Chapter 7

In Vitro Fertilization Activators for Future

Hiromitsu Tanaka, Morimasa Wada,
Nguyen Huu Tung, Shunsuke Fujii,
Takuhiro Uto and Yukihiro Shoyama

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Abstract

Artificial insemination is an indispensable technology for cattle breeding and is used for treating infertility in humans. Thus, new or improved methods are needed to increase the efficiency of artificial insemination. *In vitro* fertilization (IVF) has been developed in mice. Although many mouse lines produced using IVF have been preserved by freezing embryos and/or fertilized eggs, the more efficient IVF using freezing preservation or long-term refrigeration of sperm is expected to preserve mouse lines more easily. In this chapter, we introduce the active compounds in licorice to improve the rate of IVF. We previously reported that the rate of IVF in mice was improved by adding a water extract of licorice (*Glycyrrhiza uralensis*), but not glycyrrhizin, to the artificial insemination culture medium. Recently, we analyzed the active ethyl acetate fraction containing high levels of flavonoids. This fraction was further purified by bioassay-guided separation to isolate isoliquiritigenin and formononetin, which contributed to the improved rate of IVF. Isoliquiritigenin and formononetin may be useful therapeutic agents for infertility treatment.

Keywords: *in vitro* fertilization, activator, licorice, isoliquiritigenin, formononetin

1. Introduction

Glycyrrhiza species (Leguminosae), commonly known as licorice, are perennial plants that grow up to 1.5 m high and are distributed in drylands from Western Europe to Russia, and are particularly abundant in China and Mongolia. Licorice mainly consists of dried roots and stolons of *G. uralensis* Fisch and *G. glabra* Linne. Therefore, two species are listed in pharmacopoeia worldwide. Licorice is one of the most important crude drugs prescribed with other

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herb medicines in traditional Chinese medicine (TCM) for the treatment and prophylaxis of sore throats (as an antitussive), coughs and bronchial catarrh (as an expectorant), inflammation, allergic reactions, rheumatism and arthritis, liver disease, tuberculosis and adrenocorticoid insufficiency [1–5]. Nearly 500 components have been identified in licorice. Among them, glycyrrhizin is controlled at a concentration of more than 2.0% as a standardization marker component because the phamacological properties of licorice might be depend upon glycyrrhizin, and furthermore a flavonoid liquiritin has a quantitative limitation in Japanese pharmacopoeia [1]. Glycyrrhizin is a protein-kinase inhibitor with anti-ulcer and anti-viral activities [6]. In Japan and China, nowadays, glycyrrhizin is used for the treatment of hepatitis [4, 7–9]. It has been reported that glycyrrhizin has interferon-inducing activity and anti-HIV-1 activity [10–12]. Moreover, glycyrrhizin is well known as a natural sweetener and used in food additives and cosmetics [13]. On the other hand, flavonoids have also various pharmacological activities such as anti-hepatotoxic, anti-inflammatory, anti-ulcer, anti-allergenic and anti-viral as well as cardioprotective activities [2]. **Figure 1** indicates major components in licorice.

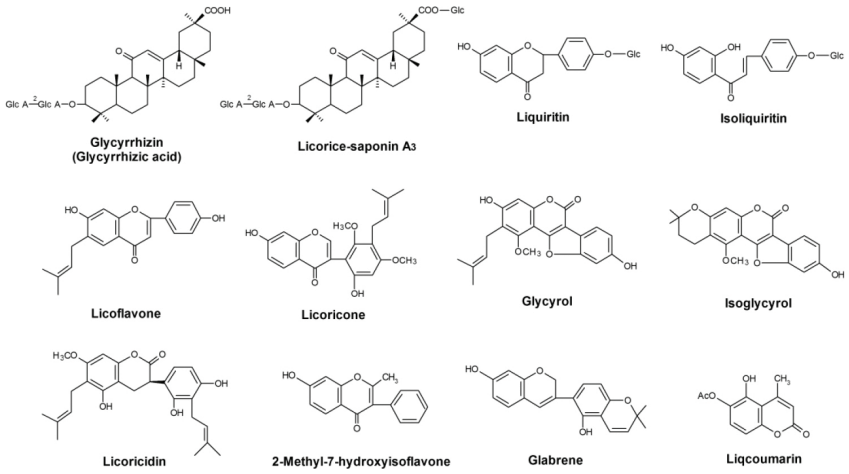


Figure 1. Major components in licorice.

In order to control the quality of licorice, we investigated and succeeded the preparation of monoclonal antibody (MAb) against glycyrrhizin and set up an enzyme-linked immunosorbent assay (ELISA) as quick, simple and reproducible assay system [14]. Furthermore, a new staining system, eastern blotting was developed [15] and immunoaffinity isolation of glycyrrhizin resulting in glycyrrhizin-knockout extract, which can be used to survey the real activity of glycyrrhizin in licorice [16, 17]. Regarding flavonoid, anti-liquiritin MAb [18] and eastern blotting [19] was developed. Since the above knowledge of components in licorice have been accumulated and also Tanaka et al. have been investigating about proteins [20–22] and mitochondria [23] related to fertilization using reproducing assay system, we have started to survey *in vitro* fertilization (IVF) active components in licorice.

2. *In vitro* fertilization of mouse sperm by licorice crude extract and fraction

Although previous studies have investigated whether licorice can increase pregnancy rates or not, the clear result has not been found yet [24]. However, since it is known that glycyrrhizin acts as the modulator of 11β-hydroxysteroid dehydrogenase (Type 1 and 2) which are the enzyme related to steroidal hormone [25], a speculation of relationship between licorice and testosterone and/or estrogen comes out. In fact Hajirahimkhan et al. searched three licorice species, *G. uralensis*, *G. glabra* and *G. inflata*, and found that isoliquiritigenin indicated strong estrogen-like activity, suggesting that this compound may be cyclized to liquiritigenin, which is an active flavonoid, under physiological conditions [26].

The sperm from wild-type C57BL/6 mice has high capacity for fertilization when cultured in standard medium (e.g., HTF) supplemented with bovine serum albumin for IVF or with polyvinyl alcohol (PVA) and methyl β-cyclodextrin (MBCD). On the other hand, IVF efficiency using the sperm of aged BALB/cA mice (>48 weeks of age) is low, albeit fluctuated between mice [27]. However, we found that the licorice crude extract improved the fertilizing ability of BALB/cA mouse sperm *in vitro* as indicated in **Figure 2** and that the fertilized eggs developed normally [28]. We performed five separate experiments using five different BALB/cA mice. The fertilization rate for HTF medium containing PVA plus MBCD and licorice extract (0.12 mg/ml) was between 15.6 and 84.0% (average: 43.0 ± 11.0%). On the other hand, the fertilization rate was between 0.0 and 43.5% (average: 15.0 ± 7.8%) when licorice extract-free medium was used. The optimal concentration of licorice extract in HTF medium was 0.3 mg/ml.

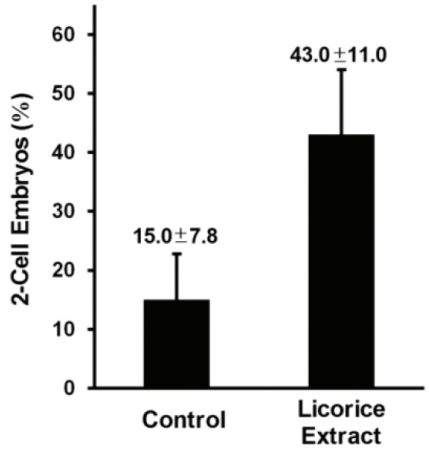


Figure 2. Influence of licorice extract on the IVF. Sperm from BALB/cA mice was preincubated in conditioned medium with or without licorice extract (0.12 mg/mL). Two-cell embryos were effectively obtained with preincubation medium containing licorice extract. The extract had a significant effect on IVF ($n = 5$, $P < 0.05$).

Park et al. reported that licorice extract increased cyclophosphamide teratogenicity and upregulated the mRNA expression of cytochrome P-450 2B in rats [29]. The results suggest

that licorice root contains various bioactive components. However, they may not affect gene expression because transcription rates in spermatozoa are low [30]. Our findings indicate that components in licorice other than glycyrrhizin, or together other components and glycyrrhizin, can improve the IVF rates without damaging fertilized eggs.

In order to confirm the property of active component in licorice, the crude extract was fractionated with EtOAc and *n*-BuOH successively. **Figure 3** showed the fertilization level indicated by the percentage of two-cell embryos describing that the EtOAc fraction was more effective compared to that of *n*-BuOH fraction. From this result, active components might be not conjugated compounds like saponins including glycyrrhizin but free compound like flavonoids as indicated in **Figure 1** depending on their solubility.

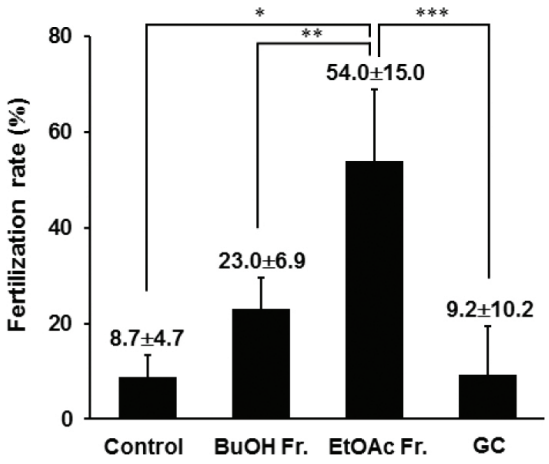


Figure 3. Active fraction of licorice extract and glycyrrhizin against IVF. *n*-BuOH (1.6 mg/mL) and EtOAc (1 mg/mL) fractions of licorice and glycyrrhizin (1.6 mg/mL) were added to the conditioned medium. Activity was observed in the EtOAc fraction containing flavonoids.

3. Survey of active components in licorice

The above active EtOAc fraction was bioassay-guided separated by silica gel column chromatography with a $CHCl_3$-MeOH solvent system as an eluent to give five fractions (1–5). Fraction 2 was further purified using a reversed-phase C18 column eluting with MeOH-H_2O to yield active compound **1**. Fraction 4 was also purified by the same way of fraction 2 to give active compound **2**.

Compound **1** was a yellow powder indicating an ion peak at *m*/*z* 257 $[M+H]^+$ in mass spectrometry having strong yellow fluorescence. From these evidences, compound **1** was supposed to be a non-conjugated flavonoid like a chalcone derivative, which has strong fluorescence in general. Finally, compound **1** was determined to be isoliquiritigenin (**Figure 4**) [31] by ^{13}C

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nuclear magnetic resonance (NMR), 1-dimensional (1D; ^{1}H and ^{13}C) and 2D correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple-bond coherence (HMBC) spectroscopies.

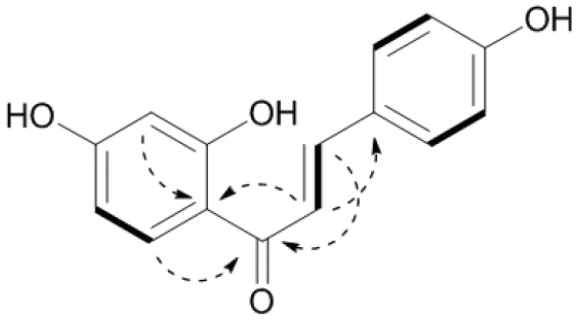


Figure 4. The structures of isoliquiritingenin with key COSY (bold lines) and HMBC correlations (dash arrows).

Compound **2** was a pale yellow powder indicating an ion peak at *m*/*z* 268 $[M+H]^{+}$ that the mass fragment pattern resembled to a flavonoid compound having a methoxyl group in a molecule. ^{13}C NMR showed a typical lower-shifted C-3 carbon (124.9 *m*/*z*) suggesting that compound **2** might be an isoflavone. The spectra of ^{1}H, ^{13}CNMR and 2D (COSY, HMQC and HMBC) confirmed that compound **2** was formononetin [32] (**Figure 5**).

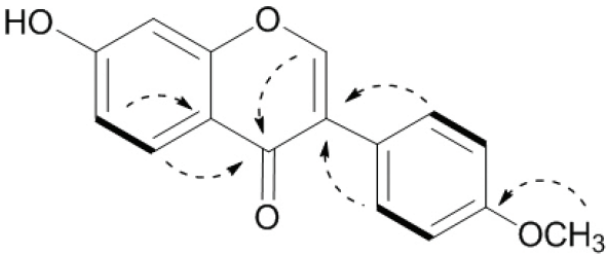


Figure 5. The structures of formononetin with key COSY (bold lines) and HMBC correlations (dash arrows).

4. Confirmation of IVF activity for two isolated components

The HTF medium containing PVA and MBCD with isoliquiritigenin or formononetin at a concentration range from 0 to 0.04 mg/ml was tested for IVF [33]. The optimal concentration of isoliquiritigenin was found at 0.02 mg/ml resulting in 47.2 ± 16.8% of IVF ratio. In the case of forononetin, the ratio was slightly higher (50.2 ± 9.5%) but not significantly, than that of isoliquiritigenin as indicated in **Figure 6**.

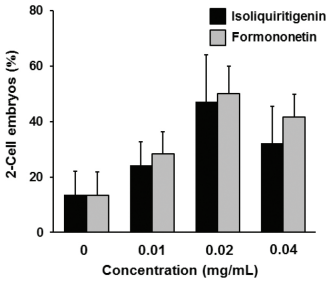


Figure 6. Optimal concentration of licorice extract for IVF. Black and gray bars indicated isoliquiritigenin and formononetin, respectively.

We examined the viability of embryos treated with isoliquiritigenin (**Figure 7A**) or formononetin (**Figure 7B**) to confirm that both embryos were morphologically normal similar to the previous observations using licorice crude extract [28].

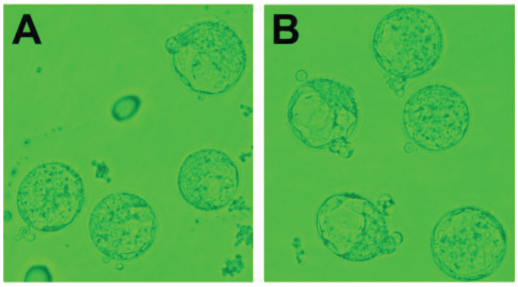


Figure 7. Morphology of blastocysts incubated with isoliquiritigenin or formononetin.

5. Licorice in future

It is considered that the decrease of population is now a serious problem in advanced countries depending on diversification of course of life (late marriage, unmarried, etc.). Carlsen et al. reported interesting evidence related to human semen [34]. It became evident that approximately 30% decrease of sperm number during 40 years from 1950 to 1990 occurred. From this data, it suggested that the number of sperm was half in 2005 compared to that in 1950. This evidence may be deeply related to the decrease of population. In order to increase the fertilization ratio, the activation of sperm is necessary against the decrease of sperm number and also the mechanism of increase of IVF by isoliquiritigenin or formononetin should be evident.

We investigated the incorporation and distribution of isoliquiritigenin or formononetin in mice sperm in order to obtain some information related to the mechanism. **Figure 8** showed the fluorescence staining in mice sperm using fluorescence microscope. Staining distributed in

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almost all part of sperm. It seems to be that this phenomenon might be related to activation of sperm although the mechanism is still unknown and should be solved from now.

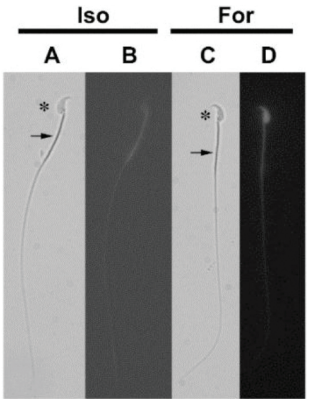


Figure 8. Fluorescence imaging of mice sperm in the preincubation medium with isoliquiritigenin (Iso) and formononetin (For). Signals were observed intensely in postacrosomal region (star) and mid piece (arrow).

Licorice used to be used for approximately 70% of TCM formula. From this current status, the pseudohyperaldosteronism often occurred by over uptake of licorice. It is well known that this phenomenon was occurred by taking of much glycyrrhizin. Glycyrrhizin is hydrolyzed to give glycyrrhetic acid (aglycone) by enteric bacteria, resulting the reabsorption of glycyrrhetic acid. This compound is changed to mono glycoside in liver. Glycyrrhetic acid monoglycoside inhibits 11β-hydroxysteroid dehydrogenase (HSD), which catalyzes the conversion of cortisol to cortisone resulting in the accumulation of cortisol which reacts to aldosterone receptor.

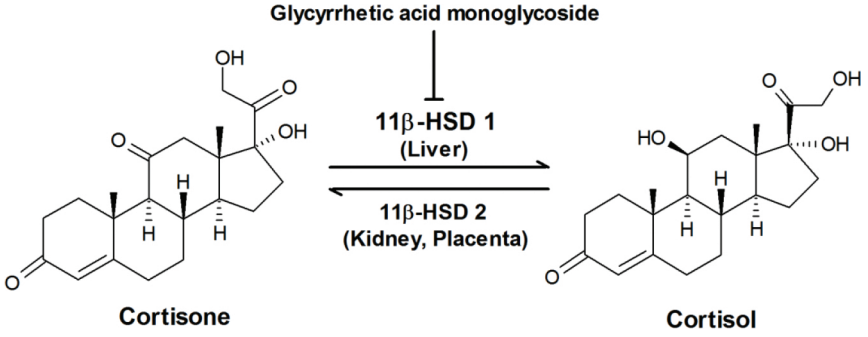


Figure 9. The inhibition of 11β-hydroxsteroid dehydrogenase (HSD) by glycyrrhetic acid monoglycoside inhibits in liver.

This phenomenon activates the reabsorption of sodium ion, and on the other hand potassium ion is eliminated inducing pseudohyperaldosteronism. Therefore, if we take too much licorice

extracts for promotion of fertilization, such disease may be occurred. Previously, we purified glycyrrhizin from the crude extract of licorice using an immunoaffinity column conjugated with anti-glycyrrhizin monoclonal antibody, and prepared glycyrrhizin-knockout extract [17]. The glycyrrhizin-knockout extract contains all components except glycyrrhizin. Thus, glycyrrhizin-knockout extract can function for promoting fertilization **Figure 9**.

Author details

Hiromitsu Tanaka, Morimasa Wada, Nguyen Huu Tung, Shunsuke Fujii, Takuhiro Uto and Yukihiro Shoyama*

*Address all correspondence to: shoyama@niu.ac.jp

Faculty of Pharmaceutical Sciences, Nagasaki International University, Huis Ten Bosch, Sasebo, Nagasaki, Japan

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