Effect of *Glycyrrhiza glabra* extract on *in vitro* sperm activation and embryonic development following intra-peritoneal insemination in mice: experimental model for mammals

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Abstract:

Background

Glycyrrhiza glabra(Gg) is a herbal medicine used in the treatment of different syndromes. Recently, the effect of this plant on the reproductive system has been studied. However, its role in *in vitro* still not clear.

Objectives

The present study aims to investigate the possibility of using *Glycyrrhiza glabra* extract for *in vitro* sperm direct activation, and its effect on *in vivo* fertilization rate (FR) and early embryonic development (ED) following intra-peritoneal insemination (IPI).The mice were used as an experimental model for mammals.

Materials and Methods:

Glycyrrhiza glabra extract (2 mg/ml culture medium) was used for *in vitro* direct sperm activation technique. The female mice were divided into two groups: spontaneously ovulated (SOM) and superovulated groups (SuOM).Each group was divided into two subgroups, the first: the IPI was accomplished by epididymal sperm activated in vitro by adding 20%Gg to the culture medium.The second subgroup: the IPI is accomplished without adding Gg to the culture medium.

Results

In vitro activation of epididymal sperms with 20% Gg has shown positive effects on sperm concentration, sperm motility, and grade activity of progressive forward movement. There was a significant ($P<0.05$) increase in FR with adding 20%Gg compared with that free of Gg of spontaneously ovulated groups after 24 hours of insemination. The FR in the right oviduct showed highly significant ($P<0.001$) increment compared to the left. Embryonic development rate significantly ($P<0.05$) increased after 24 and 48 hours of insemination in grouping with adding Gg. The study showed that the quantity and quality of embryos generated from IPI with adding Gg were higher than that of groups inseminated without adding Gg.

Conclusion

It is concluded from the results of the present study that adding the 20% Gg to the culture medium of the epididymal sperm and IPI leads to an improvement in certain sperm function parameters and supports the FR in SOM and SuOM with an increase in the embryonic development rate. These data can be utilized for artificial insemination programs.

Key Word: intra-peritoneal insemination, *Glycyrrhiza glabra*, *in vitro* sperm activation

Introduction

Assisted Reproductive Technologies (ART) have been used for decades to aid couples that are having difficulty in creating and maintaining pregnancy. The most ART procedures known are; *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and artificial inseminations (AI), (1,2). Artificial insemination is categorized as an assisted reproductive technology (3). Indeed, AI has become an option for many infertile couples prior to considering more expensive assisted reproductive treatment such as IVF (4). Artificial insemination is the oldest technique used in human and animal (3).According to Sherman (5), the procedure has a long history that

belongs to the eighteenth century. Artificial insemination includes Intra-peritoneal Insemination (IPI), Intra-follicular Insemination (IFI), Intra-tubal Insemination or Intra-Fallopian Insemination , Intrauterine Insemination (IUI) and Intracervical Insemination(ICI)(6).

On the other hand ,in recent years, there has been a worldwide interest in the extraction of various medicines from several plants for the treatment of different diseases, since they are natural products, easy to get and also cheap, (7).One of these plants is the Gg which is a herb of great versatility. Some of the traditional uses of licorice include: anti-inflammatory action similar to cortisone, anti-allergic, expectorant, antibiotic, anti-viral, anti-bacterial, anti-spasmolytic and thirst quenching (8). The plant contains different compounds that enhance sperm parameters *in vitro* and increases the reproductive efficiency in human and mice *in vivo* (9,10).However, there are few literatures regarding in vitro effect of Gg on sperm activation and *in vivo* fertilization rate and embryonic development following artificial inseminations. Therefore the aims of this study were to find out:1- The effect of Gg extract on epididymal sperm activation in vitro.2-The effect of activated sperm on the embryonic development following the insemination Intra-peritonealy. The study investigated:- *In vivo* FR and *in vivo* preimplantation ED.

Materials and Methods

This study was conducted in the Institute of Embryo Research and Infertility treatment, Al-Nahrain University. One hundred and twelve mature mice Balb/C St Can BR Strain (80 females and 32 males) 8-12 weeks old and 25-35 gm weight, were obtained from the colony of the animal house of the Institute were included in this investigation.

They have been kept in a room supplied with an air conditioner to keep the temperature between 22-24 °C; the air of the room was changed continuously using ventilating fan. Photoperiod was automatically controlled of 13±2 hrs light from 6A.M. to 7 P.M. daily regimen.

Preparation of *Glycyrrhiza glabra* Extract:

The major method for preparation of Gg extract is aqueous method. Water extract, (1000 gm) of licorice in granular powder moistened with boiling water and percolated until the licorice was exhausted. Then ammonia solution was added to the percolate, filtrates and evaporates until black pilular mass having a characteristic of sweet taste powder is prepared. The percentages of Glycyrrhizin in these preparations were about 6-8% due to the age of the plant. The Glycyrrhiza has been stored in well-closed container protected from light and moisture as described by Al-Ahliya Flavours & Fragrances Co. Ltd. IRAQ.

Preparation of *Glycyrrhiza glabra* for mice sperm activation *in vitro*

A stock solution of Gg was prepared by dissolving 200 mg/100ml culture medium . The concentration of Gg (20%) was prepared by adding (2) ml of Gg solution to (8) ml of sEBSS medium in plastic test tube contained broad spectrum antibiotic (Ampicillin 0.004 gm) to prevent bacterial growth. The solution was filtered using Millipore (0.45µM). All prepared media have been fixed at pH 7.4-7.8 at room temperature.

Superovulation induction

Superovulation was performed by IP injection of 7.5 I.U. of pregnant mare serum gonadotropin (PMSG,Folligone®-Holland) and then followed by IP injection of 7.5 I.U. of hCG(Pregnyl®,Serono-Italy), 48 hours later.

In vitro Sperms Activation Techniques.

After preparation of spermatozoal suspension, one drop of caudal epididymal spermatozoa were examined under the microscope for certain sperm function parameters as mentioned earlier. Thereafter, the sperm activation was performed, using direct activation technique. The epididymal contents were squeezed out by making 5-7 slashes with a 30-gauge needle syringe (that allows the sperms to swim-out) and the residual caudal tissue was discarded (11). This technique for sperm activation is characterized by direct effect of culture medium on sperm parameters

Intra-Peritoneal Insemination.

Intra-peritoneal Insemination by 2X106/ ml of prepared sperms was performed for two main animal groups: a-Spontaneously -ovulated female mice (control group). b-Superovulated female mice.

Embryo Recovery and Viability:

The inseminated females have been sacrificed by dislocation and after opening the abdominal cavity (12):Fertilized ova were diagnosed by the existence of spermatozoa head at perivitelline space and the observation of two pronuclear and two polar bodies. The morphology of 2 to 8 blastomeres embryos were divided into 4 grades according to the criteria of Khalili and Anvari, (13).

1. Grade A: equal blastomeres, round, with no fragmentation, smooth cytoplasm, and bright yellow zona.
2. Grade B: slightly different blastomeres in size, up to 10% fragmentation with granules in cytoplasm.
3. Grade C: unequal blastomeres, up to 50% fragmentation with large granules and vacuoles in cytoplasm.
4. Grade D: unequal sized blastomeres, extreme fragmentation, with dark and large granules and

Statistical Analysis:

The following statistical tests were used for the analysis of the data; student's t-test and Chi-square depending on the nature of the data. When P values reach the 0.05; the results were considered significant (14).

Results

1. *In Vitro* Sperm Activation Technique:

The results of certain sperm function parameters (sperm concentration, sperm motility, grade of activity , and sperm normal morphology) following in vitro direct activation and incubation of caudal epididymal region for 10 minutes with and without adding 20%Gg are shown in Table 1 .

The mean sperm concentration of caudal epididymal sperms following direct activation by adding 20% Gg was (31.134±0.3858) which is significantly higher (P<0.0001) than that with free Gg media (20.197±0.2429) that used for activation. The percentage of sperm motility (grade A and B) in the group containing 20% Gg was significantly (P<0.0001) improved compared to that of the epididymal sperms activated without adding Gg. No significant (P>0.05) different was registered in morphologically normal sperms following the addition of free-Gg medium when compared with 20% Gg medium.

2.Fertilization Rate

The relationship between the number of a corpus luteum and the number of fertilized ova (right and left oviduct) in the two groups was illustrated in Tables 2 and 3. There were 45 fertilized ova out of 87 total ova, therefore total fertilization rate in the right and left oviduct of SOM group was 50.9% following 24 hrs of IPI of the epididymal sperm activated in vitro by adding Gg free medium. In the right oviduct, the FR revealed highly significant increase (P<0.001) compared to the left oviduct (Table 2). The total FR of SuOM group was 39.8% (99 fertilized oocytes out of 248 oocytes). The FR in the right oviduct did not show a statistically significant decrease (P>0.05) compared to the left one . There was a significant increase (P<0.05) in the FR of SOM group compared to SuOM group by adding Gg free medium as shown in Table (2).

Table 3 shows the FR in SOM group and SuOM group after IPI with adding 20% Gg into the medium to activate in vitro the epididymal sperms. The FR in SOM group was 74% (62 fertilized oocytes out of 82 oocytes). In the right oviduct, the FR was significantly higher (P<0.05) than that of the left oviduct. Whereas, the FR in the right oviduct of SuOM group was revealed no significantly differences (P>0.05) compared to the left. There has been a significant (P<0.05) increase in the FR of the SOM group (74%) compared to SuOM group (58%; 150 fertilized ova out of 258 ova).

Table 1: Certain sperm function parameters of mice caudal epididymal sperms following *in vitro* direct activation with and without adding 20%Gg

Certain Sperm Function Parameters	*In vi tro* Sperm Activation		
	Grouping with and without G.g.	After 10 minutes incubation Mean ±SE	P value
Sperm Concentration (million/ml)	Without Gg	20.197 ± 0.2429	0.0001
	With Gg	31.134 ± 0.3858	
Sperm Motility Grade A (%)	Without Gg	10.353 ± 0.2472	0.0001
	With Gg	17.650 ± 0.4047	
Sperm Motility Grade B (%)	Without Gg	16.097 ± 0.1609	0.0001
	With Gg	25.863 ± 0.2562	
Progressive Motility (A+B) %	Without Gg	26.450±0.301	0.0001
	With Gg	43.512±0.536	
Morphologically Normal Sperms (%)	Without Gg	25.521± 0.2888	0.154
	With Gg	26.959 ± 0.2681	

Values are mean ± SEM
No. Mice =30 Gg: Glycyrrhiza glabra.

Table 2: Fertilization rate after 24 hrs of IPI with epididymal spermatozoa activated *in vitro* by Ggfree medium.

Mice Group	No. Corpora Lutea			No. Fertilized Ova			Fertilization Rate (%)		
	Right Ovary	Left Ovary	Total	Right Oviduct	Left Oviduct	Total	Right Oviduct	Left Oviduct	Total
Spontaneously ovulated	49	38	87	28	17	45	57.1*	44.7	50.9*
p value	0.238			0.101			0.001		
Superovulated	121	127	248	46	53	99	38	41.7	39.8
p value	0.703			0.309			0.736		

*P<0.05: Significant difference between spontaneously and superovulated mice groups

Table 3: Fertilization rate after 24 hrs of IPI with epididymal spermatozoa activated *in vitro* by 20% Gg.

Mice Group	No. Corpora Lutea			No. Fertilized Ova			Fertilization Rate (%)		
	Right Ovary	Left Ovary	Total	Right Oviduct	Left Oviduct	Total	Right Oviduct	Left Oviduct	Total
Spontaneously ovulated	46	36	82	40	22	62	86.9*	61.1	74*
p value	0.269			0.022			0.033		
Superovulated	126	132	258	71	79	150	56.3	59.8	58
p value	0.803			0.514			0.710		

*P<0.05: Significant difference between spontaneously and superovulated mice groups

3. Embryonic Developments

3.1 Embryonic development after 24 hours

Table 4 illustrate the effect of IPI with epididymal sperms activated in vitro by adding 20% Gg to culture medium on embryonic development after 24 hrs of spontaneously and superovulated mice. The normal development of 1-cell embryo of SOM (35.5%) following 24 hrs of IPI with the epididymal sperm activated *in vitro* by adding Gg free medium was significantly higher (P<0.001) than that of adding 20% Gg (8%). In SuOM the percentage of 1-cell embryo developed without adding Gg (24.4%) was significantly (P<0.001) increased compared to adding Gg (8%) following 24 hrs of IPI. The normal development of 2-cells embryos of SuOM (58%) with adding 20% Gg to the culture medium of sperm activation *in vitro* was significantly increment (P<0.009)increased compared to without adding Gg (40.4%).No significant difference (P>0.05) was observed in the percentage of 2-cells embryos of SOM between adding 20% Gg or without adding Gg to activate the epididymal sperms *in vitro*. The SuOM have shown a significant improvement (P<0.005) in the rate of normal development of 3-4 cells when IPI accomplished by epididymal sperms activated in vitro by adding 20% Gg of the culture medium (33.3%) compared to IPI without adding Gg to the culture medium (17.1%). The rate of total number of normal embryonic development by adding 20% Gg was significantly higher (P<0.001) in both SOM (62/82=75.6%) and SuOM (150/258=58.1%) compared to their corresponding group without adding Gg (spontaneously; 45/87= 51.7%, and superovulated; 99/248=39.9%, respectively). the total number of normal embryonic development with adding 20%Gg to culture medium significantly (P<0.05) increases in SOM group compared to SuOM group.

3.2.Embryonic development after 48 hours

In SuOM mice(picture1), the rate of the normal development of 2 and 5-8 cells embryos following 48 hrs of IPI with sperms activation in vitro by adding 20% Gg was significantly (P<0.05) increased compared to adding Gg free medium. No significant (P>0.05) changes were observed in the percentage of 3-4 cells between adding Gg free medium and 20% Gg . The total number of normal embryonic development of SOM was significantly higher(P<0.001) than that of SuOM following IPI with adding 20%Gg to culture medium (Table 5).

Table 4: Effect of IPI with epididymal sperms activated *in vitro* by adding 20% Gg to culture medium after24 hrs of embryonic development of spontaneously and superovulated mice

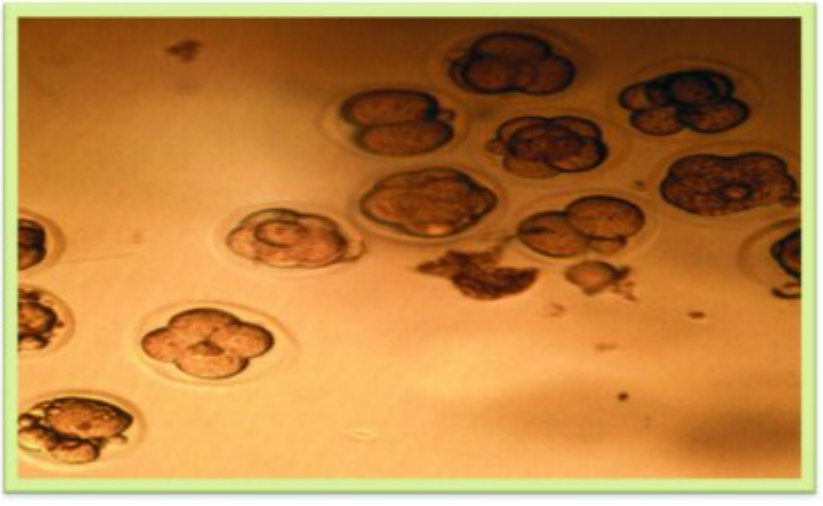
Different Embryonic Stages	Female Mice following IPI						
	Grouping with and without Gg	Spontaneously Group	Percent (%)	Significance	Superovulated Group	Percent (%)	Significance
One cell stage	Without G.g	16/45	(35.5)	0.001	42/99	(42.4)	<0.001
	With G.g	5/62	$(8)^{ns}$		13/150	(8)	
Two cells stage	Without G.g	19/45	(42.2)	0.198	40/99	(40.4)	0.009
	With G.g	34/62	$(54.8)^{ns}$		87/150	(58)	
3-4 cells stage	Without G.g	10/45	(22.2)	0.100	17/99	(17.1)	0.004
	With G.g	23/62	$(37)^{ns}$		50/150	33.3	
Total number of embryos after 24hrs.	Without G.g	45/87	(51.7)	0.001	99/248	(39.9)	0.001
	With G.g	62/82	(75.6)*+		150/258	(58.1)	

*P<0. 05: Significant difference between spontaneously and superovulated mice groups nsP>0. 05: Non significant difference between spontaneously and superovulated mice groups + P<0.05 Significant difference than superovulated groupGg: Glycyrrhiza glabra.

Table 5: Effect of IPI with epididymal sperms activated *in vitro* by adding 20% Gg to culture medium after 48 hrs of embryonic development of SOM and SuOM mice

Different Embryonic Stages	Female mice following IPI						
	Grouping with and without Gg	Spontaneously ovulated group	Percent (%)	P value	Superovulated group	Percent (%)	P value
Two cells stage embryos	Without G.g	10/37	(27)	0.128	33/89	(37)	0.002
	With G.g	8/56	(14.2)^{ns}		16/94	(17)	
3-4 cells stage embryos	Without G.g	18/37	(48.6)	0.526	39/89	(43.8)	0.205
	With G.g	31/56	(55.3)^{ns}		50/94	(53.1)	
5-8 cells stage of embryos	Without G.g	9/37	(24.3)	0.523	17/89	(19.1)	0.093
	With G.g	17/56	(30.3)^{ns}		28/94	(29.7)	
Total number of embryos after 48hrs.	Without G.g	37/75	(49.3)	0.009	89/260	(34.2)	0.120
	With G.g	56/80	(70)**+		94/229	(41)	

**P<0.001: Significant difference between spontaneously and superovulated mice groups ns P>0.05: No significant difference between spontaneously and superovulated mice groups.+ P<0.05 :Significant difference than superovulated groupGg: Glycyrrhiza glabra



Picture-1:Different stages of embryos after 48 hrs of IPI

Discussion

1. *In Vitro* Sperm Activation Technique.

The present study has investigated the positive effect of adding 20% Gg to culture medium by *in vitro* direct activation technique on certain sperm function parameters following 30 minutes. This improvement can be attributed to the constituents of Gg. It has been found that Gg has an estrogenic activity by the presence of glabridin (isoflavones) which is known to be phytoestrogenic and has the ability to bind to human estrogen receptors (15).

Estrogens are known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyperactive motility due to increasing cAMP, which has shown to be a very important factor in sperm motility percent; (16). Rather, Ca^{++} ion (which is one of the components Gg) has a role in increasing sperm motility and hyper activation by preventing the degradation of cAMP via inhibition of phosphate diesterase enzyme (17).

2- Intra-Peritoneal Insemination.

2.1 Fertilization Rate.

In SuOM, the result of FR was tending to be significantly higher in the left oviduct compared to the right by adding 20% Gg and without adding Gg. This observation can be related to several reasons; first, caudal epididymal spermatozoa have the capability of migration and fertilization of viable oocytes at the left genital tract following IPI in the right side (18). Secondly; the superovulation with exogenous gonadotropin and insemination with high concentration (2 million) of sperms, can lead to high rate of fertilization with increased ROS products of spermatozoa at the right side (19).However, the number of sperms reach the left side will be less to have this phenomena. Consequently, the FR in the left Fallopian tube was lower than that in the right of SuOM group, this result in agreement with Al-Dujaily and Albarazanchi (20).

The total FR of the combined right and left oviducts increases with adding 20% Gg to the epididymal sperms activated in vitro of the SOM and SuOM groups compared with the groups of Gg free culture medium. The Gg contains different carbohydrates such as glucose. The glucose metabolism is essential in the control of meiosis in the mouse oocyte (21).

Glucose metabolism is tied to environmental oxygen tension and reactive oxygen species (ROS) concentration within the oocyte. Excessive oxidative stress appears to contribute to reduce development of oocytes and embryos in vitro (22).

The increase in FR here is not due to increments in the sperm concentration but it belongs to the effect of the direct activation technique with 20% Gg to the culture medium (11). Moreover, adding Gg which affects sperm motility is necessary phenomenon for fertilization and conventional methods of assisted reproduction. The Gg also affects the percentage of sperm morphology positively, which considered as an essential factor to have a successful fertilization process. Sperm normal morphology had been proven to be a good predictor for fertilization in vivo (23) and assisted reproduction (24).

The percentage of total FR in SOM group significantly improved than that the SuOM group. This finding is in agreement with Al-Dujaily and Albarazanchi, (20) result.

2. Embryonic Development.

In SuOM group, the best stage of normal embryonic development is one-cell and 2-cell embryos following 24 hrs of IPI without adding Gg. This finding may resulted from the large number of superovulated ova which have more chance to fertilize at a quite time after hCG injection (20). While with adding 20% Gg the number of normal 2-cell embryos has increased. This fact can be due to that Gg accelerates the maturation process of oocytes by activation the Calipain which is Ca2+ dependant cystien proteinase. The effect of Calipain results in activating of maturation promote factor leading to complete maturation when the oocyte maturation arrests at metaphase II, (25). At the same time, the total number of ED was higher in SOM and SuOM after 24hrs of IPI with adding 20% Gg. Also, there was an increase in grades A and B embryos of SuOM and SOM when the epididymal sperms activated *in vitro* by adding 20% Gg and inseminated the females with medium that contains the Gg .This phenomenon can be explained by the effect of Gg to minimize and/or prevent the polyspermic fertilization leading to increase the number of normal embryonic development. The memorable effect of Gg that was recognized may be augmented by a number of active ingredients like: ascorbic-acid, fructose, glucose, magnesium, maltose, thiamin, vitamin E, etc.; each of which has different effects on oocytes and early developed embryos (8). Moreover, Gg powder extracts contain Glycyrrhizin (10%) as calcium and potassium salts of glycyrrhizic acid. Therefore, in this study, the first cleavage and development of embryos may occur because the medium composed of a wide range of ions such as sodium, magnesium, calcium, and potassium concentrations (26).

After 48 hrs, the rate of embryonic development revealed a significant change at the right side compared to the left side of SOM with and without adding Gg. As mentioned earlier, the maturation rate of the oocytes influenced by maturation process which increased by corona radiata and cumulus cells by supplying the oocyte with steroids and maturation factors (27)

.This in turn can increase the ED in the side of IPI by increasing the influence of the medium on the oviductal epithelium environment leading to have more number of normal ED at the right side than the left. The other explanation may be due to the presence of 20% Gg in the medium used to inseminate the activated sperm intra-peritonealy as mentioned before. The constituent of Gg may sustain the embryonic development at early cleavage stages when the embryos depend at this stage on the pyruvate as a source of energy which is found at the Gg medium (9).

It is concluded from the present work that addition of Gg to the culture medium can improve certain sperm function parameters,the FR and ED of mice following IPI. This results can be utilized in AI of infertile couples after further researches for human culture medium and assisted reproduction.

References

1-Allamaneni SS, Bandaranayake I and Agrawal A. Use of semen quality scores to predict pregnancy rates in couples undergoing intrauterine insemination with donor sperm. Fertil.Steril. 2004;82:606-611.

2-Ng EH, Makkar G,Yeun GW, Ho PA. Randomized comparison of three insemination methods in an artificial insemination program using husbands semen .J . Reprod. Med.2003;48:542- 546.

3-Bernstein G. The socio-legal acceptance of new technologies: a close looks at artificial insemination. Washington Law Review . 2002;77:1035-1120.

4- Tesarik J and Mendoza C. Gamete for assisted reproduction: past, present and future .J.Androl .2003;24:321-328.

5-Sherman JK. History of artificial insemination and the development of frozen semen banks .In: Rinfret, A and Petricciani,J.(eds).The integrity of frozen spermatozoa.N.Acad.Science.1978; p:201-211.

6- Hafez E S. Physico-anatomical parameters of andrology .In Techniques of Human Andrology. E. S. E. Hafez (eds), Elsevier Biochemical press North-Holland. 1977;p: 39-79.

7-Johnston L. Herbal Medicine for Physical Disability. Available from:http://www. Healing therapies .info/herbal.html. 2005.

8-Taylor L. Database File for Licorice. Raintree Nutrition Inc. Available from :http://www .rain - tree.com/licorice.html. 2004.

9- Al-Dujaily SS, Al-Janabi A and Nori M. Effect of Glycyrrhiza glabra extract on in vitro sperm activation of asthenospermic patients.J. Babylon Uni. 2006;11(3):477-483.

10-.Al-Mula I. Effect of licorice extract on ovulation induction in immature female mice . Thes is of High Diploma ,IVF Institute ,Baghdad university. 2006

11-Al-Dujaily SS and Alsaadi AS .The Effect of Glycyrrhiza glabra on the IVF in Mice. J.Biotech. Research Center. 2009; 3(2):81-88 .

12-Nagy A, Gertsenstein M, Vintersten K and Behringer R. Manipulating the Mouse Embryo, A Laboratory Manual. 3rd Edition. Cold Springer Harbor Press. New York. 2003; p: 31-61.

13- Khalili M. and Anvari M. The effect of in vitro culture on cleavage rates and morphology of the in vivo developed embryos in mice.Iranian. J.Reprod. Med. 2007;5:17 -22.

14-Sorlie DE .Medical biostatistics: Examination and board review. Norwalk. Connecticut. Appleton and Lang. 1995;p: 47-88.

15-Tamir S, Eizenberg M, Songen DM, Shelach R, Kaye A. and Vaya J. Estrogenic and Anti- proliferative Properties of Glabridin from Licorice in Human Breast Cancer cells. Cancer research. 2000; 15(60): 5704.

16-AL-Jarah IAN. Study of the some exogenous hormones on sperm in vitro activation of asthenozoospermic patients. MS.c Thesis. College Science. University of Babylon. 2002.

17-Nassar A, Mahony M ,Blackmore P, Morshedi M and Ozgur K. Increase of intra cellular calcium is not cause of pentoxifylline - induced hyperactivation or acrosome reaction in human sperm . Fertil. Steril. 1998; 69:745-749.

18- Al-Dujaily SS and Albarazanchi, M. In vitro epididymal sperm activation and intra- peritoneal insemination experimental model for vasal obstructive in mice. 4th Asian Symposium on Animal Biotechnology, (ASAB), Kamiina-Japan Oct.7-9, 1998; pp110-113.

19-Dumoulin J, Evers J, Bras M., Pieters M and Geraedts J. Positive effect of taurine on preimplantation development of mouse embryos in vitro. J. Reprod.Fertil. 1992;,94:373-380.

20-Al-Dujaily SS and Albarazanchi M. In vitro epididymal sperms activation and intra-bursal insemination in mice: model for human vasal obstruction. The 3rd Asian Symposium on Animal Biotechnology(ASAB),Seoul-Korea,Dec. 1997;11-14.

21-Downs S, Daniel S ,and Eppig J. Induction of maturation in cumulus cell -enclosed mouse oocytes by follicle stimulating hormone and epidermal growth factor : evidence for a positive stimulus of somatic cell origin .J. Exp.Zoo. 1988;245:68-96.

22-Haraguchi H, Yoshida N, Ishikawa H, Tamura Y, Mizutani K. and Kinoshita T. Protection of mitochondrial functions against oxidative stresses by isoflavones from *Glycyrrhiza glabra*. J. Pharmacy and Pharmacology. 2000; 52: 219-223.

23-Van Zyl J, Kotze T and Menkveld R. Predictive value of spermatozoa morphology in natural fertilization In :Acosta ,A,..et al (eds), Human spermatozoa in assisted reproduction. Williams &Wilkins,Baltimore.1990;Pp:319-324.

24-Coetzee K ,Kruger T, Lombard C. Predictive value of normal sperm morphology: a structured literature review .Hum. Reprod. 1988;4: 73-84.

25-Ganong WF. The gonads: Development and function of the reproductive systems. In: Review of Medical Physiology. W.F. Ganong, 22th edition .Lange, California ,2005; p: 437.

26-McKiernan S and Bavister B. Environmental variables influencing in vitro development of hamster 2-cell embryos to the blastocyst stage. Biol. Reprod.. 1990;43: 404.

27-Fukui Y and Sakuma Y. Maturation of bovine oocytes cultured in vitro :relation to ovarian activity and the presence or absence of cumulus cells. Theriogenology. 1980;22:669-675.