradation was firstly measured by enzymatic coupling method and UV-Vis spectroscopy. As shown in Fig. 1, different concentrations of L-Ala samples in 1-6 were colourless, which indicated that L-Ala was not catalyzed to H₂O₂ by DAAO. In contrast, samples 7-11 of D-Ala were striking red, and the colour became darker with the increase of concentrations of D-Ala. These results confirm that H₂O₂ is produced by DAAO from DAA rather than LAA, and that it could be detected by enzymatic coupling in combination with UV-Vis spectroscopy, which have high sensitivity and specificity (0.1 mM of DAA). Our results draw the same conclusions as our previous research, where colour of reaction sample was significantly darker with concentrations of DAA ranging from 0.1 mM to 10 mM (27).

It has long been known that the reaction involving H₂O₂ with "redox-active" iron or copper (i.e. Fenton reaction) would produce ·OH, leading to DNA damage (19,31). To further assess the effects of H₂O₂ derived from DAA on the production of ·OH and DNA damage, we performed the studies on DNA damage with D-Ala, DAAO and Fe²⁺. When concentrations of DAAO and Fe²⁺ were constant, the band became more diffuse with the increase of the concentration of D-Ala (Fig. S1), indicating that DAA can act as an effective disruptor for DNA in the presence of DAAO and Fe²⁺. Other research on DNA damage with salmon sperm DNA and pBluescript K⁺ plasmid had proven that the presence of hydroxyl radicals leads to DNA breaks (19).

Our final attention was paid to the formation of H2U from DAA, although we cleared that H₂O₂ was produced from DAA in the presence of DAAO, followed by the damage of DNA in the presence of "redox-active" iron, caused by the production of ·OH ions. A research reported that the produced hydrogen peroxide was able to quench the QDs fluorescence, which was proportional to uric acid concentration, so the formation of H2U has relative relationship with H₂O₂ (32). Thus, the determination of H2U could be carried out by detection of H₂O₂ using chromogenic agent, because the new products of H₂O₂ indirectly show the product of H2U in the process of converting nucleic acids into uric acid when the H₂O₂ formed in the first step is removed by CAT.
It was found that the H2U concentration increased by 2 times when the concentration of D-Ala was in the range of 0.5 mM to 2.5 mM (Fig. 2), which suggested that DAA can produce ·OH in the presence of DAAO and Fe$^{2+}$ and destroy DNA to cause excessive formation of H2U. The results indicate that the DAA was oxidized to form ·OH in the presence of Fe$^{2+}$ ions, which induced DNA damage and further formed purine bases and pyrimidine bases, and xanthine oxidase can catalyze the oxidation of purine bases to yield H2U.

**The effect of DAA in beer on the formation of uric acid**

To investigate important role of DAA in beer in inducing H2U, DAA content in the solution of beer samples (Yanjing, Tsingtao and Harbin) was determined by HPLC and compared with standard amino acids (Cys 0.75 mM, Ser 1.00 mM, Asn 0.54 mM, Phe 1.00 mM, His 0.65 mM, Thr 1.80 mM, Ala 1.89 mM, Pro 0.90 mM and Arg 0.80 mM). PITC reacted with amino acid to form stable phenylthiocarbamyl derivatives (28), which can effectively be used to analyze and quantify amino acids in beer. As shown in Fig. S2, eight types of amino acids in beer were observed at different retention times. The total contents of amino acids in beer and the remaining LAAs after catalysis of all DAAAs by DAAO were determined by HPLC. The content of DAAAs in beer was detected according to difference of HPLC peak areas between the samples treated with DAAO and control ones with no-treatment and calculated by comparing above difference of HPLC peak areas of two samples with the standard amino acid. The concentrations of DAA in Yanjing beer, Tsingtao beer and Harbin beer were (0.80±0.04), (0.34±0.02) and (0.29±0.01) mM, respectively (data not shown).

The DAA in beer may be produced from microbial metabolites in beer or by racemization of free and protein-bound LAA during food processing (12). We used circular dichroism spectrophotometer to detect active chiral molecules, such as L/ D- AA. The effect of temperature and metal ions on the racemization of L-Ala was studied based on the simulation of beer processing. During cooking or heating, Cu$^{2+}$ ions can catalyze the racemization of L-Ala at pH=7 and 100 °C. Racemization of L-Ala increase with the increase of the ratio of L-Ala to Cu$^{2+}$, which proves that the rate of racemization depends on Cu$^{2+}$ concentration and it could be as high as 40.1 % when the molar ratio of L-Ala to
Cu\(^{2+}\) was 2:1 (Fig. S3). Hans Brückner group detected D-Pro in matured wine vinegar by chiral gas chromatography, which proved that relatively high amounts of D-Pro were mainly attributed to the Maillard reaction (33). Therefore, the presence of D-AA in beer could be attributed to Maillard reaction caused by heating during beer production.

Based on our studies on DAA in aqueous solution, we know that DAA could be used as a potent DNA-damaging agent in the presence of DAAO and Fe\(^{2+}\), which induced the formation of H2U. To prove that DAA can induce DNA damage, we excluded the possible factors including ethanol, adenine and adenosine (5-8). Adenine and adenosine in the beer solution were effectively removed using ADA, PNPase and XOD, and the ethanol was also removed using vacuum drying oven (DZF-6050; Beijing Land and Technology Co., Ltd.) at 45 °C. Detection of H\(_2\)O\(_2\) indirectly prove the production of H2U, because H\(_2\)O\(_2\) and H2U were formed together during metabolic degradation of adenine and adenosine.

Fig. 3 shows that there was no colour change of samples 1 and 2 with different volumes of beer when they were treated with ADA and PNPase, indicating that adenine and adenosine could be completely removed from the beer. Adenine and adenosine in samples 3 and 4 were removed by the same method, and incubated in the presence of Fe\(^{2+}\) and DAAO. Values of \(A_{512 \text{ nm}}\) of samples 3 and 4 increased with the increase of the volumes of the beer, which showed that the concentration of H2U increased by 27 % when the volumes of beer increase by 2 times (Fig. 3). This confirmed that the mixture of beer with DNA, Fe\(^{2+}\) and DAAO could cause DNA damage and form H2U. Blood accounts for about 8 % (by mass) of the human body (34). The average body mass of the adult is 75 kg, and the average blood volume is 60 dL. Normal levels of H2U in blood are 2.18-7.0 mg/dL (30), and if increased after the consumption of large volumes of beer it could easily result in gout.

The effect of vitamins and I\(^{-}\) ions on the formation of uric acid induced by DAA

A previous study reported that vitamin B\(_2\) content in beer was in the range of 0.5-1.0 μM, and the active form of vitamin B\(_2\) is coenzyme of DAAO (21). In the presence of DAAO, D-Ala was oxidized to pyruvic acid, which has strong UV absorption. We thus evaluated the effect of riboflavin on DAAO
by quantification of pyruvic acid. Fig. 4 shows that the concentration of pyruvic acid increased with the increase of riboflavin concentration from 0 to 1.0 mM, which indicated that DAAO activity increased up to 1.7 times due to the presence of riboflavin.

Vitamin E, as an ingredient of beer, can promote DNA damage induced by H$_2$O$_2$ and Fe$^{3+}$ or Cu$^{2+}$ ions via Fenton reaction (35). H$_2$O$_2$ produced by DAA and DAAO could more easily react with Fe$^{2+}$ than with Cu$^+$ to form ·OH, and thus we studied the reduction of Fe$^{3+}$ by vitamin E to Fe$^{2+}$, which caused the DNA damage in the presence of DAA and DAAO. Fig. 5 shows the DNA damage in the presence or absence of vitamin E. DNA band was slightly dispersed in the presence of 0.2 mM vitamin E in lane 3 and it still existed in the absence of vitamin E in lane 2, suggesting that vitamin E accelerated DNA damage with DAA and DAAO. Also, vitamin C could directly react with H$_2$O$_2$ to generate ·OH and damage DNA even without the assistance of metal ions, since the reduction ability of vitamin C was stronger than vitamin E (36). Thus, vitamin C in beer can promote formation of H2U.

But just as importantly, I$^-$ exists in daily diet together with vitamins, and its impact on DNA damage induced by DAA should not be neglected due to its strong reduction ability to Fe$^{3+}$ and Cu$^{2+}$. Several isomeric tyrosines can be produced in the reaction of phenylalanine with ·OH and total amount of products was proportional to the amount of ·OH in the system (29,30). Fig. 6 indirectly displays that formation of ·OH from 0.1 mM D-Ala in PBS containing different concentrations of I$^-$ and 0.75 U/mL DAAO in the presence of Fe$^{3+}$ or EDTA-Fe$^{3+}$, which eliminates interference with Fe$^{3+}$ by the chelation of EDTA. The retention times of 7.95, 9.37 and 12.14 min correspond to p-tyrosine, m-tyrosine and o-tyrosine, which were generated from phenylalanine and ·OH. Three peak areas for the products (p-tyrosine, m-tyrosine and o-tyrosine) in the presence of Fe$^{3+}$ were 18054, 5854 and 9944 (Fig. 6a) and the corresponding peak areas for the products in the presence of EDTA were 17881, 5032 and 8719 (Fig. 6b), respectively.

Actually, chelation by EDTA-Fe$^{3+}$ decreases formation of ·OH, indicating that the reduction reaction of I$^-$ to Fe$^{3+}$ promotes the formation of ·OH. In addition, DNA damage induced by 0.1 mM D-Ala in PBS containing different concentrations of I$^-$ and 0.75 U/mL DAAO in the presence of Fe$^{3+}$ (lanes 1-5) or EDTA-Fe$^{3+}$ (lanes 6-10) was further investigated, as shown in Fig. S4. Lanes 1-5 showed DNA
damage levels of 30.0, 47.0, 50.6, 51.1 and 51.1 %, respectively. However, when Fe$^{3+}$ was chelated by EDTA, lanes 6-10 showed the DNA damage levels of 33.3, 33.4, 33.4, 33.5 and 33.6 %, respectively. This confirms that the complex of EDTA-Fe$^{3+}$ can prevent production of reduction product (Fe$^{2+}$) by I$^-$, thereby inhibiting the conversion of H$_2$O$_2$ into ·OH and reducing DNA damage.

Gout occurs as a result of H$_2$U accumulation in the blood, caused by purine metabolism disorder. Exogenous (about 20 %) and endogenous (about 80 %) purines are present in the body. The former originate from daily diet, and the latter are formed by the oxidation of nucleic acids, which is the key for the formation of purine. Previous surveys show that excessive intake of beer can increase the risk of gout, which could be attributed to high level of purines in beer (5-8). However, recent reports showed that content of H$_2$U in the serum remains the same or even decreases when purine-rich beans and vegetables are being consumed (9,10). Factors other than purine-rich diet can also promote the accumulation of uric acid in the blood. In the metabolic pathway of purine, the oxidation of nucleic acid leads primarily to the production of purines, but further oxidation gives a balanced amount of H$_2$U as a final product. Once hydrogen peroxide and free radicals are produced, the nucleic acids are oxidized faster and produce more purines, which leads to the increase of H$_2$U concentration. The DAA in food is absorbed in liver, kidney, or through the stomach and intestine, and it can be oxidized and deaminated to NH$_4^+$, α-keto acid and H$_2$O$_2$, which can react with Fe$^{2+}$ or Cu$^+$ to generate highly reactive ·OH (18). Therefore, we propose that there is a direct linkage between DAA and H$_2$U concentrations.

In aqueous solution, DAA produces H$_2$O$_2$ in the presence of DAAO, which could be monitored by the chromogenic reaction. Assay of DNA damage shows that oxidation of nucleic acids is related to DAA content, which proves that relatively high content of DAA increases the degree of DNA damage. Beer, as a fermented drink, has a relatively high content of DAA. Next, we have investigated the influence of DAA presence in the beer on the formation of H$_2$U, a key factor that induces gout. Removing purines by ADA and PNPase, H$_2$U formed from reaction of beer power dissolved in water with DAAO and Fe$^{2+}$.

In addition, our experiments show that vitamins and I$^-$ can promote the formation of H$_2$U by DAA,
which confirms the key role of DAA in formation of H2U in beer. Therefore, the molecular mechanism by which DAA forms H2U is summarized. The DAA catalyzed by DAAO is oxidized to H2O2, which further reacts with Fe^{2+} or Cu^{+} to produce highly reactive ·OH. And the nucleic acid is damaged by ·OH and produces a large amount of hydrazine, which is oxidized to H2U by a series of enzymes. The study explains why excessive intake of beer can cause gout, and purine-rich foods are one of the most important factors leading to gout.

CONCLUSIONS

In the report, the effect of DAA in beer on formation of H2U was discussed. It was found that DAA could be oxidized to H2O2 by DAAO, and further oxidation gave highly active hydroxyl radicals, which would accelerate metabolism from nucleic acid to purines and elevate H2U level, accompanied by gout. We conclude that DAAs in beer were one of key roles in inducing increase of H2U, and some food ingredients, such as vitamins and I^- ions, promote the effect.

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CONFLICT OF INTEREST

No conflict of interest has been declared.

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Fig. 1. Chromogenic reaction of different concentrations of L/D-AA and UV absorbance of the reaction mixtures of L, D-Ala oxidized by DAAO. 1-6=0, 0.001, 0.01, 0.1, 1.0 and 10 mM L-Ala respectively, 7-11=0.001, 0.01, 0.1, 1.0 and 10 mM D-Ala respectively
Fig. 2. Influence of DAA concentrations on H2U formation. 1-6= 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mM D-Ala, respectively.
Fig. 3. Chromogenic reaction and UV absorbance of the samples treated with ADA and PNPase. 1 and 2=0.5 and 1.0 mL of Yanjing beer respectively, 3 and 4=0.5 and 1.0 mL of Yanjing beer in the presence of Fe$^{2+}$ and DAAO respectively

![Graph of chromogenic reaction and UV absorbance](image)

Fig. 4. The effect of riboflavin (vitamin B$_2$) on DAAO activity. 1-4=1.00, 0.75, 0.50 and 0 mM of riboflavin

![Graph showing effect of riboflavin on DAAO activity](image)

Fig. 5. DNA damage induced by 0.8 mM D-Ala with 0.2 mM Fe$^{3+}$ and 0.8 U/mL DAAO with or without vitamin E using 1 % agarose gel. M=molecular marker, lane 1=DNA sample, lane 2=DNA damage without vitamin E, lane 3=DNA damage in the presence of 0.2 mM vitamin E

![Agarose gel showing DNA damage](image)
Fig. 6. Consumption of ·OH by phenylalanine (10 mM) in the presence of 0.1 mM I⁻, 0.75 U/mL DAAO, 0.1 mM D-Ala and a) 0.1 mM Fe³⁺ or b) 0.1 mM EDTA-Fe³⁺.

Supplementary material

Fig. S1. The effect of D-Ala on DNA damage using 1 % agarose gel in the presence of 0.1 mM Fe²⁺.
and 0.1 U/mL DAAO. M=molecular marker, lanes 1-6=0, 0.05, 0.1, 0.2, 0.4 and 0.6 mM D-Ala respectively.

Fig. S2. Determination of amino acids in beer by HPLC: a) sample of standard amino acids: Cys 0.75 mM, Ser 1.00 mM, Asn 0.54 mM, Phe 1.00 mM, His 0.65 mM, Thr 1.80 mM, Ala 1.89 mM, Pro 0.90 mM and Arg 0.80 mM, b) beer sample.
Fig. S3. The effect of heating and metal ion on the racemization of L-Ala. The concentration of L-Ala in samples was 0.03 mmol/L, and the ratios of L-Ala to Cu²⁺ were 2:1, 4:1 and 8:1.

Fig. S4. The effect of I⁻, 0.1 mM Fe³⁺ (EDTA-Fe³⁺), 0.1 mM D-Ala and 0.75 U/mL DAAO on DNA damage. M=molecular marker, lanes 1-5=DNA damage in the presence of Fe³⁺ and I⁻ at different concentrations (20, 40, 60, 80 and 100 μM respectively), lanes 6-10=DNA damage in the presence of EDTA-Fe³⁺ and I⁻ at different concentrations (20, 40, 60, 80 and 100 μM respectively), lane 10=DNA damage in the presence of 100 μM I⁻, lane 12=DNA sample.