ORIGINAL Aichi Gakuin Dent Sci 34 : 19–23, 2021

EXPERIMENTAL EFFECTS OF LICORICE SOLUTION ON FETAL MORTALITY AND CLEFT LIP AND/OR PALATE IN *A/J* MICE

ICHINNOROV CHIMEDTSEREN[1)], TERUYUKI NIIMI[1,2,3)], HIROO FURUKAWA[2,3,4)], HIDETO IMURA[1,2,3)], MASAAKI ITO[1,2,3)], YASUNORI AKIYAMA[1,2,3)] AND NAGATO NATSUME[1,2,3)]

[1)] *Division of Research and Treatment for Oral and Maxillofacial Congenital Anomalies, School of Dentistry, Aichi Gakuin University, Nagoya 464-8651, Japan*

[2)] *Cleft Lip and Palate Center, Aichi Gakuin University Dental Hospital, Nagoya 464-8651, Japan*

[3)] *Division of Speech, Language, Hearing Therapy, Aichi Gakuin University Dental Hospital, Nagoya 464-8651, Japan*

[4)] *Department of Health Science, Faculty of Psychological and Physical Science, Aichi Gakuin University, Nisshin 470-0195, Japan*

SYNOPSIS

Licorice dry root extract and its major compounds have been reported to exhibit varying degrees of estrogen receptor agonism in various tissues *in vitro* and *in vivo*. We evaluated the effects of licorice extracts on incidence of cleft lip and/or palate using A/J mice. The male and female A/J mice in the experimental group were provided with a 0.89 mg/kg/day licorice solution instead of normal tap water for 2 weeks, then mated, and 18-day-old fetuses were removed and examined. The percentages of fetuses with cleft lip and/or palate were 4.3% of 117 live fetuses and 8.9% of 101 live fetuses for control and experiment groups. The experimental group had 8.1% more fetal resorption than the control group. There was no significant difference in CL/P between the experiment group and the control group.

Key words: estrogen, licorice, cleft lip and/or palate

INTRODUCTION

The genus *Glycyrrhiza*, commonly referred to as licorice, is composed of about 30 species. *G.glabra* is the most widely cultivated, although *G.uralensis, G.inflata, G.aspera, G.korshinskyi,* and *G.eurycarpa* are also commonly used. Known as the grandfather of herbs, licorice has a long history of usage, and it has been widely adopted as a flavor and sweetener for products as diverse as tobacco, chewing gum, candies, toothpaste and beverages[1)]. In addition, dried licorice root has been valued for its nutritional and medicinal values[2)], with people in places as diverse as Turkey, Spain, Iraq, Russia, Mongolia, North Korea and Japan using licorice therapeutically. In the Japanese Pharmacopoeia, two licorice species, *G. glabra* and *G. uralensis*, are listed for use in root, powder and extract form.

Recent pharmacological studies of licorice have demonstrated antioxidant activity *in vitro*[3,4)], as well as antibacterial effects[5,6)], with some components of licorice root exhibiting anti-ulcer, anti-inflammatory, anti-viral, anti-atherogenic and even anti-carcinogenic properties[1)]. Licorice appears to be of particular relevance to research and treatment for congenital anomalies such as cleft lip and/or palate (CL/P) since root extract has been reported to exhibit estrogen receptor agonism in certain tissues[7)].

The incidence of cleft lip and/or palate is common among humans, with prevalence of 1 per 500 to 1,000 live births[8-10)]. Since the risk of CL/P recurrence within the family is 28-40 times greater than the risk for the

Corresponding author at: Nagato Natsume, Division of Research and Treatment for Oral and Maxillofacial Congenital Anomalies, School of Dentistry, Aichi Gakuin University
Received for publication September 16, 2021
Accepted for publication December 24, 2021

general population, it is believed that genetic factors contribute to the development of this disorder, and it has been suggested that non-syndromic cleavage in humans is likely due to a combination of genetic and environmental factors as described in a multifactorial threshold model[11-12].

There have been multiple reports which indicate estrogen has a preventive effect on CL/P. Fetal expression with CL/P and cleft palate only (CPO) has been reported in A/J mice, which are model animals for spontaneous expression of cleft lip and palate at a rate of 5-10%[13]. It has been reported that a dose of 12.5 mg/kg estradiol benzoate, injected into *A/J* mice, has a preventive effect on CL/P development[14]. Licorice root extract and its major active component, glabridin, which is an isoflavan, exhibit varying degrees of estrogen receptor (ER) agonism in various tissues in vitro and *in vivo*[15].

The purpose of this study is to determine whether administration of licorice extract to *A/J* mice that spontaneously develop cleft lip and palate has the effect of preventing CL/P and fetal mortality.

Materials and Methods

1. Ethics

Aichi Gakuin University Animal Research Committee approved all the *in vivo* studies in compliance with the ethical principles of the Declaration of Helsinki (World Medical Association, 2013).

2. Animal models

Eight-week-old A/J mice were purchased from (SLC, Japan), and 48 mice (30 female and 18 males) were housed at a temperature of 23 ± 1 ℃ and a humidity of 60 ± 10%. The mice were kept under 12 h day/night cycle, and drink/food consumptions were checked at daily observations.

3. Preparation of Licorice solution

Licorice root G.uralensis was purchased from Daikoshoyaku, (Nagoya, Japan) (Figure 1). Three grams of licorice root was boiled in 1 liter of tap water for 1 hour. After 1-hour of boiling, all licorice roots were removed and the remaining solution was further boiled to until the volume of the solution became 500 ml. The twice-concentrated extract was stored in a sealed bottle at 4 ℃ and was diluted with the same amount of tap water to become 0.89 mg/kg/day.

4. Experimental conditions

The 16 female and 9 male mice of the control group and the 14 female and 9 male mice of the experiment group were separated into males and females and kept in separate cages in the animal laboratory. The control group mice were given tap water whereas the experimental group mice were provided with 0.89 mg/kg/day of licorice extract. After 2 weeks, male and female mice were mated overnight, and the day when a vaginal plug was observed was taken as gestation day 0. On the eighteenth gestation day, the pregnant mice were sacrificed by cervical dislocation. Mice were fed licorice solution until the day of sacrifice. Then the fetuses were removed from the uterus, and the fetal deaths and resorptions were examined and recorded. Live fetuses were placed in physiological saline and their anatomy observed under a stereoscopic microscope. Fetuses with oral and maxillofacial defects were fixed in a mixed solution as in previous research[16].

5. Statistical analysis

Statistical significances between control and experimental groups were evaluated using a one-way analysis of variance (ANOVA), and the results were analyzed using the adjusted chi-square test.

Results

1. Fetal deaths and mean number of fetuses

In the control group, 7 (5.6%) fetal resorptions occurred among 124 fetuses from 16 dams, whereas in the experimental group, 16 (13.7%) fetal resorptions

Fig. 1. Licorice root

were observed among 117 fetuses from 14 dams. The experimental group had 8.1% more fetal resorption than the control group (Table 1). Mean numbers of fetuses per litter were 7.8±1.8 for the control group and 8.4±1.8 for the experimental group respectively. The mean weight of live fetuses was 0.65±0.05 g for the control group and 0.58±0.08 g for the experimental group, respectively. Each mouse drank approximately 0.89 mg/kg of extract solution per day.

2. The incidence rate of cleft lip and/or cleft palate

There were no significant differences in the incidence of CL/P between the experimental group (fed with 0.89 mg/kg/day of licorice solution) and the control group. The incidence rate of CL/P was 8.9% (9 fetuses) among 101 live fetuses for the experimental group, and 4.3% (5 fetuses) among 117 live fetuses for the control group (Table 2).

Among the 5 fetuses with CL/P in the control group, 4 fetuses had cleft palate only (CPO) and 1 fetus had unilateral cleft lip and palate (UCLP). In the experimental group, 7 out of 9 affected fetuses had CPO, 1 had bilateral cleft lip and palate (BCLP) and 1 had UCLP (Figure 2).

Table 1. Mortality and mean weight of fetuses

Subject	Dams	Fetuses	Average number of fetuses per litter	Live fetuses	Fetal resorptions
	n (+)	n (+)	n (+)	n (+)	n (+) (%)
Control group	16	124	7.8±1.8	117	7 (5.6%) (*)
Experimental group	14	117	8.4±1.8	101	16 (13.7%)

(*) p value < 0.05, with adjusted chi-square test
(+) n means number

Table 2. Comparison of incidence rate of cleft lip and/or palate among live fetuses

Subject	Dams	Live fetuses	Unaffected fetuses	Affected fetuses		
				CPO	UCLP	BCLP
				n (%)	n (%)	n (%)
Control group	16	117	112 (95.7%)		5 (4.3%)	
				4 (3.4%)	1 (0.9%)	0(*)
Experimental group	14	101	92 (91.1%)		9 (8.9%)	
				7 (6.9%)	1 (1.0%)	1 (1.0%)

(*) p value < 0.05, with adjusted chi-square test
CPO: Cleft palate only
UCLP: Unilateral cleft lip and palate
BCLP: Bilateral cleft lip and palate

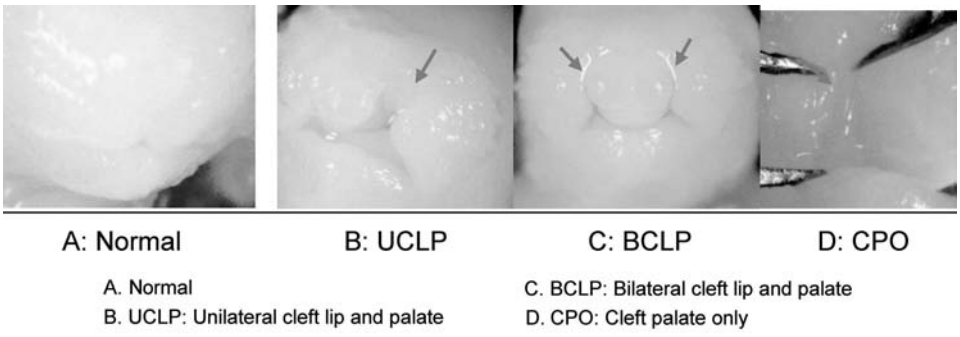

Fig. 2. *A/J* mouse fetuses

Discussion

Licorice root extract and its significant isoflavone, glabridin, have exhibited varying degrees of estrogen receptor (ER) agonism in different tissues *in vitro* and *in vivo*[15]. Congenital malformations are severe congenital disabilities associated with substantial morbidity and mortality, often with unknown causes. Previous research reported that administration of 0.1 mg/kg (body weight) of Bisphenol-A (BPA), a compound with an estrogen-like activity on animals, resulted in lower prevalence of CL/P in *A/J* mice[17]. In this study, licorice solution at 0.89 mg/kg/day did not have an effect on the prevention of CL/P in *A/J* mice. However, since an *in vitro* study has reported that high levels of estrogen lowered the incidence of CL/P[18], it seems possible that licorice may have a similar estrogen-like effect if administrated appropriately. Therefore, determining the dosage of licorice solution that will increase estrogen level may help to suppress the occurrence of CL/P. Because licorice root or licorice extract is a plant product with possible estrogen replacement effects, many American women consider licorice root as a natural alternative to pharmaceutical hormone replacement therapy. However, whether dose and response are linearly associated should be interpreted with caution and confirmed in future studies. The most useful starting dose for drug selection is to know the shape and average dose-response curve of both the desired and undesired effects. Although the dose of licorice solution used in this study (approximately 0.89 mg/kg/day) did not clearly show the desired effect, we hypothesize that the development of CL/P might be suppressed if licorice solution is used appropriately.

Conclusion

In conclusion, we investigated the effects of the licorice extract on fetal mortality and cleft lip and/or cleft palate with 241 fetuses of *A/J* mice. Unfortunately, we did not get a conclusive result as we expected. The licorice solution of 0.89 mg/kg/day used in this study had no significant, and possibly even a negative, effect on CL/P and fetal viability. More research with other dosages of licorice should be performed.

The authors have no financial conflict of interest to disclose concerning the paper.

References

1) Wang ZY, Nixon DW: Licorice and cancer. Nutr Cancer, **39**(1): 1-11, 2001.
2) Nazari S, Rameshrad M, Hosseinzadeh H: Toxicological effects of Glycyrrhiza glabra (licorice): a review. Phytother Res, **31**(11): 1635-1650, 2017.
3) Vaya J, Belinky PA, Aviram M: Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. Free Radic Biol Med, **23**(2): 302-313, 1997.
4) Belinky PA, Aviram M, Fuhrman B, Rosenblat M, Vaya J: The antioxidative effects of the isoflavone glabridin on endogenous constituents of LDL during its oxidation. Atherosclerosis, **137**(1): 49-61, 1998.
5) Friis-Møller A, Chen M, Fuursted K, Christensen SB, Kharazmi A: *In vitro* antimycobacterial and antilegionella activity of licochalcone A from Chinese licorice roots. Planta Med, **68**(5): 416-419, 2002.
6) Demizu S, Kajiyama K, Takahashi K, Hiraga Y,

Yamamoto S, Tamura Y, Kinoshita T: Antioxidant and antimicrobial constituents of licorice: isolation and structure elucidation of a new benzofuran derivative. Chem Pharm Bull, **36**(9): 3474-3479, 1988.

7) Tamir S, Eizenberg M, Somjen D, Izrael S, Vaya J: Estrogen-like activity of glabrene and other constituents isolated from licorice root. J Steroid Biochem Mol Biol, **78**(3): 291-298, 2001.

8) Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC: Cleft lip and palate. Lancet, **374**(9703): 1773-1785, 2009.

9) Burg ML, Chai Y, Yao CA, Magee W, Figueiredo JC: Epidemiology, etiology, and treatment of isolated cleft palate. Front Physiol, **7**: 67, 2016.

10) Murray JC: Gene/environment causes of cleft lip and/or palate. Clin Genet, **61**(4): 248-256, 2002.

11) Carter CO: Genetics of common disorders. Br Med Bull, **25**(1): 52-57, 1969.

12) Fraser FC: The multifactorial/threshold concept - uses and misuses. Teratology, **14**(3): 267-280, 1976.

13) Kalter H: The history of the A family of inbred mice and the biology of its congenital malformations. Teratology, **20**(2): 213-232, 1979.

14) Miura S, Natsume N, Horiuchi R, Nakamura T, Kanoh Y, Fujiwara H, Furukawa H, Inagaki S, Marukawa T, Kawai T: Experimental study on cleft lip and palate. Preventive effects of estradiol on cleft lip and/or palate in *A/J* mice. J Jpn Cleft Palate Assoc, **15**(2): 122-131, 1990.

15) Tamir S, Eizenberg M, Somjen D, Stern N, Shelach R, Kaye A, Vaya J: Estrogenic and antiproliferative properties of glabridin from licorice in human breast cancer cells. Cancer Research, **60**(20): 5704-5709, 2000.

16) Duy LT, Niimi T, Furukawa H, Imura H, Natsume N: Effects of a Mongolian dairy product on fetal mortality and cleft lip and/or cleft palate in *A/J* mice: an *in vivo* study. Aichi Gakuin Dent Sci, **32**(7): 1-7, 2019.

17) Toyama Y: Experimental effects of cleft lip and/or palate and thymic anomalies estimated bisphenol-A (BPA) in *A/J* mice. Aichi Gakuin J Dent Sci, **43**(3): 409-420, 2005. (In Japanese)

18) Zava DT, Blen M, Duwe G: Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. Environ Health Perspect, **105**(suppl 3): 637-645, 1997.