

## Effect of *Glycyrrhiza glabra* extract on IVF outcome in Mice : Experimental model for mammals

تقييم تأثير مستخلص عرق السوس على معدل الاخصاب والنمو الجنيني  
في الزجاج باستخدام الفئران كموديل للبانن

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### Abstract

**Objective:** To evaluate and examine the effect of *Glycyrrhiza glabra* extract (GgE) on the fertilization rate and embryonic development *in vitro* using the mice as a model for mammals. **Design:** Prospective study. **Methods:** Seven hundred forty ova were collected from superovulated (SUO) females mice. *Glycyrrhiza glabra* extract was prepared and used for *in vitro* activation of caudal epididymal sperm. *In vitro* fertilization was performed using both 10% GgE mixed with IVF medium for 367 ova (treated group) and IVF medium alone for 373 ova (Control group). Fertilization rate and normal development of different early embryonic cleavages stages was recorded. **Results :** There was a significant ( $P < 0.05$ ) increase in the fertilization rate (FR) of SUO mice oocytes (53.89%) by using 10% GgE compared to IVF medium alone in SUO group (36.82%). Addition of 10% GgE to the IVF medium, the number of 2-cell and 4-cell embryonic stages of SUO mice was significantly ( $P < 0.05$ ) higher than that of control mice embryos cultured with IVF medium alone (60% and 60% Vs. 51% and 54%, respectively). **Conclusion:** The investigation showed that the *Glycyrrhiza glabra* extract may contain many growth factors, and energy sources that supporting the FR and normal development of early cleavage stages of mice embryos *in vitro*. This result can be utilized for IVF program in mammals.

### المستخلص

استخدمت سبعماناة واربعين بويضة جمعت من فئران محفزة مبايضها هرمونيا . حضر واستخدم عرق السوس في تنشيط النطف البريخية في الزجاج . انجزت عملية الاخصاب في الزجاج باستخدام 10% من مستخلص عرق السوس لمجموع 367 بويضة . كما اجريت عملية الاخصاب في الزجاج باستخدام وسط زرع خاص للاخصاب في الزجاج لا يحوي عرق السوس لمجموع 373 بويضة . سجلت معدلات الاخصاب والنمو الجنيني لمختلف مراحل الانقسام المبكر . النتائج: سجلت الدراسة زيادة معنوية ( $P < 0.05$ ) في معدل الاخصاب للبيضات المحفزة وباستخدام 10% من مستخلص عرق السوس مع الوسط الزرع الخاص بالاخصاب في الزجاج ( 53.89% ) بالمقارنة بالوسط الزرع الخاص بالاخصاب في الزجاج وحده (36,82) % . عند اضافة 10% عرق سوس الى

**Keywords :** *Glycyrrhiza glabra* , IVF , Fertilization rate , embryonic development *in vitro*

الوسط الخاص للاخصاب في الزجاج, لوحظ بان عدد الخلايا الجنينية في مرحلة ( 4 و 8 ) خلية اعلى من استخدام الوسط الزراعي وحده ( 60 و 60 ) %مقابل ( 51 و 54 ) % على التوالي . الاستنتاجات: اظهرت نتائج الدراسة الحالية بان مستخلص عرق السوس ربما يحتوي على عدد من عوامل النمو ومصادر طاقة التي ساعدت معدلات الاخصاب والنمو الطبيعي للجنة في مختلف مراحل الانقسام المبكر في الفرنان في الزجاج . هذه النتيجة يمكن الاستفادة منها في برامج الاخصاب في الزجاج في اللبائن .

## Introduction

In 1978, the first *In vitro* fertilization baby (Louise Brown) was born. Since that time, this procedure has been used with increased success rates to produce offspring from patients with various [infertility](#) problems. The birth of the first baby conceived *in vitro*, opened up a completely new frontier in treatment of infertility [1].

The indications for IVF were tubal factor infertility, which accounted for over 60% of cases in most series, followed by male factor infertility and combined tubal and male factors. Unexplained infertility and endometriosis were not so common indications and accounted for less than 20% of indications for IVF [2].

Over the following 25 years, the pregnancy rates (PR) in IVF procedures have constantly improved and nowadays the success rates of around 20-30% per cycle are routinely reported by majority of Assisted Reproductive Techniques (ART) centers. Unfortunately, the need to increase the PR has led to transfer of multiple embryos, resulting in unacceptably high (~30%) multiple pregnancy rate [3]. The potential adverse effects of multiple pregnancies have stressed the need to limit the number of embryos transferred in each cycle [4].

On the other hand, it has been found that many causes of infertility can be remedied simply by the correction of diet, and supplementation of vitamins and herbs [5]. The medical use of herbal medications and nutritional supplements in their natural and unprocessed form undoubtedly began when noticed that certain food plants altered particular body functions, human culture institutionalized these observations, [6].

The medicinal use of *Glycyrrhiza glabra* (licorice) in both Western and Eastern cultures dates back several thousand years. Licorice is known to exhibit many pharmacological actions, such as estrogenic, anti testosterone, aldosterone-like; anti-inflammatory (cortisol-like); antiallergic; antibacterial, antiviral; and many others, [7]. Although much of the pharmacology focuses on glycyrrhizin and glycyrrhetic acid; Licorice has many other components, such as flavonoids, that act as an antioxidant [8]. However, very limited information is mentioned in the literatures about the effect of *Glycyrrhiza glabra* on the fertilization rate of IVF and early embryonic development in human and animals. Therefore, the aim of the present study is to find out the effects of addition of 10% *GgE* extract in the culture medium that used for IVF on *In vitro* Fertilization rate (FR) and *In vitro* preimplantation embryonic development (ED) using the mice as an experimental model for mammals.

## Materials and Methods

### 1. Management of experimental animals:

Males and females mature Balb /C mice 8-12 weeks old and 25-30 gm of weight were obtained from the Animal House of the Institute of Embryo Researches and Infertility

Treatment/ Al-Nahrain University. They were kept in an air conditioned room (~25°C) with a photoperiod (13±2) hours. The animals were housed in box cages of opaque plastic measuring (29x15x12) cm. Tap water and diet were freely available for the animals (ad libitum) [9].

## **2. *Glycyrrhiza glabra* preparation:**

The extract was provided as a water soluble powder form (Food Industry Company Iraq). Storage of *Glycyrrhiza* was done in well-closed container protected from light and moisture [10].

*Glycyrrhiza glabra* extract was used for the treatment groups as 10% concentration, was prepared by adding 0.1ml of Gg suspension (0.1% the stock solution) to 0.9ml of mouse IVF media (HTF, Millipore, USA and KSOM-AA Millipore). The 10% solution was filtered by using millipore size 0.45µm and 0.22µm (Millipore) and then pH was adjusted to reach 7.2-7.4.using pH meter.

## **3. Preparations before *in vitro* fertilization**

- **Male vasectomy:** The mature male mice were vasectomized as the procedure was described by [11].

- **Detection of female estrous cycle:** Stages of the mice estrous cycle were detected and reported using vaginal smears. The smears were performed daily between 8:00 am and 1:00 pm. The procedure was done according to [9].

-**Superovulation induction:** Superovulation was induced by intra peritoneal injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG, Folligon<sup>®</sup>,Holland), then followed by IP injection of 7.5 IU of human chorionic gonadotropin (hCG, Pregnyl<sup>®</sup>, China) after 42-48 hours. Ovulation was taken place between 12±2 hours after injection of hCG, then after 5 hours of hCG injection, one vasectomized male mouse was placed with three superovulated female mice to enhance ovulation induction procedure, [9].

The number of collected oocytes that cultured for one hour before IVF program in GgE - free HTF medium was 367 oocytes (the control). Whereas, the number of collected oocytes that cultured for one hour before IVF in 10%G.g-HTF medium was 373 oocytes (the treated group).

-**One day before IVF procedure:** Sperms and oocytes collection and culture dishes were prepared as the following:

**A. Epididymal sperm and Egg collection dishes:** central well Falcon culture dishes were contained 1 ml of GgE-free HTF medium for the control group and another dishes contained 10% GgE-HTF medium for the treatment group.

**B. Microdrops Culture Dishes Preparation:** Drops of culture media (in two groups: GgE-free HTF medium and 10% GgE-HTF medium) were dispensed in an array on the bottom of 35-mm sterile plastic tissue culture dishes. The dishes were flooded with embryo-tested light mineral oil.

**C. Fertilization dishes:** Were contained 250 µl of GgE-free HTF medium for the control group and another dishes contained 10% GgE-HTF medium for the treated group. Culture dishes (1 per each fertilization dish): Were contained 5 drops x 100µl of GgE-free KSOM-AA medium for the control group and another dishes contained 10% GgE- KSOM-AA medium for the treated group in a 35-mm dish

also. The dishes were transferred to an incubator at 37°C in an atmosphere of 5% CO<sup>2</sup> and 100% humidity.

#### 4. IVF procedure:

-Mature male mouse was sacrificed by cervical dislocation. The caudal epididymis region was isolated and put in previously prepared 1ml of HTF medium in a central well Falcon culture dishes [11].

All treated and control epididymal sperms were obtained and evaluated under the high power of light microscope to determine the final sperm concentration, motility and morphology before insemination as described by Alsaadi, [12].

- ***In vitro* Fertilization:** A mature female mouse was sacrificed by cervical dislocation at 13 hours post-hCG administration. The oviducts were dissected out and placed them into a culture dish contain 1ml of oocyte collection medium with the aid of dissecting microscope. All the cumulus masses were transferred to a single fertilization dish. After one hour of incubation, sperm was gently added to each fertilization dish by using a wide-bore pipette tips to give a final motile sperm concentration of  $1 \times 10^6$  ml. Fertilization dishes were Incubate at 37°C, 5% CO<sub>2</sub> and 100% humidity. After four to six hours of insemination, oocytes were washed through several drops of HTF medium "in wash dish" to remove the excess of sperm and debris. The washed fertilized oocytes incubated overnight in 100µl fresh HTF microdrops under mineral oil. The culture medium KSOM-AA was used for the culture of developing embryos [11, 13].

Fertilization rate was assessed by recording the number of zygote and two cells stage embryos 6-12 hours after insemination *in vitro*. Early embryo morphological evaluation was assessed on warmed microscopic stage of a Wild inverted microscope. Early embryos with 2 to 8 blastomeres were evaluated morphologically according to, [14].

All statistical analysis was performed with SPSS statistical program, LEAD Technologies. Results were expressed as mean  $\pm$ SEM and analyzed using paired sample t-test and Chi-square test depending on the nature of the data. When P-value reach the degree of 0.05, it was considered significant [15].

### Results

#### -*In vitro* activation technique:

In table 1, the mean sperm concentration ( $\times 10^6$  sperm/ml) following direct activation with 10% GgE-HTF medium was significantly ( $P < 0.05$ ) higher than before activation. Active sperm motility (grade A and grade B) was significantly ( $P < 0.05$ ) increased compared with before activation. The percentage of morphologically normal sperms following the addition of 10% GgE-HTF medium showed no significant ( $P > 0.05$ ) difference when compared with the GgE-free HTF medium.

#### - Fertilization rate: (FR)

Fertilization rate was gained by dividing the numbers of preimplantation embryos after 24 hours after insemination on the number of collected oocytes. The FR in control groups was 36.82% (134 embryos out of 367 oocytes), While the FR in treated groups was 53.89% (200 embryos out of 373 oocytes). There was a highly significant ( $P < 0.001$ ) difference between the two groups, as shown in table-2.

**-Embryonic developments after 24 hrs and 48 hrs**

This study was concerned on the numbers of mouse preimplantation embryo development after 24 and 48 hours of insemination and on the percentage of normal embryonic developments. There were 134 embryos after 24 hours of insemination and incubation with GgE-free HTF culture medium (control group), the stages of embryonic developments was 69 embryos at two cells stage and 65 embryos at three-four cells stage. While after the same period of insemination and incubation with 10% GgE-HTF culture media (treated group), there was 200 embryos divided into 119 embryos at two cells stage and 81 embryos at three-four cells stage. There was a highly significant ( $P<0.001$ ) difference in the total number of embryos and at the total number of two cells stage of embryos after 24 hrs as shown in table (2).

There were 134 embryos after 48 hours following insemination and incubation with GgE-free KSOM-AA culture medium (control), the stages of embryonic developments were 36 embryos at two cells stage, 72 embryos three-four cells stage and 26 embryos at five-eight cells stage. While after the same period of insemination and incubation with 10% GgE-KSOM-AA culture media (treated groups), there was a (200) embryos divided into 30 embryos at two cells stage, 121 embryos at three-four cells stage and 49 embryos at five-eight cells stage. And there was a highly significant ( $P<0.001$ ) difference at the total number of embryos and at the total number of three-four cell stage and five-eight of embryos after 48 hrs as shown in table( 3) .

**Table (1): Comparison between the effect of addition of G.g-free HTF medium and 10%**

Grouping with and without G.g.		<i>In vitro</i> activation	
		After one hour incubation	Significance
Sperm concentration ( $10^6/ml$ )	Without G.g	24.36±1.405	0.039
	With G.g	34.33±4.323	
Sperm motility grade A (%)	Without G.g	11.40±2.315	0.046
	With G.g	19.73±0.918	
Sperm motility grade B (%)	Without G.g	21.33±2.622	0.042
	With G.g	29.73±2.472	
Progressive motility (A+B)%	Without G.g	32.73±4.362	0.022
	With G.g	49.46±3.065	
Morphologically normal sperms (%)	Without G.g	69.43±4.410	0.741
	<b>With G.g</b>	<b>70.93±3.084</b>	

**G.g-HTF medium on certain sperm function parameters following *in vitro* direct activation.**

**.Values are Mean± SEM**

**P<0.05 is significant**

**Table (2): Comparison in fertilization rate and embryonic development between GgE-free HTF medium (control group) and 10%G.g-HTF medium (treated group) after 24 hours of insemination**

Grouping with and without G.g.		Fertilization rate(%)	Significance
Total number of fertilized oocytes after 24 hrs	Without G.g	134/367 (36.82)	0.001
	With G.g	200/373 (53.89)	
Grouping with and without G.g.		Embryonic development rate(%)	Significance
Total number of two cells stage of embryo after 24 hrs	Without G.g	69/134 (51.5)	0.001
	With G.g	119/200 (60)	
Total number of 3 - 4 cells stage of embryo after 24 hrs	Without G.g	65 /134(48.5)	0.082
	With G.g	81/200 (40)	

$\chi^2$ . table

**Table (3): Comparison in embryonic development between GgE-free KSOM-AA medium (control group) and 10%G.g-Ksom-AA medium (treated group) after 48 hrs. of insemination.**

Grouping with and without G.g.		Total embryonic development rate (%)	Significance
Total number of embryos after 48 hrs	Without G.g	134/367 (36.82)	0.001
	With G.g	200/373 (53.89)	
Number of total two cells stage embryos after 48 hours	Without G.g	36/134 (27)	0.302
	With G.g	30/200 (15)	
Total number of 3 - 4 cells stage of embryos after 48 hrs	Without G.g	72/134 (54)	0.001
	With G.g	121/200 (60)	
Total number of 5 - 8 cells stage of embryos after 48 hrs	Without G.g	26/134 (19)	0.001
	With G.g	49/200 (25)	

$\chi^2$ . table

## Discussion

### 1. -In vitro sperm direct activation:

In this study, there was an enhancement in certain sperm function parameters following in vitro activation by adding GgE to the culture medium used, and that was attributed firstly to the direct activation technique with HTF medium. The medium provided the same culture components which found in the female genital tract and that will trigger the sperm hyperactivity motility [11]. Secondly, adding of GgE to the culture media enhances different sperm function parameters following 60 minutes of activation, namely; sperm concentration, total sperm motility percentage and grade activity of forward progressive movement. And the booster effect of GgE powder extract on epididymal sperms to move

out and to release from the epididymal tissue. *Glycyrrhiza glabra* has an estrogenic activity due to the presence of glabridin which is known to be a phytoestrogenic and has the ability to bind to human estrogen receptors [16]. Estrogens are known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyperactive motility [17]. Moreover, it has been found that the G.g contains  $\text{Ca}^{+2}$ , potassium, glucose, fructose, vitamin E, vitamin C and many other substances e.g.:  $\text{Zn}^{+2}$ , sucrose, amino acid[7], all these substances stimulate sperm motility and the grade activity of forward movement. The sugars are considered to be a source of energy for sperm motility, the protein and amino acids, which sustain and maintain sperm osmolarity and in turn integrity of sperm cell membrane [18].

## **2. Fertilization rate:**

The results of this study showed a significant increase in the FR after 24 hours of insemination, and the differences in the means are 17% between the treated and control groups. Several factors may affect the FR in the present work. First, all the ovulated oocytes are inseminated with the same sperm concentration ( $1 \times 10^6$  sperm/ml). Therefore, the differences in FR are not due to the increments in the sperm concentration but it may be belonged to the effect of the direct activation technique with 10% GgE and to the decrease in the insemination volume to each fertilization well. This reduction may reduce the decapacitation factors and contaminants from the seminal plasma [19].

The study found that the FR increases with an increasing in sperm motility and grade activity of progressive forward movement. This finding is in agreement with [20], who emphasized that the only parameter that could predict treatment outcome was the percentage of motile spermatozoa after appropriate sperm preparation. The last factor that may interfere with the increases in FR was the addition of GgE powder extract to the insemination medium. The GgE extract provided a wide range of active ingredients that provide a nourishment and/or protection to the oocytes and early cleaved embryos. The licorice contains antioxidant compounds like: isoflavones, hispaglabridin A and B, which inhibit  $\text{Fe}^{+3}$  induced mitochondrial lipid peroxidation [21].

## **3. Embryonic developments:**

A significant improvement in ED and in embryo quality was observed after 24 of insemination with 10% Gg-HTF medium. This improvement can be attributed to the addition of 10%GgE with HTF and KSOM-AA medium, because all variables during the insemination and culture procedures were fixed and controlled. The positive effects of GgE that recognized in this study may be augmented by a number of active ingredients like: Ascorbic-acid, Asparagine, Carotenes, Fructose, Glucose, Magnesium, Maltose, Manganese, Sucrose, Sugar, Thiamin, Vitamin E etc., each of which have different effects on oocytes and early developed embryos [7]. Moreover, GgE contains Glycyrrhizin (10%) as calcium and potassium salts of glycyrrhizic acid. Therefore, in the present study, the first cleavage and development of early developed embryos occur in a wide range of sodium, magnesium, calcium, and potassium concentrations[22]. It may also be explained the developing embryo is exposed to gradients of carbohydrates as it develops, together with amino acids. It has been noticed that carbohydrates are the main energy substrates for the embryo[23]. The plant is rich in glucose and the addition of GgE extract to HTF

medium enhanced the EDs after 24 hours of insemination. That may be explained that the mice cumulus cells surrounding the oocyte and early embryo readily produce both pyruvate and lactate from glucose [24]; whilst glucose uptake increases with development[25].

At this work, there was early developmental arrest of mouse embryos. This finding may be associated with elevated levels of free oxygen radicals, indicating that oxidative stress might be the reason for such high incidence of arrest [26]. Oxidative stress may be generated endogenously or exogenously, but either way it can affect the oocyte and embryo. Therefore, IVF culture media, may be the exogenous site of oxidative stress generation ,affecting the oocytes and the preimplantation embryo [27]. Higher day one ROS levels in culture media were associated with delayed embryonic development [28], high fragmentation and development of morphologically abnormal embryo after prolonged culture. In treated group, the antioxidant system is influenced GgE powder extract which contains a wide range of vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene, and carotene which may play a critical role in conversion of ROS to H<sub>2</sub>O[29,30]. Vitamin C and vitamin E is a chain breaking antioxidant that stops the propagation of the peroxidative process. Thus, treatment with 10% GgE increased the total antioxidant capacity in culture media of oocytes that later were successfully fertilized.

It is concluded from the present study that the addition of GgE to the culture media of sperm and oocyte can enhance the fertilization rate and embryonic development in mice. This result can be utilized for other mammalian IVF programs.

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