Embryonic development following the insemination with epididymal sperms of vasectomized mice activated in vitro using Glycyrrhiza glabra (Licorice) extract : model for obstructive azoospermia

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Abstract:

Background

Many studies found that the Glycyrrhiza glabra (G.glabra) showed a positive effect on the activation of sperm in vitro specifically improve the activity of progressive movement. However, it was not known the impact of this stimulant on the certain sperms parameters in vasal obstructive mammals.

Objectives

This experiment was designed to examine the possibility of fertilization ova by epididymal sperm of vasectomized mice activated by G. glabra and study the early embryonic development, as a model for obstructive azoospermia

Methods

Glycyrrhiza glabra aqueous extract (0.3 mg/ml culture medium) was used for in vitro direct sperm activation technique . The superovulated female mice (SUO) were divided into two groups , the first group; the insemination of flushed ova was performed with epididymal sperm of vasectomized male mice activated by adding30% of G. glabra to the culture medium (treated group) .While the second group ,the ova were inseminated by epididymal sperm of vasectomized male mice activated by G. glabra - free medium (control).

Results

This study showed a highly significant (P <0.01) improvement in certain sperm function parameters i.e the sperm forward movement , morphologically normal sperms (MAS) . There was a significant (P<0.05) increase in fertilization rate (FR) of SUO mice oocyte using 30 % G. glabra medium(75.84%) compared to G. glabra-Free medium (Hams -F12 medium) alone (54.43%).By adding 30% G. glabra to the medium , the number of 3-cell and 4-cell embryonic stage of SUO mice was significantly (P <0.05) higher than that of grouping G. glabra- Free medium mice embryo(62.10 % and 65.78% vs. 58.88 and 66.29% , respectively).

Conclusion

This study showed a highly significant (P <0.01) improvement in certain sperm function parameters i.e the sperm forward movement , morphologically normal sperms (MAS) . There was a significant (P<0.05) increase in fertilization rate (FR) of SUO mice oocyte using 30 % G. glabra medium(75.84%) compared to G. glabra-Free medium (Hams -F12 medium) alone (54.43%).By adding 30% G. glabra to the medium , the number of 3-cell and 4-cell embryonic stage of SUO mice was significantly (P <0.05) higher than that of grouping G. glabra- Free medium mice embryo(62.10 % and 65.78% vs. 58.88 and 66.29% , respectively).

Key word:Embryo,Licorice,Epididymal sperm,azoospermia

Introduction

Infertility is a relatively common problem that affects couples worldwide. It is estimated that approximately 1 in 6 couples will experience difficulties in reproduction(1). Infertility is either male and female or both factors. One of male factor infertility is azoospermia is defined by the complete absence of sperm cells in the ejaculate. It affects approximately 1% of the male population and can be easily detected and diagnosed by using simple microscopy techniques following centrifugation(2).

Obstructive azoospermia is a condition where there is a mechanical obstruction in the male fertility track. Such obstructions are usually located between the epididymis and the ejaculatory duct, and they block semen from being expelled into the vaginal tract. Causes of obstructive azoospermia include congenital disease, infection, and vasectomy(3).

Assisted reproductive technologies(ART) have become more accessible to the general population, enabling infertility couples to hope that they can materialize their aspirations for healthy offspring later in life. It is well established that the main limiting factor to fertility and good reproductive outcome has been female's age(4,5).

On July 25, 1978, Louise Brown was the first human to be successfully conceived outside the body or, as it been more commonly described, she became the first successes in vitro fertilization (IVF)(6) . This medical procedure came about as a result of this tireless efforts of a British team led by Robert Edwards and Patrick Steptoe (7).

In vitro fertilization is being used increasingly as primary treatment for infertility. This is largely the result of the commercialization of the assisted reproductive technology (ART) and its services. The industry is well capitalized and is lucrative. Up till now it is important to mention that even if couples were to undergo three successive cycles of IVF, nearly 50% would fail to obtain a baby (8). On the other hand , G. glabra (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 (9). The aim of the present study is to examine the possibility to fertilize ova using epididymal sperm of vasectomized mice, as a model for obstructive azoospermia through: 1-prepare epididymal sperms obtained from vasectomized male mice . 2- Conventional IVF technique and embryonic development of early cleavage stages.

Materials and Methods

This study was conducted in the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies /AL-Nahrain University. Seventy mature mice (40 females and 30 males) mice of albino-Swiss between 8-12 weeks old and 25-35 gm were obtained from colony of the animal house at the institute . Each cage contains four animals and Tap water and diet were freely available for the animals. cages were cleaned and sterilized with 70% ethyl alcohol once a week regularly.

Preparation of Glycyrrhiza glabra Extract for in vitro sperm activation:

The concentration of Glycyrrhiza glabra working solution was prepared by adding 10 mg from Glycyrrhiza glabra extract to 10 ml PBS (0.1%) in plastic test tubes contained broad spectrum antibiotic (Ampicillin 0.004g) to prevent bacterial growth (10). The media used for activation was prepared by adding 0.3 ml of Glycyrrhiza glabra suspension (30%) to 0.7 of Ham's F-12 Media . The solution was filtered using Millipore filter (0.45 µM) and (0.25µM) , and then PH was adjusted to reach 7.2-7.4 .

Male vasectomy:

A white (albino-Swiss) healthy fertile male mouse was weighed and anesthetic using thiopentol sodium (0.075- 0.125) mg. The anesthetized male mouse was placed on its back; The mouse scrotum was shaved and cleaned with 70% alcohol. The mature fertilized male mice were vasectomized as described by Foley(11).

Detection of female estrus 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 (12).

Superovulation program :

Superovulation (SUO) program starts by intraperitoneal injection of female mice with 7.5 I.U. of PMSG (pregnant mare serum gonadotropin, Folligon®, Holland) followed by 7.5 I.U.of HCG (Pregnyl®, Holland) after 48hours. Oocytes were recovered 13hours post HCG(12).

***In vitro* sperm activation technique:** vasectomized male mice were sacrificed approximately 13 hours after females receiving HCG. The caudal epididymal was isolated and placed on a transfer dish with 1 ml of phosphate buffer solution (PBS) for washing. The caudal epididymal was isolated and placed on a transfer dish with 1 ml of Ham's F-12 medium only or with G. glabra extract Making a snip in the cauda , the spermzoa were flushed out, and then dispose the cauda epididymis collected sperm were incubated for at least 30 minutes. Because it is purely sperm (no seminal fluid), they become capacitated by being incubated in the culture medium. Then the sperms were assessed (13).

***In vitro* fertilization:** After 2-3 hours of oocytes incubation , an aliquot of capacitating sperms were gently added to each well of 4- well dish. Each well contains 4 oocytes flooded with 0.7 ml Ham's F-12 medium .Two out of four IVF wells filled with licorice extract- Ham's F-12. The other two wells loaded with Ham's F-12 medium alone. All wells covered with 0.2

ml paraffin oil. Insemination of mature oocytes was done by adding 1-2×105/ml of the incubated sperm to the IVF well containing 4 oocytes. Fertilization dishes were incubated at 37oC, 5% CO2 and 95% humidity overnight.

Evaluation and grading system of embryos : Early morphological evaluation was assessed on warmed microscopic stage of an inverted microscope . Early embryos with 2 to 8 blastomeres were divided in to 4 grades was done according to the criteria of Khalil and Anvari (14).

Statistical Analysis:
All statistical analysis was performed using version 16 .0 Minitab statistical program (15). Data were expressed as mean ±SEM were analyzed by using paired sample t-test and Chi- square test were used depending on the nature of data . P-value<0.05 was considered significant in this study.

Results :

In vitro sperm activation :
The mean sperm concentration following direct activation technique with 30% G.glabra extract - Hams F-12 medium (41.6±3.26) was highly significant (P<0.001) increased compared to G.glabra extract -free Hams F-12 medium (22.2±1.06) as shown in table -1. The percentage of progressive sperm motility (grades A and B) was significantly (P<0.001) improved using in vitro G.glabra extract more than that of epididymal sperms activated without adding licorice extract .Significant (P<0.001) enhancement was recorded to G.glabra extract- Hams F-12 medium (64.13±2.00) in the percentage o f MNS, when compared to G.glabra extract - free Hams F-12 medium following the activation in vitro (33.0±1.68).

Table (1): Effect of G.glabra (licorice) extract on certain sperm function parameters following *in vitro* direct activation technique

Certain sperm function parameters		After 30 min .incubation		
		Control	Treated	P value
Sperm Concentration (million/ ml)		22.2±1.06	41.6±3.26	· 0.001
Sperm motility	Grade A(%)	7.33±0.69	25.50±2.46	· 0.001
	Grade B(%)	35.87±2.64	44.17±2.61	0.011
	Grades A+B (%)	43.20±2.58	69.70±2.84	· 0.001
Morphologically Normal sperms (%)		33.00±1.68	64.13±2.00	· 0.001
Values are expressed as Mean± SE (significant difference using paired t- test)				

Fertilization Rate (FR):
Fertilization rate was obtained by dividing the number of fertilization ova after 24 hours of insemination on the number of collected oocytes.
The FR in treated group was 75.96% (380 out of 502 oocytes) , while the FR in control group was 54.4 % (272 out of 500 oocytes). There was highly significant (P.0.001) difference between the treated group and control group ,as shown in Figure (1).

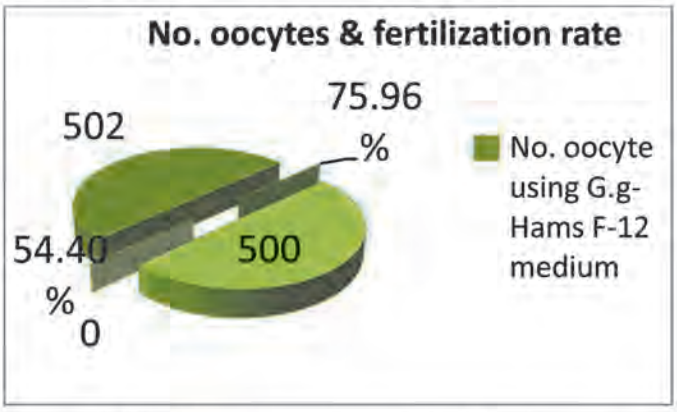

Figure .1. Number of oocytes and fertilization rate.

Embryonic Development:
3.1. Embryonic Development after 24 hours: After 24 hours of insemination and incubation, using medium contains G. glabra, there were 228 embryos at the 2- cell stage out of 380 embryos developed, and the rest 152 embryos were at the three-four cell developmental cleavage stage while using G.glabra extract free medium, the number of embryo developed were 138 at 2- cell stage out of 272 embryos and the rest 134 embryos were at the three - four cell stage. Therefore a significant (P<0.001) improvement in the total number of embryos was assessed in the treated group compared to the control group. Also a significant (P<0.001)difference in the total number of two cells and significant difference in the total number of three-four cells stage of embryo was observed in treated group compared to the control group as shown in table (2).

Table 2: Comparison of embryo grading score between treated and control media after 24 hours of *in vitro* fertilization .

Embryos stages	Medium used	No. Developed embryos	Embryonic grading score				P value
			GradeA	GradeB	GradeC	GradeD	
2Cell stage	With*G.glabra* medium	228	98 42.98%	76 33.33%	30 13.15%	24 10.54%	· 0.001
	Without *G.glabra* medium	138	30 21.73%	42 30.42%	30 21.73%	36 26.52%	
3-4 Cell stage	With*G.glabra* medium	152	72 47.36%	40 26.31%	25 16.44%	15 9.86%	· 0.001
	Without *G.glabra* medium	134	27 20.14%	44 32.83%	25 18.44%	38 28.45%	
Chi-square test at 0.05 level of significance.							

3.2. Embryonic Development after 48 hours: After 48 hours of insemination using in vitro fertilization technique, there was 380 embryos developed within a medium contains G. glabra, which was divided into 30 embryos at 2- cell stage, 250 embryos at three to four cells stage and 100 embryos at five to eight cells stage . On the other hand, the developed embryonic using Hams- F12 medium only at the same period of insemination and incubation, there was 272

embryos divided into 60 embryos at two cell stage, 160 embryos at three to four cells stage and 52embryos at five to eight cells stage. The statistical analysis showed significant (p?0.05), as shown in the table (3).

Table 3: Comparison of embryo grading score between treated and control media after 48 hours of in vitro fertilization

Embryos stages	Medium use	No. developed embryos	Embryonic grading score				P value
			Grade A	Grade B	Grade C	Grade D	
2Cell stages	With *G.glabra* medium	30	8 26.66%	12 40%	6 20%	4 13.33%	0.45
	Without *G.glabra* medium	60	10 16.66%	13 21.66%	17 28.33%	20 33.33%	
3-4Cell stages	With *G.glabra* medium	250	88 35.2%	102 40.8%	40 16%	20 8%	· 0.001
	With out *G.glabra* medium	160	40 25%	53 33.12%	30 18.75%	37 23.12%	
5-8Cell stages	With *G.glabra* medium	100	35 35%	42 42%	12 12%	10 10%	· 0.001
	Without *G.glabra* medium	52	15 28.8%	18 34.61%	7 13.64%	12 23.07%	
Chi-square test at 0.05 level of significance.							

.Discussion

In this study, there was an enhancement in certain function parameters of epididymal sperms obtained from vasectomized male mice following in vitro activation by adding G.glabra to the culture medium used, and that was attributed to the direct activation with G.glabra added to Hams-F12 medium .Thus, the culture medium enhances different sperm function parameters following 30 minutes of incubation. Moreover, culturing of epididymal sperms with 30% licorice extract - Hams-F12 medium for 30 minute before insemination result in a significant increase in the percentages of sperm motility and grade activity of forward movement (grade A and grade B) of epididymal sperm sample. This results are similar to the results reported by other studies (16-21) .

The G. glabra has estrogenic activity(22). Estrogens are known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyperactive motility(23). Furthermore, it has been found that the G.glabra contains Ca+2, potassium, glucose, fructose, vitamin E, vitamin C and many other substances e.g.: Zn+2, sucrose, amino acid(9). All these substances can stimulate sperm motility and the grade activity of forward movement. The sugars are considered to be a source of energy for sperm motility(9). Fructose is one of the principle energy substrate for spermatozoa and an activator factor of mammal spermatozoa(24) , the protein and amino acids, which sustain and maintain sperm osmolarity and in turn integrity of sperm cell membrane(25). Moreover, vitamins E and C are major chain - breaking antioxidants in sperm membranes and appears to have a dose dependent protective effect (26).

2. Fertilization rate:

In this study, there was a significant increase in FR after 24 hours of insemination by epididymal sperms obtained from vasectomized male mice .This result might be attributed to the improvement of certain sperm function parameters when incubated in a medium containing G.glabra .

The present study found a significant increase in the total active sperm concentration after direct activation with 10% G.glabra - HTF medium. Sperm concentration had the largest reliability coefficient for conception, followed by motility and morphology, and that sperm concentration has influence on fertilization if patients were treated with IVF (27). The present study found increment in FR when the percentage of total sperm motility and grade activity of progressive forward movement improved. This finding is in agreement with other studies (16,17,28).

Sperm morphology is the other important factor .This factor was known to be the best predictive factor in natural fertilization, intrauterine insemination and conventional in vitro fertilization. There was a positive significant relationship between the percentage of FR and the percentage of morphologically normal spermatozoa find in this work (29)

The sperm morphology parameter is very important to determine fertilization success because morphologically abnormal sperm appears less effective to reach ovum at the infundibulum and/or penetrate an egg to achieve fertilization (30). The main factor that may interfere with the increases in FR was the addition of G .glabra powder extract to the insemination medium. The G. glabra 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 Fe + 3 , induced mitochondrial lipid peroxidation, which may decrease the effect of ROS production by vasectomy on epididymal sperms(31).

In mammalian oocytes, a series of spikes in [Ca2+] or so called Ca2+ oscillations, are triggered upon sperm entry. These Ca2+ oscillations are needed to relieve MII arrest and to provoke all the other events of oocyte activation, such as the cortical reaction, maternal mRNA recruitment, pronuclear development and mitotic cleavage (32).

3. Embryonic development :

The present study showed a significant improvement in ED and in embryo quality after 24 and 48 hours of insemination and incubation with licorice extract medium .There was an increase in the number of grades A and B of 2-cell stage , three-four cell stage and five- eight cell stage embryo . This improvement in ED and embryo quality can be attributed to the addition of licorice extract to the medium as compared to the control medium. The positive effects of licorice extract in this study may be augmented by a number of active ingredients like: Ascorbic-acid, Asparagine, Carotenes, Fructose, Glucose, Magnesium, Maltose, Manganese, Sucrose, fructose, Thiamin, Vitamin E etc., each of which have different effects on oocytes and early developed

embryos (9).
It may be also explain the developing embryo is exposed to gradients of carbohydrates, together with amino acids. It has been noticed that carbohydrates are the main energy substrates for the embryo(33). However, any modifications to the in vitro culture environment can have profound effects on the quality of the resulting embryos measured (34).Most of culture media contain carbohydrates, pyruvate, lactate, and glucose. The addition of 30% licorice extract to Hams-F12 medium enhanced embryonic developmental potential and so embryonic quality. Licorice -Amino acids exert beneficial effects on the development of the preimplantation embryos as they are known to have multiple functions including : precursors for biosynthesis , energy sources, regulators of energy metabolism , osmolytes , buffers of intracellular pH, and chelators of heavy metals(35).
The G.glabra 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 H2O(36). Thus, treatment with G.glabra extract increased the total antioxidant capacity in culture media of oocytes that later were successfully fertilized.It is concluded that G.glabra extract have supporting effects on epididymal sperms leading to increase FR and embryonic development.

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