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Two activators of *in vitro fertilization* in mice from licorice

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ABSTRACT

Systems for artificial insemination have been established in some animals. However, due to limited availability of sperm and oocytes, more effective treatment methodologies are required. Recently, it was demonstrated that the rate of *in vitro* fertilization (IVF) in mice was improved by adding a water extract of licorice (*Glycyrrhiza uralensis*), but not glycyrrhizic acid, to the artificial insemination culture medium. In this study, we examined licorice extract for active compounds using bioassay-guided separation. The results indicated that isoliquiritigenin and formononetin were the active molecules in licorice that contributed to the improved rate of IVF.

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1. Introduction

Artificial insemination is an assisted reproductive technology (ART) in which pregnancy is achieved by artificially introducing semen into the female genital tract. At present, artificial insemination is an indispensable technology for cattle breeding and is used for infertility treatment in humans. Methods to increase the efficiency of artificial insemination are required [1]. A system for *in vitro* fertilization (IVF) has been developed in mice, and many mouse lines produced using this system have been preserved by freezing embryos and/or fertilized eggs. However, efficient IVF utilizing freezing preservation or long-term refrigeration of sperm would allow mouse lines to be preserved more easily. After ejection, sperm begin movement immediately. An ejected sperm does not have fertilization ability. A sperm cell undergoes maturation after a definite period of time (capacitation), causes an acrosome reaction, and fertilizes an egg. However, there is still insufficient knowledge regarding the molecular mechanism of sperm maturation *in vivo*. Although natural mating is possible, there are lines in which the efficiency of IVF is known to be very poor. Such restrictions necessitate improvement of IVF.

Licorice (genus *Glycyrrhiza*) root has medicinal properties and is used in at least 70% of traditional Chinese medicine (TCM) formulas for various diseases, including gastrospasm, stomachache, sore throat, gastric ulcers, and duodenal ulcers [2]. Licorice is also widely used as a sweetener in the production of confectioneries and soy sauce. Nearly 500 compounds have been identified in licorice root, among which glycyrrhizin (GC) and several flavonoids are major components [2]. The global demand for licorice is high. Previously, we reported synergistic effects of the major constituent of licorice, GC, and other constituents using knockout extracts [3], interfacial behavior of GC [4], and screening of higher GC-containing *Glycyrrhiza* species using the eastern blotting technique [5]. Recently, we found that licorice extract improved IVF using mouse sperm [6]. The results indicated that addition of the fraction without GC to the sperm prior to culture yielded an improved fertilization rate.

Here, we report the isolation of two active components of licorice for IVF using bioassay-guided purification.

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2. Materials and methods

2.1. Animals

Female ICR mice (10 weeks old) and male BALB/cA and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The animals were killed by cervical dislocation just before the experiments. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Nagasaki International University, Nagasaki, Japan). The mice were kept under conditions of controlled temperature and lighting throughout the experiments and were provided with food and water *ad libitum*.

2.2. Preparation of licorice extract and purification of active components

2.2.1. Extraction and isolation

Licorice (*Glycyrrhiza uralensis*) roots cultivated in Genkai-cho, Saga Prefecture, Japan were collected in August 2011 and stored at the Faculty of Pharmaceutical Science, Nagasaki International University. An air-dried sample (350 g) was sliced and then extracted using hot distilled water (500 mL × 8 h × 3 times) at 40 °C under sonication. The combined extracts were filtered and concentrated under reduced pressure. The residue obtained was lyophilized to produce a crude aqueous licorice extract (deep yellow powder, 51.8 g). The crude aqueous extract was suspended in water and then partitioned successively with Ethyl acetate (EtOAc) and butyl alcohol (BuOH). The EtOAc and n-BuOH fractions were evaporated under reduced pressure to obtain residues.

The EtOAc fraction (4.20 g) was then subjected to silica gel column chromatography using $CHCl_3$–MeOH (20:1, v/v) as the eluent by bioassay-guided purification to give five sub-fractions (Fr. 1–5). Active fraction 2 (Fr. 2) (350 mg) was further chromatographed over a reversed-phase C_{18} column using MeOH–H_2O (6:5, v/v) to yield isoliquiritigenin (147 mg). Similarly, formononetin (11 mg) was purified from active fraction 4 (Fr. 4) (280 mg) using a reversed-phase C_{18} column with MeOH–H_2O (1:1, v/v).

2.2.2. Isoliquiritigenin

Yellow powder; ESI-MS: *m/z* 257 $[M + H]^+$; ^{13}C NMR (CD_3OD, 100 MHz): δ_C 114.7 (C-1), 166.3 (C-2), 103.8 (C-3), 167.5 (C-4), 109.1 (C-5), 133.4 (C-6), 193.5 (C-7), 118.3 (C-8), 145.6 (C-9), 127.8 (C-1′), 131.8 (C-2′,6′), 116.9 (C-3′,5′), 161.5 (C-4′).

2.2.3. Formononetin

Pale yellow powder; ESI-MS: *m/z* 269 $[M + H]^+$; ^{13}C NMR (CD_3OD, 100 MHz): δ_C 152.6 (C-2), 124.9 (C-3), 175.9 (C-4), 128.2 (C-5), 115.2 (C-6), 161.4 (C-7), 101.2 (C-8), 157.9 (C-9), 118.4 (C-10), 124.2 (C-1′), 130.5 (C-2′,6′), 114.6 (C-3′,5′), 159.8 (C-4′), 55.8 (4′-OCH_3).

2.3. Sperm collection

Mice were sacrificed by cervical dislocation just before the start of the experiments. Mature caudal epididymal sperm cells (~8×10^6) from each mouse were incubated in 200 μL human tubal fluid (HTF) medium without bovine serum albumin (BSA) (LifeGlobal® medium; IVFonline, Guilford, CT, USA) covered with paraffin oil. After 5 min, each sperm suspension was transferred to conditioned medium for preincubation. The control conditioned medium for sperm preincubation was HTF medium containing 1 mg/mL polyvinyl alcohol (PVA; Sigma, St. Louis, MO, USA) and 1.0 mM methyl-beta-cyclodextrin (MBCD; Sigma) [7]. Aliquots of 20 μL of the sperm suspension in HTF without BSA were transferred to 20 μL of each conditioned medium containing twice the concentration of PVA, MBCD, and licorice extract and kept at 37 °C in a humidified incubator under 5% CO_2/95% air (motile sperm concentration: ~10000/μL). After 50 min, 2–4 μL sperm from each conditioned medium was used for insemination (final motile sperm concentration: 150/μL). Motile sperm swimming at the periphery of each drop were used for insemination as described previously [7].

2.4. IVF

Female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (Asuka Inc., Tokyo, Japan), followed 46–48 h later by 5 IU human chorionic gonadotropin (Asuka Inc.), and then euthanized 14–16 h later. The mice were sacrificed by cervical dislocation just before the start of the experiment. Ovaries with oviducts were transferred to dishes 30 mm in diameter filled with paraffin oil (Nacalai Tesque, Kyoto, Japan). Cumulus-oocyte complexes were obtained from the ampullae of uterine tubes and transferred to dishes, each containing a 200-μL drop of HTF medium covered with paraffin oil, under a stereomicroscope. Two to four cumulus-oocyte masses were transferred to each 200-μL drop of HTF medium covered with paraffin oil for insemination. A sperm suspension cultured in conditioned medium was transferred to the insemination drop. At 24 h after insemination, the fertilization rate was determined as the proportion of 2-cell-stage embryos among all of the oocytes.

2.5. Statistical analysis

Differences between the experimental and control conditions were determined by one-way analysis of variance (ANOVA) and Fisher's protected least significant difference tests. Significant differences were determined as $P < 0.05$.

3. Results

3.1. Identification of active components for IVF

To identify the active components in licorice extract, the fertilization rates were examined in HTY medium containing PVA and MBCD to which individual fractions were added. As the ethyl-acetate fraction showed the strongest stimulation of IVF efficiency as reported previously [6], this fraction was further purified using bioassay-guided separation. Finally, we isolated Fr. 2 and 4 as the fractions that significantly stimulated fertilization rate (Fig. 1).

Compounds 1 and 2 in Fr. 2 or 4, respectively, were authenticated by spectroscopic data and comparison with reported data [8].

Compound 1 in Fr. 2, a yellow powder, showed an ion peak at *m/z* 257 $[M + H]^+$ and strong yellow fluorescence suggesting a chalcone derivative, which was confirmed by ^{13}C NMR, and finally identified as isoliquiritigenin by comparison with an authentic sample (Fig. 2). Compound 2 in Fr. 4, a pale yellow powder, showed an ion peak at *m/z* 269 $[M + H]^+$, suggesting that this compound is an isoflavonoid. ^{13}C NMR indicated a typical lower shifted C-3 carbon suggesting that compound 2 is an isoflavone. Therefore, we identified this compound as formononetin using an authentic sample (Fig. 2).

3.2. Optimal dose

To investigate the effects of each compound on sperm, the dose dependency of the effects on IVF were examined. The fertilization rates for HTF medium containing PVA plus MBCD and each isoliquiritigenin or formononetin at a concentration of 0, 0.01, 0.02, or 0.04 mg/mL were 13.6 ± 8.5, 24.2 ± 8.5, 47.2 ± 16.8, and 32.2 ± 13.3

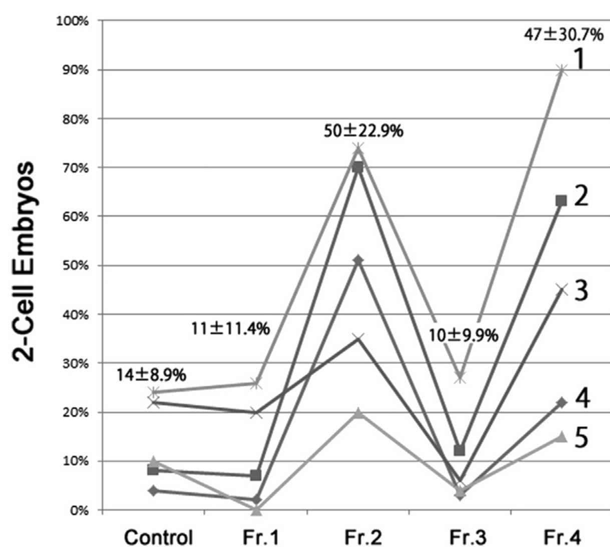

Fig. 1. Effects of licorice extracts on IVF rate. Sperm from BALB/cA mice were preincubated in conditioned medium with or without licorice extract (0.02 mg/mL). The fertilization rates varied among male BALB/cA mice. In five experiments (1–5), more embryos were obtained from preincubation medium containing Fr. 2 (compound 1) and Fr. 4 (compound 2) of licorice extract than from preincubation medium lacking licorice extract. The extract had a significant effect on IVF ($P < 0.05$).

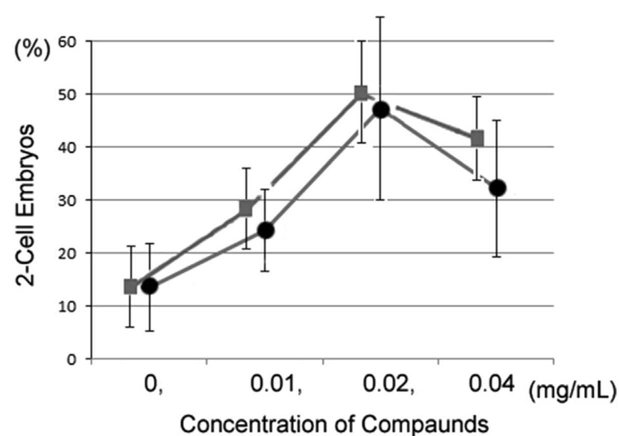

Fig. 3. Optimal concentration of licorice extract for IVF. The data are given as the means ± 95% confidence intervals of five experiments. Circles and squares indicate isoliquiritigenin and formononetin, respectively.

or 13.4 ± 8.5, 28.3 ± 8.1, 502 ± 9.8, and 41.6 ± 8.2, respectively (Fig. 3).

3.3. Effects on embryonic development

To examine developmental disorders associated with each compound, the viability of embryos treated with isoliquiritigenin was examined. The embryos did not show abnormalities (Fig. 4), similar to previous observations with licorice crude extract [6]. Similar results were also obtained with formononetin (data not shown).

4. Discussion

Artificial insemination is an important technology used widely in cattle breading, as well as for fertility treatment in humans. IVF is easier in cattle and fertility treatments and should be the first choice treatment in these applications. Production of genetically modified mice has been indispensable to evaluate the functions of genes *in vivo*, and many genetically engineered strains have been developed. As these mice are very important research materials, a feasible preservation method is necessary. Moreover, although natural mating is possible, further improvement of IVF culture medium and methodologies are useful, as there are lines in which the efficiency of IVF is very poor. Previously, we showed that the fertilization rate was improved by aqueous licorice extract, without defects in ontogenesis, using sperm from the mouse line BALB/c, which exhibits a low fertilization rate. The licorice extracts stimulated fertilization without a spontaneous acrosome reaction of the sperm in the medium. For understanding sperm maturation, it is important to identify the substances present in licorice that affect the efficiency of IVF.

Of the nearly 500 components in licorice root, the main active ingredients are GC and several flavonoids. However, as we reported previously that GC had no effect on the IVF system [6], the active ethyl acetate fraction was analyzed by fingerprinting, and the results indicated high levels of flavonoids. This fraction was further purified by bioassay-guided separation to isolate two active compounds, which were identified as isoliquiritigenin and formononetin. The results indicated that sperm treated with isoliquiritigenin and formononetin in preincubation medium stimulated fertilization without defects in embryonic development. Isoliquiritigenin and formononetin may be useful therapeutic agents for infertility treatment.

Although there has been some progress in research regarding the maturation of ejaculated sperm, the details remain unclear. Although the role of CatSper was clarified, and olfactory receptors and their expression patterns are known, it remains unclear what type of signal transmission contributes to the maturation process of ejaculated sperm. From these results, it is expected that the signal of acrosome reaction and motility of sperm is another even if it was association with each other. Estrogen was reported to have an influence on activation of sperm and acrosome reactions [9]. Hajirahimkhan and collaborators surveyed three licorice species,

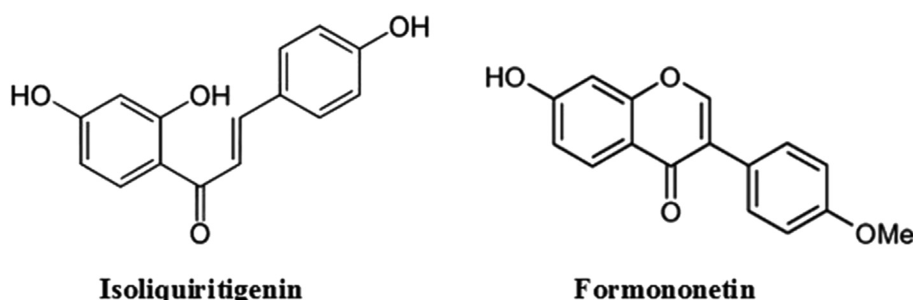

Fig. 2. Structural formulas of the compounds that improved the *in vitro* fertilization rate.

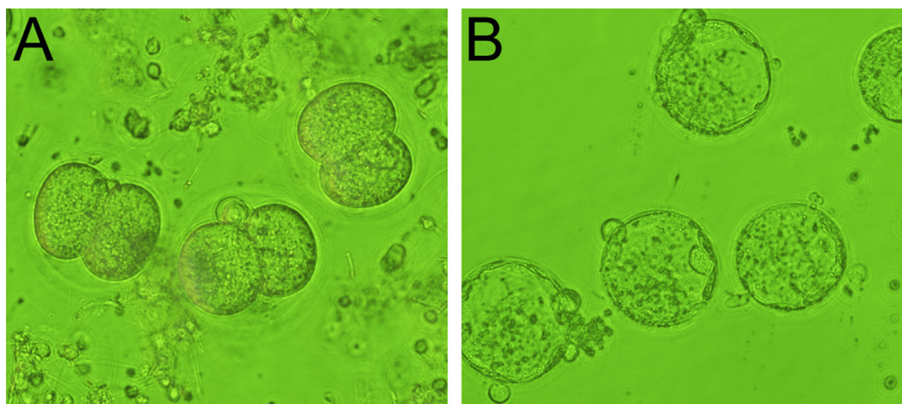

Fig. 4. Embryos from oocytes incubated with isoliquiritigenin. Two cell-stage embryos (A) and blastocysts (B).

G. uralensis, *Glycyrrhiza glabra*, and *Glycyrrhiza inflata*, and found that isoliquiritigenin showed strong estrogen-like activity, suggesting that this compound may be cyclized to liquiritigenin, which is an active flavonoid, under physiological conditions [10]. We also postulated that the hydrolysis reaction occurred in culture medium on diarylheptanoid glycoside to give free diarylheptanoid with anti-trypanosomal and apoptotic activities [11,12].

These observations suggest that isoliquiritigenin itself may not be active but may act as a precursor yielding active liquiritigenin. Furthermore, Kim and Park reported that isoflavones, including formononetin, play roles in sexual development, such as pubertal timing, impaired estrous cycling, ovarian function, and functions of the hypothalamus and pituitary [13]. Although the relationships between fertilization and estrogens are not completely clear, the two phytoestrogens isolated in this study may function as fertilization-promoting agents. Moreover, we suggest that some of the 500 components of licorice exert synergistic effects on fertilization. Fortunately, as we succeeded in preparing a knockout extract using an immunoaffinity column combined with an anti-antigen monoclonal antibody [2], it will be feasible to purify additional active components from the isoliquiritigenin and formononetin knockout extracts of licorice root [2,3]. Although many pharmacological activities of isoliquiritigenin have been investigated, such as antiinflammatory, immunoregulatory, antimicrobial, antioxidant, anticancer, hepatoprotective, and cardioprotective activities [14], it is interesting that isoliquiritigenin and formononetin affect sperm during fertilization. Analysis of the molecular mechanisms underlying the effects of these compounds on sperm may provide insight into their biological activities in other cell types.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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