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# EFFECT OF NOVEL LICORICE AND MACA COMBINED PLANT CRUDE AQUEOUS EXTRACT ON LITTER SIZE AND PUPS DNA NORMALITY IN MICE: EXPERIMENTAL MODEL FOR MAMMALS

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## Abstract

Licorice and Maca extracts has many beneficial effects that recently many researchers have revealed their interest to investigate the potential valuable effects of these plant extracts. However, there is no study about the effects of a Lico rice and Maca extracts mixture on pups and DNA normality in mice. Therefore the goal of this study is toinvestigates, for the first time, the effects of Lico rice and Maca mixture on the first and second generation of the treated female mice.

The dosages of both Licorice (Gg) and Maca (M) were orally administrated for 10 days as following; Gg group (25mg in 0.2ml daily), M group (5mg/0.5ml daily), Gg+M group (0.1ml+0.25ml/day respectively), Gg+M+SO group (0.1 ml +0.25 ml/day respectively) then undergo ovulation induction. The number, weight and teratogenicity of offspring of first and second generation were recorded. The DNA damage was evaluated by COMET assay, manually quantification and categorization into 4 categories (0-3) viavisual scoring of cells.

The results of current study found that oral administration of Licorice and Maca combined with super ovulation program in mice age 28 weeks has enhancement in pups numbers compared with 8 weeks old mice with no teratogenic effect on pups of both first and second generations and no DNA damage. It is concluded that the mixture of licorice and maca crude aqueous extract was confirm the option to be used by aged mammals to improve the reproductive fertility status. These results can be utilized for aged human females for further investigation.

***Key words:*** licorice, maca, embryonic development, comet assay.

## Introduction

For thousands of years, plants considered a resource of medicine and phytochemicals play an important role in medicine (Dixon and Wong, 2007). Female infertility is a very real medical problem (Mustafa, 2015). It has been found that many causes of infertility can be remedied simply by the correction of diet and supplementation of vitamins and herbs (Clark *et al.*, 2013).

*Glycyrrhiza glabra* (Licorice) is widely available in Italy, Spain, Turkey, Iran, Iraq, middle Asia, northern Africa and northeast Chaina. This herbal plant is an important medicinal plant (Badkhane *et al.*, 2014) belongs to Leguminosae family. It has many pharmaceutical uses as anti-inflammation, anticarcinogenic and fertility (Wang and Nixon, 2001). The U.S. food and Drug Administration (FDA) considered Licorice extract and its metabolites as safe (Mahalingam *et al.*, 2016). It has more than 20 tri-terpenoids and 300 flavonoids (Thakur and Raj, 2017). Glycyrrhizin and flavonoids are the most important metabolites (Faliah and AL-Jiboori, 2010; AL-Wailli, 2019). Glycyrrhizic acid is used for the treatment of sterility in females; furthermore, its flavonoids reduces hyperglycemia in poly cystic ovaries (Ali and Hasan, 2016). A study shows that Licorice extract rise offspring numbers and weights with no mutagenicity (Faliah and AL-Jiboori, 2010) and it supports mice embryo *in vitro* by supporting fertilization rate and normal early cleavage (AL-Dujaily and AL-Saadi, 2009).

*Lepidium meyenii* Walpers (Maca) is a perovian plant belongs to the family Brassicaceae. For many centuries it used as food and medicine in Peru. It is safe for use and do not induce hepatotoxicity *in vitro* or *in vivo* (Gonzales *et al.,* 2009). It contains free fatty acids (palmitic, linoleic, oleic acids), amino acids (arginine,

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leucine), macaridine, macaene, sterols, alkaloids, glucosinolates and macamides (Gonzales, 2012). A diet containing Maca improve reproductive performance (Di Cerbo *et al.*, 2019). Maca aqueous extract has been shown to rise sperm count in testis, epididymis and vas deference (Sanchez-Salazar and Gonzales, 2018; Del Prete *et al.*, 2018; Tafuri *et al.*, 2019), also it enhances some sperm function parameters in vasectomized male mice (AL-Dujaily *et al.*, 2018). The aim of the present study is to investigate for the first time, the effect of Licorice and Maca mixture on the first and second generation of the treated female mice.

## Materials and Methods

### Plants extraction

Licorice roots were air dried and milled into powder. A hot aqueous extract was prepared according to Harborne, (1984) as following: 250 g of dried grinded powder were extracted by using soxhlet (Sigma, USA) using 300 ml distilled water for 10 hours. Then, the filtrate lyophilized and kept at 4°C in dark containers until use. Maca powder (Healthworks®, USA) with 100% raw and organic certification was obtained from Peru. The extract were prepared according to the traditional method, dried Maca hypocotyls 100g mixed with 2 liters of water, let boiled at 100°C for 2 hr., then cooled, filtered and kept at 4°C until use (Sanchez Salazar and Gonzales, 2018).

### Animal management and treatment

Mature fertile female mice were used with two age groups 8 and 28 weeks old and orally administrated the dose by stomach tube for 10 days to the following groups; Gg group had 1g/Kg/B.W /day (25mg in 0.2ml daily), M group had 0.2g/Kg/B.W/day (2.5mg/0.25ml daily), Gg+M mixture group had 0.1ml Gg+0.25ml M/ day, Gg+M+SO group had 0.1 ml Gg+0.25 ml M/day for 10 days then subjected to super ovulation induction program (IP injectionon metestrus or diestrus females with 7.5 IU PMSG Folligon® and after 48hr. with 7.5 IU HCG pregnyle®), SO group had 0.1 ml tap water for 10 days then subjected to super ovulation induction program and untreated (CON) group had 0.1 tap water only. After 10 days of administration, female let mate with mature fertile male. The number and weight of pups of first and second generation were documented. Also if there were any dead births or teratogenic effects.

Alkaline Comet assay was carry out to determine DNA damage (single and double-stranded DNA breaks) in all first generation pups groups using Trevigen®, US comet assay kit according to (Villalba-Campos, 2016). 1mL of whole blood was withdraw by heart puncture in EDTA tubes and kept in 4°C until use. The WBCs were isolated as described by Rowland-Jones and McMichael, (1999). The DNA damage were manually quantified in cells and categorized into 4 categories (0-3) by way of visual scoring.


**Fig. 1:** Side view of 8 weeks old treated mother newborns (right) and 8 weeks control mother newborns (left) (first generation). A: M group, B: Gg group, C: M+Gg group, D: M+Gg+SO group, E: SO group.

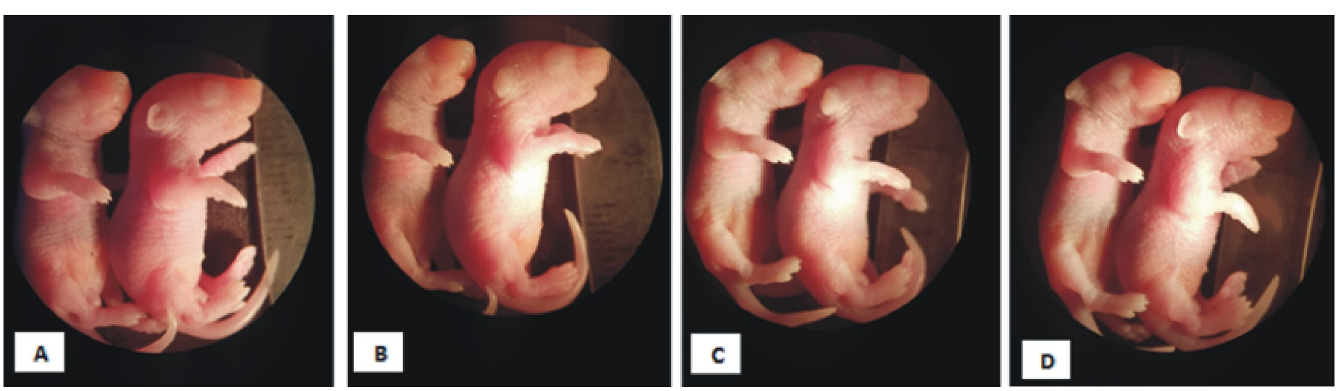


**Fig. 2:** Side view of 28 weeks old treated mother newborns (right) and 28 weeks control mother newborns (left) (first generation). A: Gg group, B: M+Gg group, C: M+Gg+SO group, D: SO group.

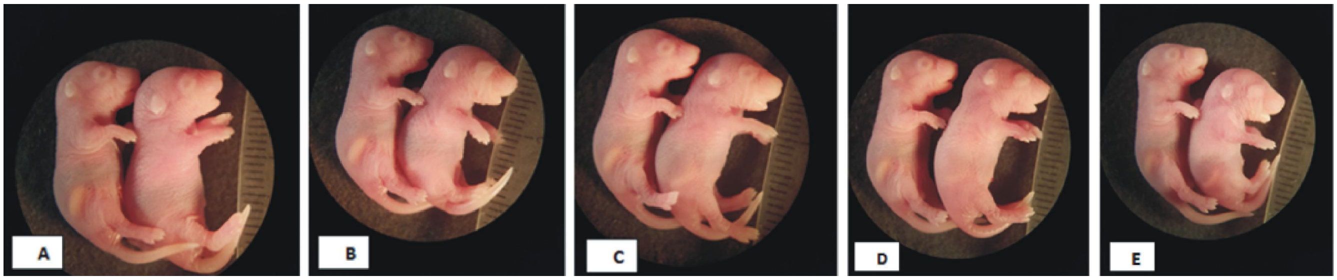


**Fig. 3:** Side view of 8 weeks old treated mother newborns (right) and 8 weeks control mother newborns (left) (second generation). A: M group, B: Gg group, C: M+Gg group, D: M+Gg+SO group, E: SO group.

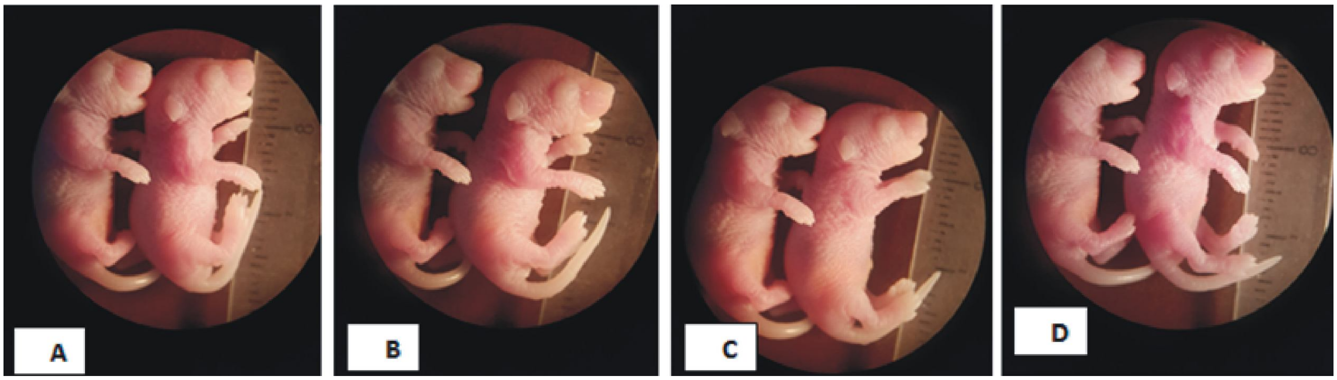


**Fig. 4:** Side view of 28 weeks old treated mother newborns (right) and 8 weeks control mother newborns (left) (second generation). A: Gg group, B: M+Gg group, C: M+Gg+SO group, D: SO group.

## Results

The present study shows that Licorice and Maca and the mixture of them has no teratogenic effect on the pups of both first and second generation with normal morphology comparing with pups of untreated female mice (control group). (Fig. 1, 2, 3 and 4).

**Changes in first generation pups weight and litter size born from treated female mice**

Concerning 8 weeks old mice, M group littersize showed a significant decrease compared with control group. While M+Gg, M+Gg+SO and SO groups showed a significant increase compared with control group. For 28 week old, Gg, M+Gg and M+Gg+SO groups showed a significant improvement compared with control group. At the same time, M+Gg+SO group, showed a significant growing compared with treated (Gg, M+Gg, SO) groups. While, M group has no litter size table 1.

**Table 1:** Changes in first generation litter size born from treated female mice.

| Mice groups | Mice ages | | | |
|---|---|---|---|---|
| | 8 weeks old | | 28 weeks old | |
| | No. | P-value | No. | P-value |
| C | 35 | - | 17 | - |
| M | 25 | 0.047 | 0 | - |
| Gg | 38 | 0.872 | 30 | 0.045 |
| M+Gg | 45 B | 0.048 | 38 | 0.039 |
| M+Gg+SO | 55 C, I, E | 0.040 | 60 I, E | 0.038 |
| SO | 53 D, L | 0.043 | 32 G | 0.045 |

NO. mice per group = 5. A:(M&G); B:(M&M+G); C:(M&M+G+SO); D:(M&SO); E:(M+G&M+G+SO); F:(M+G&SO); G:(M+G+SO&&SO); H:(G&M+G); I:(G&M+G+SO); L:(G&SO); Gg: licorice (Glycyrrhiza glabra); M: maca; C: control; SO: superovulation.

The results of first generation litter size comparison shows no significant change between 8 weeks and 28 weeks of M+Gg and Gg groups. While, M+Gg+SO group of 28 weeks shows none significant raise compared with 8 weeks. (Table 2).

The results of first generation pups weight was the following : In 8 weeks old, Gg, M and M+Gg groups shows a significant improvement in the weight compared with control group. Regarding mice age (28) weeks old,

**Table 2:** Comparison of litter size born from treated female mice (first generation) between 8 weeks and 28 weeks.

| Mice groups | Mice ages | | P-value |
|---|---|---|---|
| | 8 weeks old N. | 28 weeks old N. | |
| C | 35 | 17 | 0.042 |
| M | 25 | 0 | - |
| Gg | 38 | 30 | 0.099 |
| M+Gg | 45 | 38 | 0.087 |
| M+Gg+SO | 55 | 60 | 0.089 |
| SO | 53 | 32 | 0.041 |

NO. mice per group = 5 Gg: licorice (*Glycyrrhiza glabra*), M: maca, C: control, SO: superovulation.

**Table 3:** Changes in first generation pups weight in treated female mice

| First generation pups | Control | M | Gg | M+Gg | M+Gg+SO | SO |
|---|---|---|---|---|---|---|
| Body Weights of pups (g) 28week | 1.2975± 0.008913 | 0 | 1.642433± 0.016415 | 1.63611 $^{E,F}$± 0.00323 | 1.218907 $^{I}$± 0.031054 | 1.378179 $^{L}$± 0.024467 |
| p value | - | - | 0.048 | 0.049 | 0.998 | 0. 475 |
| Body Weights of pups (g) 8week | 1.256771± 0.004752 | 1.7674± 0.008932 | 1. 618036 $^{I,L}$± 0.004845 | 1.6425± 0.015587 | 1.251042 $^{C}$ ±0.005728 | 1.348459 $^{D}$± 0.071027 |
| p value | - | 0.047 | 0.049 | 0.049 | 0.987 | 0.447 |

Values are mean ± standard error (SE), (n=5/group).A:(M&G); B:(M&M+G), C;(M&M+G+SO), D;( M&SO), E;( M+G&M+G+SO), F;( M+G&SO), G;( M+G+SO&&SO), H;( G&M+G), I:( G&M+G+SO), L;( G&SO).
Gg: licorice(Glycyrrhiza glabra) , M:maca, C: control ,SO :superovulation.

Gg and M+Gg groups shows a significant improvement compared with control group. At the same time, M+Gg group was shown a significant increase compared with M+G+SO and SO groups. (Table 3).

**Pups DNA damage (first generation) by Comet Assay**

The DNA damage in cells was quantified and categorized manually into 4 categories (0-3) by visual scoring. Result of cells of pups of 8 weeks old treated mother mice shows a predominance in undamaged cell category (0) and low damage cells with category (1) in al all treated groups. The DNA damage categories (2) and (3) in cells of all treated groups are less than 20%. No necrotic DNA comets were observed in all treated groups. In high damage level, a significant decline was observed in the value of M, M+Gg and SO groups compared with control group. Whereas Gg and M+Gg+SO groups show no significant difference with control as illustrated in table 4.

The results of 28 weeks old treated mother mice pups shows a predominance in undamaged cell category (0) and the low damage category (1) cells at all treated groups. While the DNA damage (categories (2) and (3) in all treated groups was less than 20%. No necrotic DNA comet was observed in all treated groups. The high damage level value in all treated groups revealed no significant change compared with control group. The M group of 28 weeks old mothers has no pups (Table 5).

**Table 4:** Percentage of DNA damage in cells of pups of 8 weeks old treated mother mice and control.

| Mice groups of 8 weeks | DNA damage (%) | | | |
|---|---|---|---|---|
| | No damage | Low | Medium | High |
| Control | BC 46.585+0.985 | B39.850+0.999 | A6.3675+0.4112 | CD7.1975+0.4318 |
| M | D49.920+1.369 | A37.760+1.709 | A6.3500+0.2699 | A5.9725+0.1517 |
| Gg | C47.825+0.661 | AB 38.827+1.028 | A6.4575+0.4083 | BC6.8925+0.2016 |
| M+Gg | A43.620+1.002 | C43.638+0.758 | A6.3375+0.1852 | AB6.4100+0.7214 |
| M+Gg+SO | A44.742+1.810 | B39.500+2.448 | B 7.7225+0.3689 | D8.0350+0.9665 |
| SO | AB44.910+2.420 | C42.802+1.014 | A 6.4475+0.7524 | A5.8425+0.7010 |
| P-VALUE | 0.00021 | 0.00016 | 0.0024 | 0.0011 |
| LSD | 1.835 | 1.776 | 0.535 | 0.741 |

Different letters: Significant difference ($P \leq 0.05$) among groups.Gg: licorice (*Glycyrrhiza glabra*), M:maca, C: control, SO: superovulation.

**Table 5:** Score mean % of DNA damage in cells of pups of 28 weeks old treated mothers mice and control.

| Mice groups of 8 weeks | DNA damage (%) | | | |
|---|---|---|---|---|
| | No damage | Low | Medium | High |
| Control | A48.498+1.716 | A40.700+1.969 | A5.3900+0.1995 | A5.4125+0.2964 |
| Gg | A48.892+4.223 | A39.335+4.143 | AB5.7950+0.4449 | A5.9825+0.5390 |
| M+Gg | A46.943+3.270 | A41.032+2.781 | BC6.1875+1.0100 | A5.8375+0.5354 |
| M+Gg+SO | A48.120+1.330 | A39.510+1.230 | C6.6875+0.3772 | A5.6800+0.0883 |
| SO | A47.042+0.197 | A41.352+0.618 | A5.3625+0.3519 | A6.2475+0.0776 |
| P-VALUE | 0.766 | 0.708 | 0.019 | 0.058 |
| LSD | Non.Sig. | Non.Sig. | 0.677 | Non.Sig. |

Different letters: Significant difference ($P \leq 0.05$) among groups.Gg: licorice (*Glycyrrhiza glabra*), M:maca, C: control, SO: superovulation.

## Discussion

The results show no teratogenic effect of Licorice and Maca and mixture of them on first and second generation pups, with normal morphology compared with control group. Many studies has reported that Licorice and Maca has no toxic or teratogenic effects on embryos normal development (Itami *et al.*, 1985; Yoshida *et al.*, 2011; D'Arrigo *et al.*, 2004). The results of comet assay shows no DNA damage in first generation pups, which can be attributed to Maca and Licorice active compound. Maca alkaloids, polysaccharide and glycosylates has been reported to remove free radicals and generate antioxidant function (Sanchez *et al.*, 2018).

The litter size results in mice age (8 and 28) weeks old in M+Gg and M+Gg+SO groups showed a significant increase compared with control group with no significant difference between 8 weeks and 28 weeks of Gg, M+Gg and M+Gg+SO groups. Both Maca and Licorice has saponins which has role in normalize sex hormones secretion (Oshima *et al.*, 2003) so, Licorice and Maca mix may enhanced FSH and LH levels which rise ovulation and may increased progesterone which lead to successful implantation, as Licorice don't affect implantation (Diao *et al.*, 2013; Steffensen *et al.,* 2018) and elevate weight and number of pups (Faliah and AL-Jiboori, 2010). Moreover, Licorice has Isoloquiritigen in (flavonoid phytoestrogen), which has been found to reduce contraction in mouse uterus (Mahalingam *et al.*, 2016). An efficient amount of estrogen and progesterone is vital for implantation (Hall, 2016). Moreover, Licorice extract implies varying types of vitamins (Ody, 1993) and folate which is crucial for reproduction and fetus development. The significant decrease in M group litter size of 8 weeks old and absent of litter size in M group of 28 weeks old may due to Maca steroids as it has estrogen-like effect (Gonzales *et al.*, 2009) that would lower a renal function and then inhibit progesterone secretion in the young mice, which in old mice it would highly rise estrogen levels, plus the normally high estrogen levels in early premenopausal (Delamater and Santoro, 2018) leads to highly decrease in progesterone which prevents implantation. Mice uterus treated with high dose of estrogen become unreceptive in short time (Ma *et al.*, 2003) which affect blastocyst attachment to endometrial stroma (Ozturk and Demir, 2010). Moreover, Maca are 59% carbohydrates (Gonzales, 2012) which may increase metabolism and reactive oxygen species ROS plus the already high levels of ROS in aged females all may affects implantation and pups.

The significant enhance in body weight of Gg, M, Gg+M groups may be attributed to Licorice and Maca constituents that involve: protein, amino acids, sugar, vitamin and sterols which all essential for cell growth (Gonzales, 2012). Amino acids is a simulate factor for growth hormone secretion (Ganong, 2003). Additionally, Maca play a role on fetal development by stimulating insulin like growth factor (IGF-1) production in target tissues (Gonzales *et al.*, 2009; Hellstrom *et al*., 2016). A significant increase in pups has shown of SO and M+Gg+SO groups with no significant difference in pups weight has shown compared with other treated groups and control, although studies studies stated that super ovulation induction induction reduces birth weight (Van der Auwera and D'Hooghe, 2001).

It is concluded from this study that the oral administration of Licorice and Maca crude aqueous extract for 10 days before fertilization can enhance littersize and pups weight in young and old mice ages and it has no teratogenic effect on pups of both first and second generations. with no DNA damage in all treated groups of first generation pups so it can be used by aged mammals to improve reproductive fertility potential.

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