Study on the antipyretic activity and potential mechanism of Indigo Naturalis on lipopolysaccharide-induced fever rat model

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Research Article

Keywords: indigo naturalis, antipyretic, cytokines, possible mechanism, non-targeted metabolomics

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Abstract

Background: As a Traditional Chinese Medicine with an antipyretic effect, Indigo Naturalis (IN) has a long history of application in China. It is often used with other heat-clearing drugs to treat high fever in children, which is different from other heat-clearing medicines. But its antipyretic effect and mechanism have not been fully elucidated yet. And the heat-clearing development of IN lacks convincing current evidence.

Methods: This study aimed to uncover the antipyretic effect and mechanism of IN on infectious fever by rectal temperature, pyrogen cytokines and metabolomics.

Materials and Methods: Firstly, the antipyretic effect of IN on LPS-induced fever rats was evaluated by the changes in rat rectal temperature. Then, the content of PGE$_2$ and cAMP in the hypothalamus and TNF-$\alpha$, IL-1$\beta$ and IL-6 in the serum were determined by ELISA. Finally, the plasma and urine samples of febrile rats were analyzed in combination with UHPLC-Q-TOF-MS to explore the antipyretic mechanism of IN.

Results: Compared with pyrexia model rats, the rectal temperature of rats treated with Indigo Naturalis was suppressed after 0.5 h ($P < 0.01$). The enzyme-linked
immunosorbent assay (ELISA) showed that the expression of tumor necrosis TNF-α ($P < 0.01$) and IL-6 ($P < 0.05$) were suppressed by IN. A total of 11 endogenous metabolites were identified from the plasma samples of the IN group, the model group and the control group. Furthermore, 15 metabolites were identified in the urine samples. Pathway analysis showed that IN mainly regulated glycerophospholipid metabolism, glucuronide mutual conversion and tryptophan metabolism.

**Conclusion:** This study clarified the exact effect of IN on infectious fever. It mainly works by regulating pyrogenic cytokines and glycerophospholipid pathways. The present research provided a deeper understanding of the traditional heat-clearing effect of IN. It provided a reference for the further scientific research of IN and the selection of antipyretic drugs in clinical practice.

**Keywords:** indigo naturalis; antipyretic; cytokines; possible mechanism; non-targeted metabolomics

**Graphic abstract**
1. Introduction

Fever is a symptom associated with various clinical diseases, which is of great significance for disease judgment and treatment [1, 2]. Fever is more common in children, but the children are not fully developed and can easily cause irreversible injuries [3]. Meanwhile, wearable sensor technology and children's Day dosage forms have also received some attention [4]. The commonly used antipyretic drugs in clinic are aspirin, acetaminophen, ibuprofen, etc. [5]. However, the use of these antipyretics is easy to cause inevitable damage to gastrointestinal tract, liver and kidney function and nervous system [6, 7]. Therefore, it is necessary to find new antipyretic drugs from traditional Chinese medicine or natural medicine.

IN is a traditional heat-clearing medicine, can also be used for psoriasis, colon cancer treatment [8]. It is salty and cold in nature and tastes, and it returns to the liver meridian [9]. The herbal medicines of the past dynasties all have their original ideas on its antipyretics [10]. The first publication of IN's "Medicinal properties" (Sui Tang Dynasty 541-643 AD) recorded its effect as "relieving malnutrition fever in children"
and "Supplements to Materia Medica" (Tang Dynasty 741 AD) recorded that IN has the effect of relieving fever. Moreover, "Kaibao Materia Medica"(Northern Song Dynasty 973-974) recorded that IN can solve all kinds of poisons, fever in children, convulsions and fever[11]. Ancient books such as "Compendium of Materia Medica" (Ming Dynasty 1590 AD) and "Ben Cao Qiu Zhen"(Qing Dynasty 1796 AD) also record its antipyretic effect[12]. It can be seen that the antipyretic effect of IN has expanded from the initial malnutrition fever in children to various heat trouble. And gradually clarified the selectivity of its cold nature to diseases and the characteristics of its effect on liver meridian fire stagnation. In traditional medicine, IN is often used for antipyretic. Many common clinical antipyretic prescriptions and Chinese patent medicines contain classic prescriptions contain IN, such as Bi Yu San, Dai Ha San and Fu Fang Qing Dai Jiao Nang, which can be used for antipyretic and detoxification[13]. In addition, in today's China, IN and its Chinese patent medicines are still widely used in clinical practice. Especially in the field of pediatrics of TCM, it is often used to treat children with fever, skin diseases, and children with convulsions, etc. [14]. However, the antipyretic effect of IN has not been reported. Therefore, we investigate the potential antipyretic effects and mechanism of IN in rats with LPS-induced fever through the changes of anal temperature and the levels of related cytokines in LPS-induced rats and metabonomic research on plasma and urine.

2. Materials and methods

2.1. Material

IN pieces were provided by Ya'an Xunkang Pharmaceutical Co., Ltd. and identified
by Prof. Runchun Xu (School of pharmacy, Chengdu University of traditional Chinese medicine). The content of indigo was 2.23% and indirubin was 0.27% by HPLC. Lipopolysaccharide (LPS) was purchased from sigma company with batch number LOT039M4004V055, acetaminophen was purchased from Sichuan Good Doctor Pharmaceutical Group Co., Ltd. with batch number 190806, and indigo was purchased from Shanghai Yuanye Biotechnology Co., Ltd. with batch number Z03S10H96850, the purity is greater than 98%, indirubin was purchased from Chengdu Croma Biotechnology Co., Ltd., the batch number is CHB160930, the purity is greater than 98%. And the ibuprofen suspension was purchased from Shanghai Johnson Pharmaceutical Co., Ltd. Kits such as IL-6 were purchased from Hangzhou Multi sciences Biotech, co., Ltd. NN-Dimethylformamide (DMF) and Vaseline were purchased from Chengdu Cologne Chemical Co., Ltd., acetonitrile and methanol were chromatographically pure reagents, purchased from Merck, Germany; formic acid chromatographic pure reagents, purchased from Beijing Dima Technology, Leucine Acid enkephalin was purchased from Waters, USA; other reagents are of analytical grade.

2.2. The antipyretic effect of Indigo Naturalis on LPS-induced fever rats

Male Sprague-Dawley rats (weights 200±20 g) were supplied by Chengdu Dashuo Laboratory Animal Co., Ltd. (Chengdu, China). The protocol and experimental designs were approved by Ethics Committee of Laboratory Animal Center, Chengdu University of Traditional Chinese Medicine (Approval ID: 2022-82). The rats were reared in a breeding room under standard conditions (temperature, 22±2 °C; relatively humidity,
55 ± 5%; 12:12 h light-dark cycle) and were given standard diet and water. The experimental animals were fed adaptively for 3 days, once a day (at 9:00 am and 17:00 pm) in the morning and evening (at 9:00 am and 17:00 pm). The rats were subjected to adaptive rectal temperature measurement operations. Before each temperature measurement, the electronic thermometer probe was coated with petroleum jelly and inserted rat rectum 3 cm (mark with a marker at 3 cm of the thermometer to ensure the same depth of insertion each time), and record the body temperature after the reading is stable[15].

On the day of the experiment, the rat's body temperature was measured once every 0.5 h before modelling and administration. The average of the two times was taken as the basal body temperature. Among them, if the body temperature was more significant than 38.5 °C or the temperature difference between two adjacent times, Animals above 0.5 °C are eliminated.

Before the experiment, the animals fasted for 12 hours without water, and the animals were allowed to empty their feces before measuring the rectal temperature. On the day of the experiment, the rat's body temperature was measured once every 0.5 h before modeling and administration. The average of the two times was taken as the basal body temperature. Of these, data were removed if the body temperature was greater than 38.5 °C or if the temperature difference between two adjacent times was greater than 0.5 °C

Rats with an initial rectal temperature of 37 ~ 38°C were selected for the study, and thirty rats were randomly divided into five groups, namely the control group, the model
group, Ibuprofen suspension group, paracetamol group and IN group. The method of LPS-fever rat modeling is as follows: the control group was intraperitoneally injected with the same volume of 0.9% sodium chloride injection. Except for the control group, all the other groups were intraperitoneally injected with LPS solution (100 µg/kg) of 0.9% sodium chloride injection to replicate the fever rat model[16]. Immediately after modeling, the control group and the model group were given ultrapure water (10 mL/kg), ibuprofen suspension group (1.6 mL/kg), paracetamol group (0.05 g/kg), IN group (0.9g/kg). Because of the strong hydrophobicity of IN, it was moistened with a small amount of ethanol and then added with water to form a suspension. After administration, the body temperature changes of rats were recorded every 0.5 h, the heating value (measured body temperature-basal body temperature) of each group of rats at each monitoring point was calculated, the heating curve was drawn and the antipyretic effect was analyzed.

2.3. Sample collection and preparation

After administration, the urine of rats in each group was collected by metabolic cage. After collecting, centrifuge at 1,4000 rpm for 10 min and the upper urine was collected and frozen at −80°C. At the end of the rectal temperature measurement, rats in each group were anaesthetized with 20% ethyl carbamate (8 mL/kg). Blood was collected from the rat abdominal aorta and transferred into two tubes. One of the two samples was treated with heparin sodium. Subsequently, the blood was immediately centrifuged at 3500 rpm for 15 min at 4°C, and the upper plasma and serum were collected and frozen at −80 °C. Then quickly take out the whole brain, rinse with pre-
cooled physiological saline solution 2 to 3 times to remove blood stains, operate on ice between the optic chiasm and gray nodules to remove the hypothalamic tissue, add physiological saline to prepare a 10% brain homogenate, at 3000 rpm centrifuge for 10 min, and place the supernatant in a refrigerator at -80°C. The serum and hypothalamus homogenate were used for the cytokine assay. The plasma and urine were used for the metabolomics analysis.

Before experiment, the frozen samples were thawed at room temperature. Plasma (urine) samples (200 µL) were mixed with 600 µL of pre-cooled methanol. The mixture was vortexed thoroughly for 40 s and then centrifuged at 1,2000 rpm for 10 min to remove the protein precipitate. The supernatant (600 µL) was evaporated to dryness under nitrogen, redissolved with 100 µL methanol, and centrifuged at 1,4000 rpm for 10 min at 4°C. Finally, 3 µL supernatant was transferred for UHPLC-QTOF-MS analysis.

Quality control samples (QCs) are prepared from a mixture of each sample. 10 µL of each plasma sample was mixed to obtain the plasma quality control sample. The same method was used to obtain urine quality control samples. The coefficient of variation of ion characteristics in QCs was used to evaluate the stability of the analytical process.

2.4. Determination of cytokines in hypothalamus and serum

According to the instructions provided by the manufacturer, PGE$_2$ and cAMP in hypothalamus samples and TNF - α, IL-1 β and IL-6 in serum samples were determined by ELISA kit.
2.5. Metabonomics analysis of plasma samples

2.5.1 Chromatographic conditions

The mobile phase A was 0.1% formic acid aqueous solution, and the mobile phase B was 0.1% formic acid acetonitrile solution. Gradient elution: 0-2 min, 5% - 20% B; 2-12 min, 20% - 65% B; 12-18 min, 65% - 78% B; 18-21 min, 78% - 100% B; 21-22 min, 100% B; 22-22.5 min, 100% - 5% B; 22.5-25 min, 5% B. The column temperature was 40 °C, the flow rate was 0.3 ml min$^{-1}$, and the injection volume was 3µL.

2.5.2 Mass spectrometry conditions

ESI source, data collection in MS$^E$ continuum mode. Leucine enkephalin (ESI-m/z 554.2615) solution was used as the locked mass solution for accurate mass determination. Capillary voltage: ESI- 2.5 kV, ion source temperature: 140 °C, desolvent gas temperature: 450 °C, taper hole voltage: 40 V, taper hole gas flow rate: 50 L·h$^{-1}$, desolvent flow rate: 800 L·h$^{-1}$, collision energy (10 ~ 45 V), interval scanning time: 0.2 s. Mass scanning range: 50 ~ 1200 m/z.

2.6. Metabonomics analysis of urine samples

2.6.1 Chromatographic conditions

The mobile phase A is a 0.1% formic acid aqueous solution, and the mobile phase B is a 0.1% formic acid acetonitrile solution. Gradient elution: 0 ~ 1 min, 5% B; 1 ~ 8 min, 5% ~ 70% B; 8 ~ 12 min, 70% ~ 85% B; 12 ~ 12.5 min, 85% ~ 100% B; 12.5 ~ 14 min, 100% B; 14~ 14.5 min, 100% ~ 5% B; 14.5~ 16min, 5% B. The column temperature is 40 °C, the flow rate is 0.3 mL·min$^{-1}$, and the injection volume is 3µL.

2.6.2. Mass spectrometry conditions
2.7. Data processing and multivariate analysis

The raw data were analyzed using the Progenesis QI data analysis software (Non-linear Dynamics, Newcastle, United Kingdom) for peak alignment, picking, and normalization to obtain three-dimensional data containing the retention time \(t_R\), m/z, and peak intensity for each sample. Then, tags such as variable importance for projection (VIP) >1, \(P<0.05\), Splot >0.01 were set up to export related table. SIMCA-P (version 13.0, Sartorius Stedium Biotech, Malmö, Sweden) was used for unsupervised principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). In the OPLS-DA model, the VIP >1, as well as \(P<0.05\) for independent sample t-test were selected as differential metabolites. The selected differential compounds were imported into MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/) website for pathway analysis. This website can conduct pathway analysis of compounds through KEGG and HMDB values.

3. Results

3.1. Antipyretic effect of IN on LPS-induced fever rats

The rectal temperature change curve and antipyretic effect of different administration groups are shown in Fig.1. Compared with the control group, the temperature of the rectum increased significantly after 0.5 h of intraperitoneal injection of LPS \((P<0.01)\) and reached the peak temperature at the 2nd and 4th hours after modeling. The rectal temperature of rats in each administration group was inhibited to different degrees after 30 minutes of administration \((P<0.01)\). After 1 hour, the
The rectal temperature of each administration group was controlled below 0.5℃, and the IN group was administered 1 hour later. The posterior rectal temperature basically returned to a normal level. The antipyretic effect is better than ibuprofen at 1.5h, 3h, 3.5h, the antipyretic effect is better than the paracetamol group after 1.5h, the antipyretic effect of ibuprofen is better than paracetamol, after administration It can suppress fever in 4 hours.

**Fig.1.** The rectal temperature of febrile rats and drug-administered rats. Rat rectal temperature change degree at different time points(A), rat rectal temperature change curve(B). All the data were expressed as mean ± SD.

### 3.2. ELISA results

The ELISA results are shown in Fig.2. Compared with the control group, the contents of PGE$_2$, cAMP, TNF-α, IL-1β and IL-6 in the model group were significantly increased ($P<0.01$). Compared with the model group, IN could significantly down regulate the levels of TNF-α ($P<0.01$) and IL-6 ($P<0.05$). Ibuprofen and acetaminophen can down-regulate the levels of PGE$_2$, TNF-α, and IL-6, and ibuprofen down-regulates the above-mentioned cytokines better than acetaminophen. IN does not significantly reduce the level of pyrogenic factors in the hypothalamus, but mainly affects the levels
of TNF-α and IL-6 in the periphery.

Fig.2. The contents of PGE2 (A), cAMP (B) and serum TNF-α (C), IL-1β (D) and IL-6 (E) in the rat hypothalamus. **P < 0.01; Vs Model, ##P<0.01. Data are expressed as mean ± SD (n = 6).

3.3. Quality control evaluation of metabonomics samples

The PCA model is used to determine the difference in metabolites between the model group and the administration group and the control group. The unsupervised PCA score chart shows that in the plasma samples and urine samples, the samples are clustered more obviously in the positive ion and negative ion scanning modes, and QC has good aggregation in the PCA chart, as shown in Fig.3. Plasma negative ion sample ($R^2_X=0.866$, $Q^2=0.797$), plasma positive ion sample ($R^2_X=0.791$, $Q^2=0.709$), urine sample negative ion ($R^2_X=0.841$, $Q^2=0.679$), urine sample positive ion ($R^2_X=0.649$, $Q^2=0.246$). The good collection of QC samples indicates that the detection system is
stable and reliable, and has good repeatability. The differences obtained can reflect the biological differences between the samples, and the collected data can be further analyzed.

![PCA score chart of rat metabolites.](image)

**Fig.3.** PCA score chart of rat metabolites.

### 3.4. Multivariate statistical analysis of UHPLC-Q-TOF-MS data

In the unsupervised PCA score graph, there is a clear clustering trend. In order to further evaluate the efficacy of IN, a supervised OPLS-DA analysis was performed on the control group, the model group and the IN group. In the positive ion and negative ion mode, the urine metabolic profile of the model group and the control group was obviously clustered into 2 types ($R^2_X=0.732$, $R^2_Y=0.966$, $Q^2=0.869$), indicating that the urine of the two groups of rats after modeling There are significant differences in endogenous metabolites. There were also significant differences in metabolites between the IN group and the model group (Figure 4). In the plasma samples, the plasma model group and the control group were significantly separated ($R^2_X=0.872$, $R^2_Y=0.997$, 


$Q^2 = 0.983$), indicating that there are significant differences in the endogenous metabolites of rat plasma after modeling. The plasma metabolic profile of rats after the intervention of the IN group and the model group were obviously clustered into two categories, which was significantly different (Fig.5).

Fig.4. OPLS-DA score chart of rat urine metabolites.
Fig. 5. OPLS-DA score chart of rat plasma metabolites.

3.5 Potential biomarkers in plasma and urine

Through OPLS-DA analysis, it is found that there are significant differences in metabolites between the model group and the control. According to the accurate m/z of ion characteristic metabolites, 15 characteristic metabolites were identified from the model and IN group in urine samples (Table 1), mainly including amino acids. In the plasma samples, 11 characteristic metabolites were identified from the model and IN group (Table 2), mainly related to phospholipids.

Table 1. Differential metabolites in rat urine

<table>
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<tr>
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<th>R.T.(min)</th>
<th>Biomarkers</th>
<th>Formula</th>
<th>Mass</th>
<th>model vs control</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>indigo naturalis</td>
<td>QC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Retention Time</td>
<td>Compound</td>
<td>Molecular Formula</td>
<td>M/z</td>
<td>p-value 1</td>
</tr>
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<td>----</td>
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</tr>
<tr>
<td>1</td>
<td>2.46</td>
<td>6-Thiourate</td>
<td>C\textsubscript{5}H\textsubscript{4}N\textsubscript{4}O\textsubscript{2}S</td>
<td>204.9800</td>
<td>↑ *</td>
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<td>Indoxyl glucuronide</td>
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<td>308.0774</td>
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<td>Malonylcarnitine</td>
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<td>243.0764</td>
<td>↓ **</td>
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<tr>
<td>6</td>
<td>1.04</td>
<td>(2R*,3R*)-1,2,3-Butanetriol</td>
<td>C\textsubscript{4}H\textsubscript{10}O\textsubscript{3}</td>
<td>151.0345</td>
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<td>176.0699</td>
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*model vs control, **P < 0.01, model vs other groups, ##P < 0.01.*
<table>
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<th>Biomarkers</th>
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<th>Model vs other groups</th>
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<td>PC(20:3(5Z,8Z,11Z)/20:3(8Z,11Z,14Z))</td>
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<td>↓ ′′</td>
<td>↓ **</td>
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<td>↓ ′′</td>
<td>↓ **</td>
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<td>3.91</td>
<td>Methyl bisnorbiotinyl ketone</td>
<td>C_{9}H_{14}N_{2}O_{2}S</td>
<td>215.0858</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>11.87</td>
<td>Tetracosahexaenoic acid</td>
<td>C_{24}H_{38}O_{2}</td>
<td>357.2793</td>
<td>-</td>
<td>↑ #</td>
</tr>
<tr>
<td>8</td>
<td>13.18</td>
<td>LysoPL(18:0/0:0)</td>
<td>C_{24}H_{53}O_{3}P</td>
<td>645.2974</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>14.20</td>
<td>9-cis-Retinol</td>
<td>C_{20}H_{30}O_{2}</td>
<td>319.2635</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>18.34</td>
<td>Dibutyl phthalate</td>
<td>C_{16}H_{22}O_{4}</td>
<td>301.1418</td>
<td>↓ ′′</td>
<td>↓ #</td>
</tr>
<tr>
<td>11</td>
<td>18.54</td>
<td>SM(d18:0/16:1(9Z))</td>
<td>C_{20}H_{36}N_{2}O_{3}P</td>
<td>703.5735</td>
<td>↑ **</td>
<td>↑ ##</td>
</tr>
</tbody>
</table>

*model vs control, **P < 0.01, model vs other groups, ##P < 0.01.*
Fig. 6. Relative changes in the abundance of some metabolites in urine and plasma.

(A) Lanthionine ketimine, (B) L-Kynurenine, (C) Indoxyl glucuronide, (D) Taurocholic acid, (E) PC (20:3(5Z,8Z,11Z)/20:3(8Z,11Z,14Z)), (F) Dibutyl phthalate.

3.6 Pathway Analysis

Metabo Analyst 5.0, HBMD ID and related literatures were used for pathway analysis[17]. The results revealed that the metabolic biomarkers were responsible for tryptophan metabolism, glycerophospholipid, interconversion of pentose and glucuronate and glycosylphosphatidylinositol et al. Based on changes in potential biomarkers in metabolic pathways. The reversal of biomarkers in the IN treated group indicates that IN can exert its efficacy by regulating the abnormal function of related metabolic pathways during the development of fever.
Fig. 7. Pathway analysis of differential metabolites in rat plasma (A), pathway analysis of differential metabolites in rat urine (B). (a) glycerophospholipid metabolism; (b) glycosylphosphatidylinositol (GPI)-anchored biosynthesis; (c) linoleic acid metabolism; (d) taurine and taurine metabolism; (e) α-Linolenic acid metabolism; (f) sphingolipid metabolism; (g) arachidonic acid metabolism; (h) primary bile acid synthesis; (i) mutual conversion of pentose and glucuronate; (j) tryptophan metabolism; (K) Drug metabolism-other enzymes.

4. Discussion

In this study, the antipyretic effect of IN, and untargeted metabolomics profiling based on UHPLC-Q-TOF-MS, were evaluated using a rat model of LPS-induced pyrexia. The results suggested that IN could significantly relieve fever in pyrexia rats 0.5 h after treatment. Subsequently, we used ELISA and metabolomics to investigate the potential antipyretic mechanism of IN. These findings indicated that IN present an antipyretic effect focus on correcting the disturbed lipid and tryptophan.

It is found that IN has a good antipyretic effect on infectious fever induced by LPS, and the effect is rapid. In this study, the effective antipyretic dose of IN is 9 g (converted to the daily oral dose for adults). However, the daily dose of IN is 1-3 g, and the
preliminary test found that the antipyretic effect at the dose of 3 g and 6 g was not
significant. Looking back to the herbal literature, it is found that the IN before the Ming
Dynasty, also known as Dian Hua, is a surface foam enriched after adding lime to Malan
Soaking Liquid. While contemporary IN is far from Dian Hua, it is a sediment enriched
after adding a large amount of lime to Malan Soaking Liquid. And the content of indigo
and indirubin contained in Dian Hua is about 2-4 times that of contemporary IN.

Studies have shown that in LPS-induced rat models, most abnormal metabolic
abnormalities (such as amino acid metabolism, lipid metabolism, energy metabolism
and sugar metabolism) are closely related to inflammatory reactions[18]. Moreover,
Many natural products from Chinese herbal medicine also have good anti-inflammatory
effects[19]. The metabolic differences in plasma are mainly related to lipid metabolism
(glycerophospholipid metabolism, linoleic acid, sphingolipid metabolism). Inflammation can cause disorders of lipid metabolism, and imbalance of lipid
metabolism can also induce inflammation[20]. Lipid metabolism is a ligand for nuclear
receptors that regulate inflammation and is closely related to TNF-α and IL-6[21]. After
treating with LPS, lipids can affect energy metabolism and oxidative stress.
Glycerophospholipids (PC, PE, etc.) are the key components of cell lipid bilayers,
which are involved in metabolism and signal transduction [22]. Phosphatidylcholines
(PC) are an important product in lipid metabolism and closely related to the occurrence
of inflammation[23]. Moreover, the feature metabolites of LysoPCs and PCs in IN
closely related to PGE₂[24]. PGE₂ is the vital substance that induces pyrexia[25]. In the
process of PGE₂ synthesis, the phospholipids of the cell membrane generate
arachidonic acid with phosphatidase 2, further generating PGE$_2$ under the action of cyclooxygenase 2 (COX-2)[26]. LysoPCs could also generate PCs with lysophosphatidylcholine acyltransferase (LPCAT)[15]. In the present study, we found that the level of PC (20:3(5Z,8Z,11Z)/20:3(8Z,11Z,14Z)), PE (14:0/22:2(13Z,16Z)) and SM(d18:0/16:1(9Z)) in the control group and the IN group has the same level change trend, suggesting that IN could relieve fever in rats by suppressing the expression of PGE$_2$ and regulating lipid metabolism.

Taurine, an oxidation product of cysteine, has many biological functions including anti-inflammation, anti-oxidation and Ca$^{2+}$ transport regulation [27]. And it seems to be a metabolic marker of the defense mechanism against oxidative stress in inflammation rat. Bile acids are cholesterol-derived steroid molecules produced in the liver, which are closely associated with diverse metabolic and inflammatory pathways [28]. In this study, the level of taurocholic acid in plasm of model rats increased, and IN can significantly adjust its level. It indicated that IN could regulate the disorder of primary bile acid biosynthesis pathway as in Fig 7A. In addition, polyunsaturated fatty acids are also closely related to inflammation. n-3 fatty acids compete with arachidonic acid for cyclooxygenase and directly inhibit the activity of cyclooxygenase. n-6 polyunsaturated fatty acids can promote the production of pro-inflammatory eicosanoids (PGE$_2$, LTB$_4$ and TXA$_2$). Compared with IN group, the level of Tetracosahexaenoic acid was increased in model group, which has been reported to exhibit similar anti-inflammatory activities by inhibiting eicosanoids and pro-inflammatory cytokines [29].

Amino acids are important regulator of immune response. In fever rats, remarkable
changes were observed in the content of several free amino acids and their biogenic metabolites, including Indolylacryloylglycine, N-Acetyl-L-glutamate 5-semialdehyde, N (6)-Methyllysine, Alanyl-Lysine, Indoleacetic acid. And in present study, L-Kynurenine, the central compound of tryptophan metabolic pathway, was significant increasing in model group. The above compound mainly involved in tryptophan metabolism, which is closely related to the inflammatory response[30].

Tryptophan is an essential amino acid for the human body, and it is also the precursor of 5-hydroxytryptamine (5-HT), an important neurotransmitter in the human body[31]. Injection of 5-HT into the lateral ventricle of cats can cause fever. There are abundant monoamine neurotransmitters in the hypothalamus of mammals, which are related to thermoregulation. At present, it is believed that 5-HT is thermogenic amine[32], and IN may inhibit fever by regulating the metabolism of tryptophan.

Studies have shown that elevated levels of tryptophan in the urine of fever rats lead to increased fever response, which may be due to the increased ability of tryptophan in the urine of fever rats to synthesize 5-HT, γ-aminobutyric acid (GABA), and phosphatidyl muscle Alcohol also increases[33]. The increase in GABA may cause temperature increase through the Na\(^+\)/Ca\(^{2+}\)-cAMP pathway in the hypothalamus[34, 35]. The secretion of 5-HT may be related to increased Ca\(^{2+}\) or cAMP[36]. Thus, the disorder of tryptophan metabolism can increase the synthesis of 5-HT and cAMP by inhibiting the phosphoinositide signaling system. At the same time, it involves drug enzyme metabolism. IN can regulate drug-enzyme metabolism, which may be responsible for its characteristics of fast onset and relatively long time of effect.
Furthermore, the ELISA showed that the content of PGE$_2$ and cAMP in the hypothalamus of the model group increased significantly ($P<0.01$), and the content of IL-1$\beta$, IL-6, and TNF-α in the serum increased significantly ($P<0.01$). PGE$_2$, cAMP are important positive regulators of central fever[37, 38], TNF-α, IL-1$\beta$, and IL-6 are important peripheral pyrogenic cytokines[39]. And anti-inflammatory factors are the key factors in determining the prognosis of inflammation[40]. IN can significantly down-regulate the levels of TNF-α and IL-6. However, there is a certain connection between some cytokines that cause fever. IL-1 can activate MAPKp38, c-Jun and NF-$\kappa$B through TAK1, thereby inducing COX-2 and producing PGE$_2$[41]. IL-6 and IL-6R bind to the brain endothelium. After the cell, the expression of COX-2 can be induced by intracellular signal transduction of the STAT3 pathway to produce PGE$_2$[42, 43]. When inflammation occurs, elevated levels of TNF-α can cause disorders of glucose metabolism and gluconeogenesis[44]. At the same time, mPGES-1 is activated under the signal pathway mediated by glutathione to produce PGE$_2$[45], which leads to increased body temperature. Many researchers also believed that TNF-α induces IL-1 in vivo[46]. These results showed that IN exerted an antipyretic effect through the inhibition of PGE$_2$, IL-6 expression and then further influencing other thermogenic factors.
Fig. 8. The pathways closely associated with the antipyretic effect of indigo naturalist.

5. Conclusions

This experiment studied the antipyretic effect and mechanism of the traditional antipyretic drug IN on infectious fever from the perspective of cytokines and metabolomics. In the fever model of LPS-induced rats, IN exerts antipyretic effects by regulating lipid metabolism, tryptophan metabolism, down-regulating TNF-α, IL-6 and other pyrogenic cytokines. And it has the characteristics of rapid onset and long duration, which provide reference for the clinical use of IN for antipyretic.

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Not applicable

Author contributions

Z.D.K, and H.L. conceived and designed the experiments. X.X.R, H.S.J, L.C.H and H.Y.N performed the animal experiments. X.X.R and H.S.J performed the Non targeted metabonomics experiment. X.X.R and H.S.J drafted the manuscript and analyzed the data. Y.M and W.F. modified
the manuscript. L.J.Z and Z.D.K reviewed and edited the manuscript. All authors have read and
approved the final manuscript.

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202209).

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and
supplementary material.

Declarations Ethics approval and consent to participate

The protocol and experimental designs were approved by Ethics Committee of
Laboratory Animal Center, Chengdu University of Traditional Chinese Medicine
(Approval ID: 2022-82).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest or personal relationships that could have
appeared to influence the work reported in this paper.

List of abbreviation
Abbreviation | Full name
--- | ---
IN | Indigo Naturalis
PGE₂ | Prostaglandin E₂
cAMP | Cyclic Adenosine monophosphate
TNF-α | Tumour necrosis factor-α
IL-1β | Interleukin-1β
IL-6 | Interleukin-6
IL-10 | Interleukin-10
INF-γ | Interferon γ
LPS | Lipopolysaccharide
DMF | NN-Dimethylformamide
OPLS-DA | Orthogonal Partial Least Squares Discrimination Analysis
PCA | Principal Component Analysis
PLS-DA | Partial Least Squares Discrimination Analysis

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