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Original Research

Analgesic, anti-inflammatory and sedative/hypnotic effects of Icaritin, and its effect on chloride influx in mouse brain cortical cells

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Abstract: Inflammation and insomnia are medical problems that may severely affect work and health, thereby necessitating strategies for their effective treatment. Icartin (ICT) is a major active monomeric component of icariin . Studies have revealed that ICT possesses several pharmacological properties such anti-inflammatory, anti-tumor, anti-fibrotic, anti-osteoporotic and neuroprotective effects. The present research was carried out to investigate the anti-inflammatory, analgesic and sedative/hypnotic effects of ICT. The results obtained revealed that ICT exerted a good anti-inflammatory effect related to the downregulations of inflammatory cytokines and the inhibition of COX-2 signaling pathway. Moreover, ICT enhanced Cl- influx in mouse cortical cells in a concentration-dependent manner. These data suggest that ICT exerts a hypnotic effect in mice through a mechanism associated with increased Cl- influx in cortical cells.

Key words: Icaritin; Analgesic; Anti-Inflammatory; Sedative-Hypnotic; Cl- influx.

Introduction

The management of pain and inflammation constitutes a serious challenge to modern medical practice. In clinics, pain and inflammation are usually treated with non-steroidal anti - inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs. However, NSAIDs are associated with unwanted side effects which limit their usage. The adverse reactions of popular pain-relieving drugs such as NSAIDs may result in gastric diseases and cardiovascular complications (1, 2). Therefore, NSAIDs are limited to short-term use.

Chronic insomnia is one of the most common diseases which seriously affects normal life, work and study (3). Thus, there is need to evolve more effective and safe sleep-improving drugs for the management of insomnia. Studies have revealed that several medicinal plants are effective in the treatment of various diseases, including pain and inflammation (4, 5). Plant-based drugs are of advantage in that they are more effective, less toxic, and can be used for long periods of time without much associated health risks. Thus, pharmaceutical companies are on the look-out for effective medicinal plants for the development of new drugs.

Epimedium brevicornu Maxim is a genus of *Epimedium* which was first recorded in *Shennong Materia Medica*. Icaritin (ICT, Figure 1) is a hydrolysis product of icariin, the main active monomeric component of icariin. In traditional Chinese medicine, ICT is used for *tonifying* kidney, and for *strengthening* the heart and bones. Modern medical research have revealed that ICT possesses anti-tumor (6), anti-cancer cell proliferation (7), anti-osteoporotic (8), anti-inflammatory (9), antioxidant (10), and estrogen-receptor-like effects (11).

Some progress has been made in studies on the use of Traditional Chinese Medicine in the treatment of neuro-inflammatory diseases. A study has reported that Icaritin (ICT) exerted estrogen-like neuroprotective and anti-inflammatory effects (9). Moreover, it has been shown that at the cellular level, ICT exerted *in vitro* anti-inflammatory effects through suppression of the production of TNF-α, IL-1β, NO, and prostaglandin E2 (PGE2) by LPS-stimulated mouse RAW264.7 macrophages. In LPS-induced C57BL/6J mouse inflammation model, ICT reduced the serum levels of TNF-α and PGE2, and decreased expression of CD11b in neutrophil CD11b (12). It has been reported that ICT produced high analgesic effect on cancer-induced pain in the bone, chemotherapy-related peripheral neuropathy, hand-foot syndrome, hand-foot skin reaction pain, and other cancer-related pains (13). In the present study, the analgesic, anti-inflammatory and sedative/hypnotic effects of

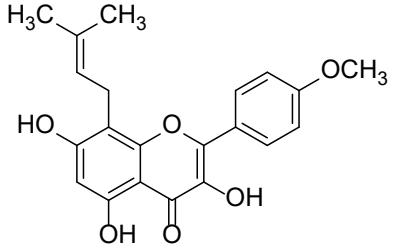

Figure 1. Chemical structure of ICT.

ICT, and its effect on chloride influx were investigated in mouse cerebral cortical cells.

Materials and Methods

Drugs and main reagents

The major drugs and reagents used, and their sources (in brackets) were: icaritin (Beijing Suolaibao Technology Co., Ltd.); glacial acetic acid (Tianjin Kemeiou Chemical Reagent Co., Ltd.); xylene (Chengdu Kelong Chemical Reagent Factory); sodium carboxymethyl cellulose (Sinopharm Group Chemical Reagent Co., LTD.); aspirin enteric-coated tablets (Bayer Schering Pharma); kits for TNF-α, IL-6, and IL-1β ELISA (Hangzhou Lianke Biotechnology Co., Ltd.), and COX-2 and -actin antibodies (Santa Cruz, CA, USA). The other reagents used were of pure analytical grade.

Experimental animals

The experimental animals used were SPF-grade ICR mice of both sexes, weighing 18 - 20g. They were obtained from Hunan Slake Jingda Experimental Animal Co., Ltd. [license number: SCXK (Hunan) 2016-0002]. The animals were housed in mouse cages at temperature range of 20 ~ 25 °C, and relative humidity of 45 - 65 %, with 8 - 10 mice/cage, and were allowed *ad libitum* access to feed and drinking water.

Acetic acid-induced mice writhing test

A total of 50 mice, with equal number of males and females, were randomly divided into 6 groups, negative control group (NS), aspirin group (ASP, 40 mg/kg), low and middle dose ICT groups (25, 50 and 100 mg/kg), and negative control group given 0.5% sodium carboxymethyl cellulose solution (20 mL/kg). The treatments were administered through gastric gavage once a day for 7 days. One hour after the last administration, 0.6% glacial acetic acid solution was intraperitoneally injected into each mouse at a dose of 10 mL/kg. The number of writhing reactions (arching of abdominal region, extension of hind limbs, and raising of rear end) in the mice within 15 min after injection was recorded, and the writhing inhibition was calculated according to the following formula:

$$Torsion\ inhibition\ (\%) = \frac{(1 - Number\ of\ torsion\ in\ treated\ group \times 100)}{Number\ of\ torsion\ in\ the\ -ve\ control\ group}$$

(-ve = negative)

Effect of ICT on xylene-induced mice ear swelling

Mice in the negative control group were given 0.5% carboxymethyl cellulose sodium solution by gavage at a dose of 20 mL/kg body weight (bwt), while the other groups were given their corresponding treatments at doses equivalent to 20 mL /kg bwt for 7 days. One hour after the last dose, the right auricles of mice were treated with 0.05 mL of xylene (dimethylbenzene, PX), while the left ears were untreated, and served as control. After 4 h, mice in each group were sacrificed by cervical vertebra removal, and the ears were cut off along the auricle baselines. Equal portions of auricles were cut off at the same part of ears with a 6-mm diameter perforator. Ear swelling was calculated as the difference in mass between the right ear and the left ear viz:

Ear swelling = right ear mass – left ear mass

The percentage inhibition of ear swelling was calculated using the equation:

$$Inhibition\ (\%) = \frac{(Difference\ in\ mean\ swelling\ between\ negative\ control\ and\ treated\ groups \times 100)}{Mean\ swelling\ in\ the\ negative\ control\ group}$$

Contents of TNF-α, IL-6, and IL-1β in xylene-induced swollen mice ear tissue

Fifty mice (half male and half female) were randomly divided into 5 groups: normal control group, model group, ICT at a dose of 25 mg/kg group, ICT at a dose of 50 mg/kg group, and ICT at a dose of 100 mg/kg group. Mice in each group were given 0.1 mL /10 g bwt by gavage for 7 days. Then, 30 min after the last treatment, the right ears of mice in each group were evenly treated on both sides with 0.05 mL of xylene. After 4h, the mice were sacrificed using cervical dislocation. Ear samples were taken for preparation of 10 % tissue homogenate in physiological saline at the ratio of 1: 9 (mass: volume). The homogenate was centrifuged at 3000 rpm for 10 min at 4°C. The clear supernatant was used for assay of TNF-α, IL-6, and IL-1β contents with ELISA kits in accordance with the manufacturer's instructions.

Expression of COX-2 protein in xylene-induced swollen mice ear tissue using Western Blot

The mice were divided into groups, and the drug treatments were the same as indicated in previous sections. The same part of ear tissue was taken and ground into powder in liquid nitrogen. The powder was transferred into a 1.5mL EP tube, and 200μl RIPA lysate was added. After adequate lysis, the lysate was centrifuged at 12000 rpm for 10min at 4°C, and total protein concentration was measured in the supernatant. The protein was denatured by boiling, and a portion (30 μg) was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and thereafter transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 5% skimmed milk for 1 h. Then, incubation with corresponding primary antibodies was carried out at 4 °C, followed by incubation with secondary antibody (1:100 dilution) at room temperature for 1 h. After washing with TBST buffer, ultra-sensitive chemiluminescence solution (ECL) was used to detect the expressions of the target protein COX-2. Actin was used as internal reference. The experiment was repeated for 3 times. Image J software was used for scanning analysis and processing.

Effect of ICT on threshold dose of pentobarbital sodium in sleep duration and sleep latency in mice

Male mice were randomly divided into 5 groups (10 mice per group). Mice in the blank group were given distilled water by gastric gavage. The other groups were diazepam group, low ICT group (25 mg/kg), low ICT group (50 mg/kg) and high dose ICT group (100 mg/kg). The treatments were given by gavage once a day for 7 days. Thirty minutes after the last dose, each group of mice received celiac injection of sodium pentobarbital at the threshold dose of 49.5 mg/kg. The number of mice with disappearance of positive varus reflex for more than 1 min after 30 min was observed, and the sleep time and waking time of each mouse were recorded as indices of sleep latency and sleep time of the

mice.

Preparation of mouse cortical cells

The mice were sacrificed using decapitation without induction of anesthesia. The cerebral cortex was quickly dissected on ice to separate the gray matter from the white matter, and the grey matter was chopped up and homogenized in a glass homogenizer. The homogenate was transferred into a 50-mL centrifuge tube containing 30 mL buffer preparation consisting of NaCl (118.5mM), KCl (4.7mM), MgSO4 (1.18mM), CaCl2 (1.0mM), HEPES (20mM), Tris (9mM) and d-glucose (10mM). The homogenate was then centrifuged at 4 °C at 1000 g for 5 min. The supernatant was discarded, and the sediment was passed through a pre-wetted nylon filter (40min).The filtrate was centrifuged at 4 °C at 300 g for l 5 min, but no clear supernatant formed.

Detection of Cl- influx

The cells were re-suspended in 10mM MQAE dissolved in chloride buffer (HEPES (10 mM), d-glucose (10mM), MgSO4 (1mM), K2HPO4 /KH2PO4 (2.4mM/0.6mM); CaSO4 (1mM) and NaCl (130 mM) for 1h at 37 °C. After loading, the suspension was centrifuged at 4°C at 300 g for 5 min.

Discard the supernatant. The pellet was gently resuspended in a chloride-containing buffer and centrifuged at 300 g for 5 minutes at 4° C for three times. Discard the supernatant and dilute the pellet to ensure a cell density of 8× 10^6 /mL. Protein concentration was measured by the Coomassie Brilliant Blue G-250 method. 20 µl of the cell lysate was added to a 96-well plate, followed by the addition of 180 mL of a chloride-containing buffer with or without a compound. The fluorescence was measured with a fluorescent microplate reader (excitation of 360 nm; emission of 460 nm). Three wells are used for each group. The initial fluorescence is Fi and the final fluorescence is Ft. The value of (Ft - Fi) in control group was considered as the maximum value 100%. The effect of each drug treatment was calculated using the following formula: $(Ft_{sam}\text{-}Fi_{sam})/(Fi_{con}\text{-}Ft_{con})* 100\%$

Statistical analysis

Measurement data are expressed as mean ± SEM, and were statistically analyzed with Student's *t*-test. Univariate analysis of variance was used for the comparison between multiple groups. For the sub-hypnotic dose of pentobarbital test, chi-square test was used for comparison of the number of sleeping mice. All statistical analyses were done with Graphpad Prism7.0 statistical software. Statistical significance was assumed at $p < 0.05$.

Results

Effect of ICT on acetic acid-induced writhing of mice

Compared with the negative control group, the number of body writhes was significantly reduced in the aspirin group (p <0.001), and the model was successfully established. As shown in Figure 2, the number of writhes induced by acetic acid in mice in the ICT-treated groups were significantly reduced in each dose group ($p < 0.05$). The results showed that ICT had significant analgesic effects in a dose-dependent manner.

Effects of ICT on the degree of auricle swelling induced by xylene in mice

Compared with the negative control group, auricle swelling of the aspirin group was significantly reduced. At the same time, compared with the aspirin group, ICT treatment at different doses produced significant degrees of reduction in auricle swelling in mice in a dose-dependent manner ($p < 0.05$; Figure 3).

Effect of ICT on TNF-α, IL-6, and IL-1β levels in xylene-induced mice ear swelling

Figure 4 shows the effect of ICT on the secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β. Compared to the negative control group, the levels of

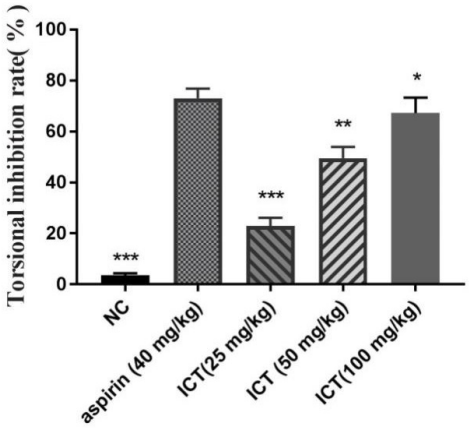

Figure 2. Analgesic effect of different doses of ICT. Each bar represents mean ± standard deviation (SD, n = 10). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, relative to aspirin group.

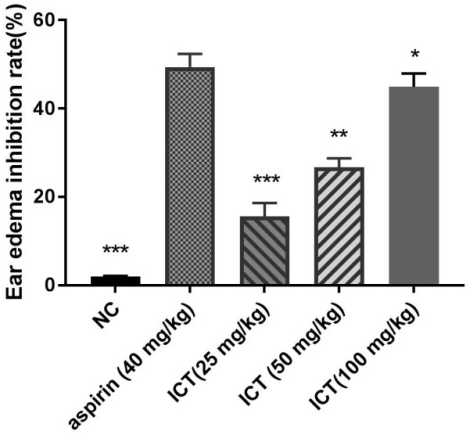

Figure 3. Effect of ICT on dimethylbenzene-induced ear swelling in mice. Each bar represents mean ± standard deviation (SD, n = 10). **$p < 0.01$, ***$p < 0.001$, compared to aspirin group.

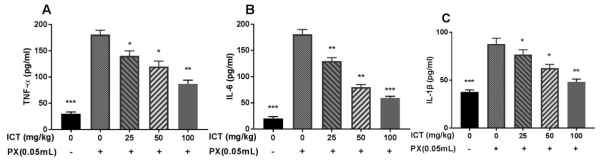

Figure 4. Effect of ICT on dimethylbenzene-induced increases in TNF-α, IL-6 and IL-1β in mouse swollen ear tissue. Mouse auricle was treated with xylene (0.05 mL/mouse) for 4 h in the absence or presence of the indicated concentrations of ICT. (A) TNF-α and (B) IL-6 (C) IL-1β in the culture supernatant were measured using ELISA. Data represent mean ± SD of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, relative to vehicle.

TNF-α, IL-6, and IL-1β were significantly increased in the model group. However, pro-inflammatory cytokines were dose-dependently decreased when treated with different concentrations of ICT ($p < 0.05$) (figure 4).

Effect of ICT on COX-2 protein expression in xylene-induced mice ear swelling

Figure 5 shows that the expression of COX-2 protein in the swollen ear tissues of mice in the model group was significantly up-regulated, when compared with the normal control group. However, ICT treatment reduced the expression of COX-2 protein in a dose-dependent manner ($p < 0.05$).

Effect of ICT on sleep duration and sleep latency induced by threshold dose of pentobarbital sodium in mice

Sleep time and sleep latency were compared amongst the groups. Compared with the blank group, the sleep time of the diazepam group was prolonged while the sleep latency was significantly shortened ($p < 0.001$). At the same time, mice in the ICT group were able to achieve similar results to varying degrees ($p < 0.05$; Figure 6).

Effect of LCT on Cl- influx in mouse cortical cells

A slight increase in fluorescence was detected in the control group during the fluorescence reading. At the end of each experiment, Nigericin, mycetin and tributyltin were added to obtain the minimum fluorescence. The addition of pentobarbital sodium (20mM) caused a significant decrease in the fluorescence of MQAE within 20 min. Diazepam and ICT significantly reduced MQAE fluorescence in a concentration-dependent manner ($p < 0.01$; Figure 7).

Discussion

Pain is one of the main manifestations of inflammation. Inflammation is often accompanied by pain, which implies that the two have mutual causal relationship (14). In order to study the characteristics and mechanisms of action of analgesics for inflammatory pain, various methods are often used (including chemical stimulation and immune methods) to replicate the animal model of inflammatory pain (15). In mice writhing method, an irritating chemical substance such as acetic acid is injected into the abdominal cavity of mice to stimulate the peritoneum, leading to inflammatory pain which is manifested as writhing phenomenon. Xylene is a chemical anti-inflammatory agent which triggers the release of certain inflammatory mediators such as kinin, histamine and fibrinolytic enzymes. These mediators increase the permeability of capillaries in local tissues, promote the infiltration of inflammatory cells, and cause acute inflammatory edema in the ear (16, 17). The results of this study showed that ICT at doses of 100 and 50 mg /kg significantly reduced the number of writhes induced by glacial acetic acid in mice, and dose-dependently reduced the degree of xylene-induced auricle swelling. These results suggest that ICT has a good anti-inflammatory effect on pain.

Tissue damage induces an inflammatory reaction leading to activation of phospholipase A2 (PLA2)

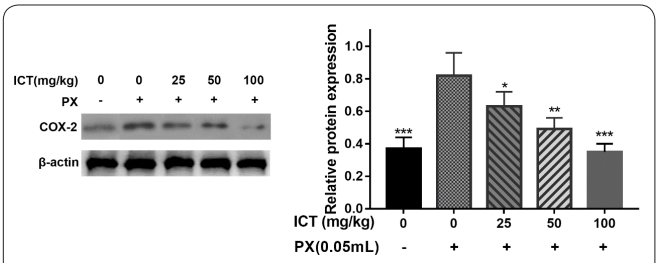

Figure 5. Effect of ICT on the expression of COX-2 protein in mouse dimethylbenzene-induced ear swelling. (A) Protein level of COX- 2 as determined by Western blot analysis. The blot was also probed with beta actin antibody to confirm equal sample loading; (B) result of data analysis performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents mean ± standard deviation (SD) calculated from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to PX group.

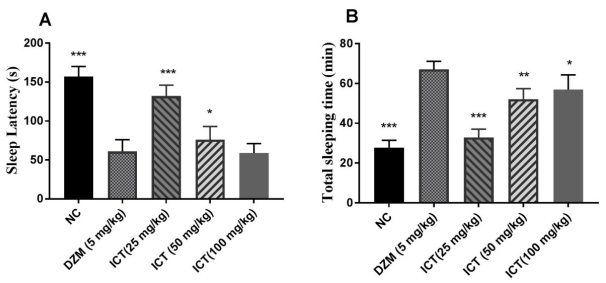

Figure 6. Effect of different doses of ICT on pentobarbital sodium-induced hypnosis. (A) Sleep latency; (B) total sleeping time. Each bar represents mean ± standard deviation (SD, n=10). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, relative to DZM.

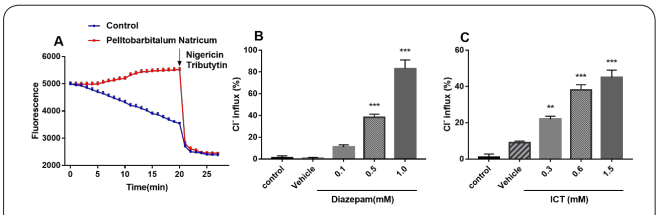

Figure 7. (A) Effect of pentobarbitalum sodium on cortical cellular fluorescence of MQAE. (B) Effect of diazepam on relative Cl-influx of the cortical cells in mice. (C) Effect of ICT on relative Cl-influx in cortical cells in mice. Data are expressed as mean ± SEM (n=6). **p <0.01, ***p <0.001, compared to the control group.

and release of arachidonic acid (AA) from membrane phospholipids. Free arachidonic acid is activated by cyclooxygenase (COX) to produce a range of prostaglandin derivatives and thromboxan A2 (TXA2). Prostaglandin E2 (PGE2) is a strong pro-inflammatory factor which causes local vasodilatation and increases the permeability of microvessels to neutrophils. Moreover, it amplifies pain induced by bradykinin or histamine, and leads to fever. It also induces the synthesis of COX-2, promotes the production and release of IL-6, TNF-α and IL-1β and other inflammatory factors; enhances the intensity and duration of inflammation and amplifies the level of inflammatory pain (17, 18). In the present study, it was found that ICT significantly reduced the levels of IL-6, TNF-α and IL-1β in the xylene-induced swollen ear tissues of mice, and down-regulated the expression level of COX-2 protein. These results suggest that the mechanism underlying the anti-inflammatory and analgesic effects of ICT is related to reduction of

inflammatory factors and the inhibition of COX-2 signaling pathway.

Synergistic sleep experiment with barbiturates is used for studying the hypnotic function of drugs. Pentobarbital sodium prolongs sleep time by interfering with the multi-synaptic transmission of the brainstem system and enhancing the inhibitory effect on the central nervous system (18). In the present study, synergistic experiments using sub-threshold and threshold doses of pentobarbital sodium showed that ICT affected sleep latency and total sleep duration in mice. Thus, ICT may be considered a sedative.

In an earlier study, Oh *et al*. (19) used Cl- fluorescence probe MQAE-loaded cerebellar granule neurons to identify compounds with sedative and hypnotic effects: these compounds increased Cl- flux in a dose-dependent manner (20,21). During sleep, the excitability of the cerebral cortex is inhibited (21). Therefore, mouse cortical cells can be used to investigate the effect of drugs on Cl- flux, thereby mimicking the mechanism of the compound *in vivo*. In the present study, there was a concentration-response relationship between the amount of fluorescence quenching and the concentration of Cl- in cells. Therefore, MQAE was used to measure the Cl- influx. During the fluorescence measurement, the cells gradually subsided, so when reading from the bottom of the plate, a slight increase in fluorescence was detected in the control group. Nigericin is the reverse transport protein of K+ and H+, while tributyltin is an OH-/Cl- reverse transporter. Under normal physiological conditions, the extracellular Cl- concentration is much higher than that in cells. When nigericin and tributyltin were added, the Cl- concentrations inside and outside the cell were quickly balanced, resulting in the lowest fluorescence. The results showed that when the Cl- fluorescence probe MQAE was loaded into the mouse cortical cells, the fluorescence was continuously decreased when pentobarbital sodium was added, relative to the control group. Diazepam can also induce influx of Cl- after administration, achieving maximum effect 20 min later. However, ICT significantly and immediately reduced fluorescence. Diazepam is a second generation hypnotic drug which increases the opening of the Cl- channel by acting on GABAA receptor-chloride channel complex, thereby producing sedative hypnotic effects. Similar effects were produced by ICT *in vivo* and *in vitro*.

The present study investigated the analgesic effect of ICT and its anti-inflammatory and sedative/hypnotic effects in mouse cortical cells. It was found that, in addition to analgesic and anti-inflammatory effects, ICT has a calming effect. It effectively controls pain and related disorders. It is a safe and effective anti-inflammatory drug.

Acknowledgements
None.

Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Chaofan Xie and Wei Xiang; Fengyu Huang, Yaodong Zhang, Yinfeng Xu, Wei Xiang, Chaofan Xie collected and analysed the data; Fengyu Huang and Yaodong Zhang wrote the text and all authors have read and approved the text prior to publication.
Fengyu Huang and Yaodong Zhang contributed equally to this work and should be considered as co-first authors.

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