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A zebrafish (danio rerio) model for high-throughput screening food and drugs with uric acid-lowering activity

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ABSTRACT

With co-treatment of potassium oxonate (PO) and xanthine sodium salt (XSS), a zebrafish larva model of acute hyperuricemia has been constructed for the first time. The results show PO 200 μM + XSS 10 μM, PO 300 μM + XSS 15 μM, and PO 400 μM + XSS 20 μM can significantly increase the level of uric acid in the zebrafish larvae ($P < 0.05$), the concentrations as described above can be used to construct the zebrafish larvae model of acute hyperuricemia. At the same time, treatment of allopurinol (APL, one of the hyperuricemia drugs) at 2000 μM ($P < 0.001$) and treatment of anserine (ASE) at 200 μM ($P < 0.05$) could significantly decrease the level of uric acid in the model group which received PO 200 μM + XSS 10 μM, which demonstrate that such model could offer a new robust approach for high-throughput screening of food and drugs with uric acid-lowering activity.

1. Introduction

Urate oxidase (uox), which is one of the most important enzymes in purine metabolism, can convert the uric acid to allantoin in most mammals except humans and certain other primates [1–3]. Due to the lack of urate oxidase in humans, the uric acid in the body cannot be converted into water soluble urea and will deposit in the blood and joints, resulting in hyperuricemia and ultimately leading to gout [4,5]. Many daily foods have been found bearing the activity of reducing the uric acid in human body. Hawthorn has been used in medications to treat hyperuricemia [6]. Besides, it has been reported that pueraria lobata which can be used as food and medicine could exert therapeutic effect on hyperuricemia [7]. Recently, two conference reports showed that Anserine (ASE, beta-alanyl-3-methyl-L-histidine), a dipeptide abundant in the skeletal muscle and brain of animals and large migratory fish, could lower serum urate levels in mice and humans [8,9].

Currently, many drugs for treating hyperuricemia and gout could cause damage to liver and kidneys. APL, which is the most commonly used medication for treating hyperuricemia, has high incidence of adverse reactions, with allopurinol hypersensitivity syndrome (AHS) as the main side effect. The incidence rate of AHS is about 0.7‰, with the associated mortality rate as 20–25%. In addition, it needs to be administrated together with thiazide diuretics, which can cause additional kidney damage [10]. Meanwhile, though uric acid enzymes, such as rasburicase (recombinant urate oxidase) [11,12] and pegloticase [13,14] can decrease the uric acid in blood robustly, acute gout can be induced at the same time. Furthermore, as these drugs (rasburicase and pegloticase) can induce hypersensitivity and resistance due to their antigenicity, it is necessary to find more effective and safe food and drugs.

Zebrafish has been used as models for many human diseases due to its short breeding cycle, production of eggs in high quantity, and high genome homogeneity compared with human genome [15]. In addition, it is relatively easy to perform genetic manipulation, drug treatment and high-throughput screening in zebrafish [16,17]. Therefore, zebrafish was used in this study to establish a hyperuricemia model to assist future studies on hyperuricemia, mechanisms underlying gout, and screening for effective and safe food and drugs for treating hyperuricemia and gout.

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2. Materals and methods

2.1. Materials

Xanthine sodium salt (XSS), Potassium oxonate (PO), Allopurinol (APL), Anserine (ASE) and Carboxymethyl Cellulose (CMC, a kind of cosolvents) were purchased from Sigma and Aldrich Chemical Co. (St. Louis, MO, USA). PO were dispersed in CMC aqueous solution and XSS were dispersed in fish water. Amplex® Red Uric Acid/Uricase Assay Kit (MolecularProbes®, USA) used in the experiments were purchased from Invitrogen Trading (Shanghai) Co. Ltd. All other reagents were commercially available and of analytical grade.

2.2. Zebrafish strains and maintenance

AB wild type Zebrafish, were obtained from Institute of hydrobiology, Chinese Academy of Sciences (Wuhan, China). All zebrafish embryos were produced by mating paired adult fish maintained at 28 °C in a circulating water system with 14 h light and 10 h dark light-dark cycle. Water supplied to the system was filtered by reverse osmosis (pH 7.5–8). Embryos were reared in holtfreter water in a humidity incubator with the temperature as 28.5 °C. Dead embryos were cleared out every day. Experiments reported in this study were carried out in accordance with local guidelines for the care of laboratory animals of Chinese Academy of Agricultural Sciences, and were approved by the ethics committee for research on laboratory animal use of the institution.

2.3. Acute toxicity experiment

300 5dpf (days post fertilization) zebrafish larvae were divided into five groups (n = 60 per group). Those groups were co-treated with PO and XSS in graded doses up to PO 40 mmol/L + XSS 2 mmol/L to assess the 24-hr mortality.

2.4. Expression of uox detected by Q-PCR

The total RNA of zebrafish larva was extracted according to the manual of RNA extraction kit (#R1002, SinoGene). cDNA was synthesized using the Thermo First cDNA Synthesis Kit (#Q1010, SinoGene). qPCR analysis was performed with StepOnePLUS, Applied Biosystems. qPCR assay with the total volume of 15 μl include the following reagent: 2 × SG Green qPCR Mix (#Q1002, SinoGene) 7.5 μl, 10 μM Forward Primer 0.25 μl, 10 μM Reverse Primer 0.25 μl, cDNA 1 μl, nuclease-free water 6 μl. Thermal cycle of the qPCR was performed as follow: initial denaturation at 95 °C for 10 min; denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, both for 40 cycles; dissociation at 95 °C for 15s, 60 °C for 30 s, 95 °C for 15 s. Primers information of *uox* is shown in Table 1.

2.5. Construction and verification of zebrafish hyperuricemia model

1200 zebrafish larvae were divided into ten groups, each group containing three culture dishes, with each culture dish including 40 zebrafish larvae. Control group was exposed to 20 mL fish water only. CMC group were treated with 0.1% CMC dissolved in fish water. To achieve acute hyperuricemia, zebrafish larvae of group PX1 to PX8 were treated with PO and XSS at the concentrations shown below: PX1, PO 100 μM + XSS 5 μM; PX2, PO 200 μM + XSS 10 μM; , PX3, PO300 μM + XSS 15 μM; PX4, PO 400 μM + XSS 20 μM; PX5, PO 500 μM + XSS 25 μM; PX6, PO 1000 μM + XSS 50 μM; PX7, PO 5000 μM + XSS 250 μM; and PX8, PO 10000 μM + XSS 500 μM. Zebrafish of all the groups were kept in an incubator with constant temperature of 28.5 °C and pH of 7.4 for one day. In verification study, APL and Anserine was used at the concentration ranging from 500 μM to 4000 μM, and from 50 μM to 500 μM, respectively.

Table 1
Primers used in quantitative polymerase chain reaction(Q-PCR).

Gene Name	Sequence(5′-3′)	Size(bp)
ACTIN F_1	AATCCCAAAGCCAACAGA	110
ACTIN R_1	CGACCAGAAGCGTACAGAG	
uox F_1	TCACCACTCTGACTGATGCA	145
uox R_1	TCGTAGGGTCCTGCAAACTT	

Note: *uox*: urate oxidase.

2.6. Uric acid level assay

Measurement of uric acid in the homogenates of total zebrafish larvae was performed using the Amplex® Red Uric Acid/Uricase Assay Kit (MolecularProbes®, USA). 1 day after the co-treatment of PO and XSS, appropriate concentration of tricaine solution was added to each culture dish. Following this, the total of 40 zebrafish larvae from the same culture dish were transferred to a EP tube about 10mins after anesthesia. All the water in the EP tube which contained the larvae need to be sucked up as much as possible. 50 μl of ice PBS was then added into the EP tube. All the above procedures were performed on ice. The zebrafish larvae were then homogenized until no obvious tissue pieces left. The homogenate was centrifuged for 10 min at 4 °C at about 15 000 g. 25 μl of the supernatant was aspirated into microplate. 25 μl of reaction buffer and 50 μL of the Amplex® Red reagent/HRP/uricase working solution were added to each microplate well. Microplate were protected from light and incubated for at least 30 min at 37 °C. Absorbance was measured at 560 nm on a microplate reader.

2.7. Statistical analysis

All data were analyzed with SPSS version 24.0 and were expressed as the mean ± standard deviation. One-way analysis of variance was performed using *t*-test and ANOVA. The p-value of 0.05 and 0.01 were used to indicate statistical significance.

3. Results

3.1. Mortalities of fish for 24 h

Mortalities of fish upon exposure to PO and XSS solutions for 24 h were demonstrated in Fig. 1, which showed that the mortality of fish exposed to 30.00 μM PO together with 1.50 μM XSS was 53%. Therefore, the medial lethal concentration (MLC) of the solution containing both drugs was 30.00 μM PO with 1.50 μM XSS.

3.2. Expression of uox and level of uric acid

Results of qPCR (Fig. 2a) demonstrated that the level of *uox* gene in zebrafish larva of 1-10dpf days exhibit significant difference, with no expressions of *uox* gene observed during the first 3 days post fertilization and the highest level of *uox* gene achieved between the 7–9 days post fertilization. As shown in Fig. 2b, level of uric acid in embryos, which was measured by the absorbance at 560 nm, increased gradually with the highest level reached at 5dpf, followed by a gradually decrease and became stabilized after 7dpf.

3.3. Construction of zebrafish hyperuricemia model

As shown in Fig. 3, the level of uric acid in zebrafish of PX2, PX3

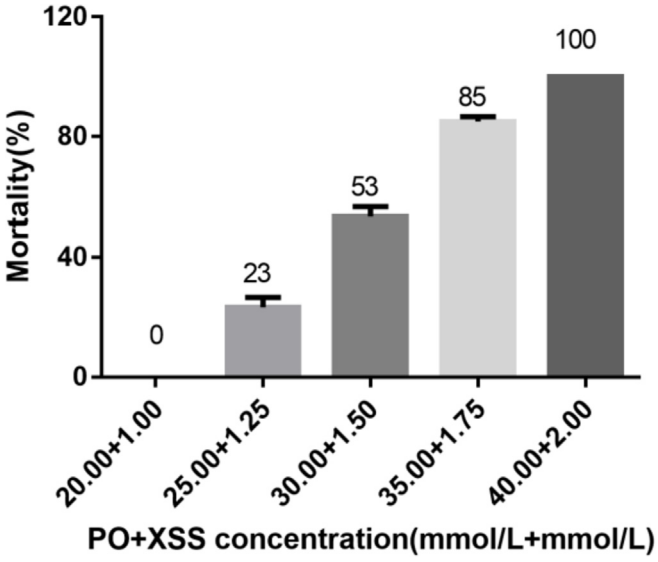

Fig. 1. The mortalities of the fish exposed in different concentration of PO and XSS solutions in 24 h.
Note: Values are group means, with STD represented by vertical bars (N = 3, n = 40). The unit of concentration of PO and XSS was mM (N = 3, n = 40). PO, potassium oxonate; XSS, xanthine sodium salt.

and PX4 groups were significantly higher than that of control group ($P < 0.05$). On the other hand, the level of uric acid in zebrafish of PX5, PX6, PX7 and PX8 groups were less than that of control group.

3.4. Verification of zebrafish hyperuricemia model

According to previous study, 200 μM PO and 10 μM XSS were used to construct the zebrafish model. As shown in Fig. 4a, APL at the concentration between 500 and 4000 μM could significantly reduce the uric acid level of the model group, with the concentration of 2000 μM being most effective ($P < 0.001$). As shown in Fig. 4b, anserine exerted good uric acid lowering activity at 200 μM ($P < 0.05$) while showed no effect at the concentration of 50 μM and 500 μM. As shown in Fig. 4c, compared with APL, ASE is less effective in reducing uric acid in fish body of APL in zebrafish larvae.

4. Discussion

It has been reported that about five days after fertilization, zebrafish organs, such as liver, are fully developed and can function [18]. Our study found that the expression of *uox* gene, which can decompose uric acid into allantoin [19], started from 4dpf and the level of uric acid in the fish was highest at 5dpf which may be due to the expression of uric acid oxidase gene mainly in liver. Uric acid can accumulate in the whole body of zebrafish before the liver is formed, prior to which *uox* gene is not expressed. After 5dpf, the *uox* gene is expressed in the newly formed liver leading to dramatically decrease of uric acid in the whole body. Therefore, embryos of 5dpf were used to establish the hyperuricemia model.

Zebrafish, described as 'the canonical vertebrate, is one of the leading models for studying human diseases. Zebrafish has short development cycle and high fecundity. Embryos of zebrafish are developed outside of the mother and are transparent, which could enable readily visualization and real-time investigation of the organs, cells and tissues in vivo. Maintenance costs of zebrafish are significantly lower than mammals. As a vertebrate organism, many of its organs and cell types are similar to that of mammals. Its major organs are developed in larvae by 5–6 dpf [20,21]. Therefore, with its small size and ease of culture, zebrafish could be developed as an efficient and robust high-throughput drug screening tool [22]. Furthermore, the zebrafish genome is similar to humans and mice. Its key regulatory genes and signals are highly conserved in mammals [23]. It has been found that the *uox* gene is expressed in the liver of zebrafish as in mice [24,25]. It is predicted that the *uox* gene retain the complete activity of urate oxidase [26]. With such unique advantages, zebrafish was used in this study to establish the acute hyperuricemia model.

PO is one of the inhibitors of urate oxidase [27]. By inhibiting the activity of uricase in the body, the uric acid level can be increased in a relatively short period. XSS is one of the precursors of uric acid. Exogenous intake of purine can lead to increased uric acid production [28]. Liu Yan et al. successfully established mouse hyperuricemia model by intraperitoneal injection of 0.3 g/kg potassium oxonate and 0.2 g/kg xanthine [29]. Xu Li et al. pointed out that the rat hyperuricemia model established upon exposure to hypoxanthine at 500 mg/kg together with potassium oxonate at 100 mg/kg has the characteristics of high serum uric acid concentration and long maintenance time [30]. At present, intraperitoneal injection or intragastric administration of PO, combined with purine

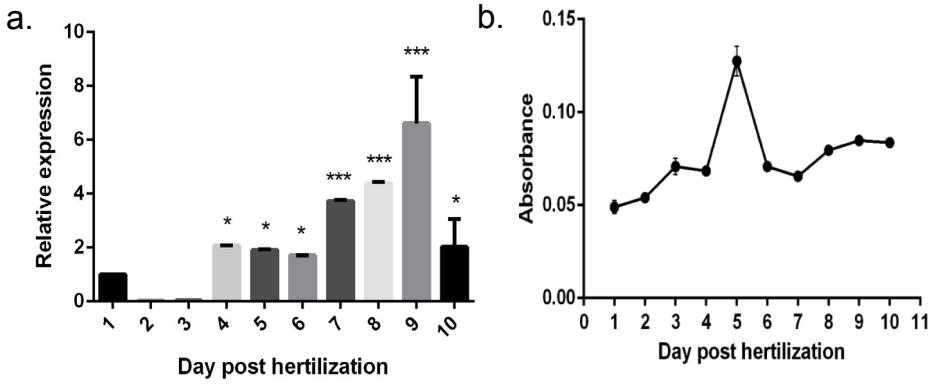

Fig. 2. Expression of uox and level of uric acid
(a) The expression of *uox* in whole body of zebrafish from 1 to 10dpf.
(b) The concentration of uric acid in embryos from 1dpf to 10dpf.
Note: Values are group means, with STD represented by vertical bars (N = 3, n = 40). *: $P < 0.05$, ***: $P < 0.01$.

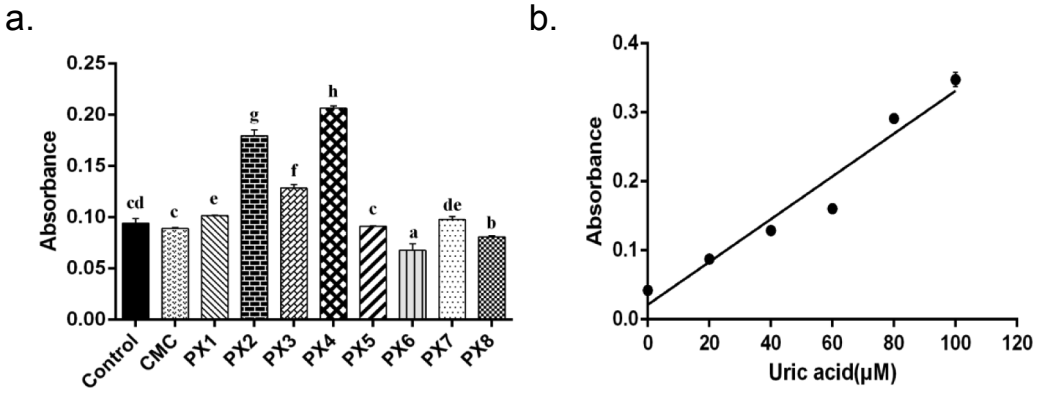

Fig. 3. The concentration of uric acid in different concentration of PO and XSS solutions.
(a) The absorbances of different concentration of PO and XSS solutions. Absorbance was measured at 560 nm. (b) The uric acid standard curve by the absorbance measured under 560 nm wave length. The relationship between uric acid content and absorbance: $A = 0.0031UA+0.0214$, $R^2 = 0.9485$.
Note: Values are group means, with STD represented by vertical bars (N = 3, n = 40). Different labeled letters denote significant differences ($P < 0.05$; ANOVA). PO, potassium oxonate; XSS, xanthine sodium salt.

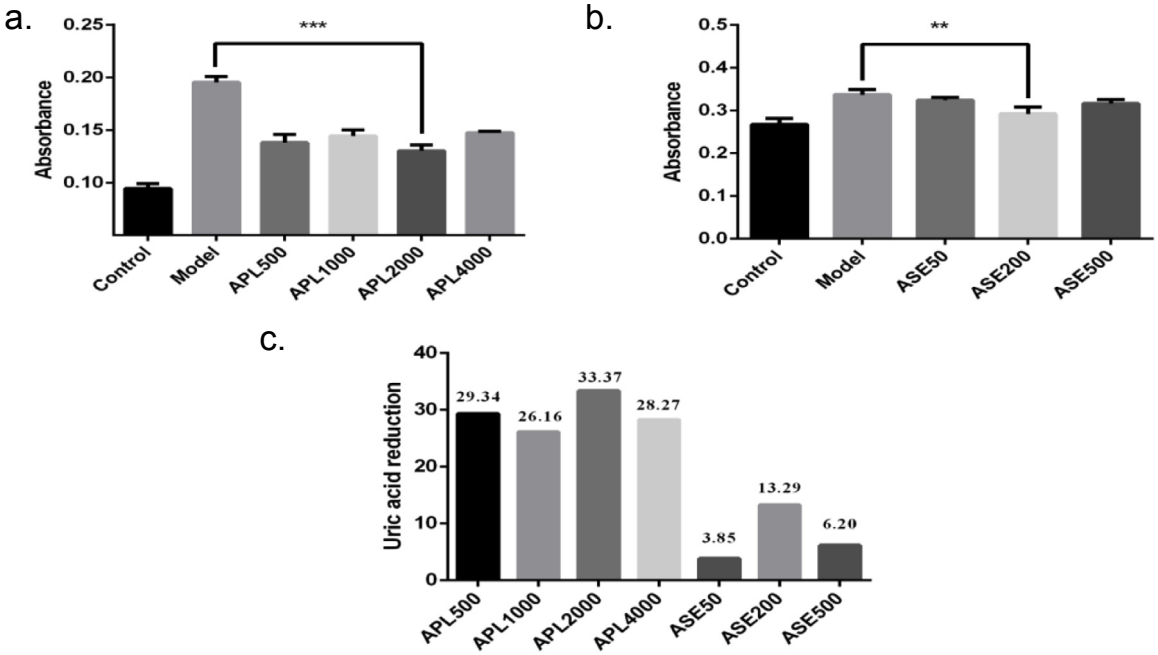

Fig. 4. Verification of zebrafish hyperuricemia model by APL and ASE.
(a) The concentration of uric acid in different concentration of APL solutions.
(b) The concentration of uric acid in different concentration of ASE solutions.
(c) The uric acid reduction of different concentration of APL and ASE solutions. The uric acid reduction = [A(Model) − A(APL/ASE groups)]/A(Model)*100%, "A" means the concentration of uric acid in different groups (N = 3, n = 40).
Note: Values are group means, with STD represented by vertical bars (N = 3, n = 40). APL, allopurinol. ASE, anserine. **: $P < 0.05$.***: $P < 0.001$.

substances, is becoming a commonly used method for the construction of mouse hyperuricemia models. Therefore, fish larvae were co-treated with PO and XSS to construct the acute hyperuricemia model in this study.

APL, a xanthine oxidase (XO) inhibitor, is one of the commonly used urate-lowering drugs. It can prevent the formation of uric acid and reduce the level of serum uric acid by decreasing purine synthesis [31]. In experimental mouse models, drug-induced hyperuricemia can be alleviated by APL treatment, which can maintain serum uric acid levels in the normal range [32,33]. In this study, 200 μM PO and 10 μM XSS were used to generate the zebrafish hyperuricemia model. Zebrafish model was verified through its response to APL. APL at the concentration ranging from 500 to 4000 μM could significantly reduce the uric acid content of the model group ($P < 0.05$). And the uric acid reduction with exposure to APL at the concentration of 2000 μM was highest compared with other concentration ($P < 0.001$). Anserine is a dipeptide containing an imidazole ring and with anti-inflammatory, anti-fatigue, and anti-oxidant effects [34,35]. It has also been reported that anserine could lower the serum urate level through its XO inhibitory effect [36]. In this study, by using our model, it demonstrated that anserine had good uric acid lowering activity at 200 μM ($P < 0.05$).

However, the effect of ASE, which was extracted from skipjack tuna (*Katsuwonus pelamis*), on lowering uric acid function is lower than that of APL. The results indicate that food containing anserine may have the effect of reducing the level of uric acid, which need further investigations. The results also demonstrated that the established zebrafish hyperuricemia model could be used for high-throughput screening of food and drugs with uric acid-lowering activity.

5. Conclusion

In conclusion, the zebrafish acute hyperuricemia model was successfully established through co-treatment with PO and XSS. We recommend constructing zebrafish acute hyperuricemia model with 200–400 μM PO and 10–20 μM XSS. Such model could offer a new robust approach for high-throughput screening of food and drugs with uric acid-lowering activity.

Ethics statement

Experiments reported in this study were carried out in accordance with local guidelines for the care of laboratory animals of Chinese Academy of Agricultural Sciences, and were approved by the ethics committee for research on laboratory animal use of the institution.

Conflicts of interest

All the authors declared no competing interests.

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