Effect of Citrullus colocynthis aqueos extract on *in vitro* fertilization and early cleavage stages of mice embryos: a model for mammals

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Abstract:

Background:

Citrullus colocynthis(CC) is an herbal medicine used in the treatment of a wide range of diseases. Recently, the effect of this plant on the reproductive system has been studied .However, its role *in vitro* still unclear.

Objective:

The present study was designed to investigate the possibility of using CC extract for *in vitro* sperm direct activation technique, *in vitro* fertilization(IVF) and early embryonic development using the mice as a model for mammals.

Materials and Methods:

Citrullus colocynthis extract (0.05mg/ml culture medium) was used for *in vitro* direct sperm activation technique. The same medium was used for oocytes insemination and for culturing the embryos after 2448-hours of insemination. The oocytes were collected from superovulated female mice and divided into two groups: first group, 343 oocytes were inseminated and cultured in CC- free Ham's F-12 medium (the control group).The second group, 345 oocytes were inseminated and cultured in 10% CC - Ham's F-12 medium (treated group). Each 4 oocytes were inseminated with the same sperm concentration (1 -2 $\times 10^5$sperm/IVF well).The fertilization rate was recorded after 24 hours of insemination ,while embryonic development rate was recorded after 24 and 48 hours of insemination.

Results:

In vitro activation of epididymal sperms with 10%CC has shown positive effect on sperm concentration, sperm motility, and grade activity of progressive forward movement. There was a highly significant ($P<0.004$) increase in FR of the treated group (61.5%) compared to control group (50.4%) after 24 hours of insemination. Embryonic developmental rate was significantly increased after 24 and 48 hours of insemination in treated group compared to control group .

Conclusion:

It is concluded that adding the 10%CC to the culture medium of the epididymal sperm and *in vitro* inseminated lead to improvements in certain sperm function parameters and sustain the FR and early embryonic development rate.

Key words: Citrullus colocynthis, *in vitro* activation, Fertilization rate ,Embryonic development

Introduction

Medical plants have played a key role in public health and the herbal drugs have been used since ancient times as medicines for the treatment of a wide range of diseases[1]. Herbal remedies are widely used for the treatment and prevention of various diseases and often contain highly active pharmacological compounds[2,3] .

Citrullus colocynthis is known to exhibit many pharmacological actions, including antioxidant, anti-inflammatory and analgesic activities[4,5]. However, very limited information was mentioned in the literatures about the effect of CC on the fertilization rate of *in vitro* fertilization (IVF) and early embryonic development following IVF in human and animals. Therefore, the aim of the present study is to find out the effects of CC extract on IVF in mice as an experimental model for mammals.The study will search on: 1- *In vitro* fertilization rate. 2- *In vitro* embryonic development rate.

Materials and Methods

This study was conducted in the Institute of Infertility Diagnosis and Assisted Reproductive Technologies, AL-Nahrain University. One hundred mature mice (60 females and 40 males) 812- week old and 25-35gm weight, were obtained from the colony of the animal house of the institute included in this investigation. Each cage contains four animals and its floor was covered with wooden shave. Tap water and diet were freely available for the animals. The animals, cages were regularly cleaned and sterilized with 70% ethanol once a week.

Preparation of *Citrullus colocynthis* Extract: Fifty grams of CC powder was added to 250 ml of distilled water and refluxed for 3 hours as described by Hasborne [6].

Stock solution of CC extract was prepared by adding 5 mg from the total yield of CC powder extract (3.77) gm. to 10 ml of mouse IVF media (Ham's F-12 Media - PBS Media). This solutions (0.05%) was filtered by using Millipore filters with pore size 0.45µm and 0.22µm.The pH was adjusted to 7.2 -7.4 [7].

Superovulation induction: Superovulation program starts by intraperitoneal injection of female mice with 7.5 I.U. of PMSG (pregnant mare serum gonado- tropin ,Folligon®,Holland)followed by 7.5 I.U. of hCG(Pregnyl®,Serono,Italy) after 48 hours. Oocytes were recovered 13 hours post-hCG.

***in Vitro* Sperm Activation Technique:1-** Male mice were Sacrifised approximately 13 hours after females receiving hCG. The caudal epididymus was isolated and placed on a transfer dish with 1ml of phosphate buffer solution (PBS).*In vitro* activation techniques was done as described by Al-Dujaily(7)Then the sperms were counted (8).

In vitro fertilization process

After 23- hours of oocytes incubation, an aliquot of capacitating sperms were gently added to each well of 4-well dish.Each well contain 4 oocytes flooded with 0.7 ml medium. Two out of four IVF wells filled with 10%CC-Ham's F-12 medium. 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× 10^5 of the incubated sperm to the IVF well containing 4 oocytes. Fertilization dishes were incubated at 37°C, 5% CO_2 and 96% humidity overnight.

Evaluation and grading system of embryos:

Early embryo morphological evaluation was assessed on warmed microscopic stage of an inverted microscope. Early embryos with 2 to 8 blastomeres were evaluated morphologically into 4 grades according to Hartshorne[9].

Statistical Analysis: Data were expressed as mean ±SEM .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[10]

Results

1- *In vitro* sperm activation :

The results of *in vitro* direct activation techniques on 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 30 minutes with and without CC are shown in Table 1.

The mean sperm concentration following direct activation technique with 10% CC –PBS medium (54.4 ±8.06) was highly significant (P<0.004) compared to CC –free PBS medium (30.8± 7.11). The percentage of progressive motility (grade A and grade B) was significantly (p<0.003) improved by activated *in vitro* with adding 10% CC more than that of epididymal sperms activated without adding CC. A significant (P<0.05) changes were recorded in morphologically normal sperm by 10% CC- PBS medium (69.6± 4.26), compared with CC- free PBS medium following the activation *in vitro* (68.0 ±4.42).

2-Fertilization Rate:

Fertilization rate was obtained by dividing the number of preimplantation embryos after 24 hours of insemination on the number of collected oocytes.(Table 2)

The FR in treated group was 61.5% (212 embryos out of 345 oocytes) ,while the FR in control group was 50.4% (173 embryos out of 343 oocytes).There was a highly significant (P<0.004) difference between the control and the treated group.

Table 1: Effects of CC on certain sperm function parameters following *in vitro* direct activation technique.

Certain sperm Function parameter	The mediem	After 30 minutes incubation Mean±SE	P value
Sperm concentration (million/ml)	CC-free medium	30.8± 7.11	0.004*
	With CC medium	54.4 ±8.06	
Sperm motility Grade A (%)	CC-free medium	4.1± 1.51	0.003*
	With CC medium	7.7± 1.42	
Sperm motility Grade B (%)	CC-free medium	14.4± 3.93	0.005*
	With CC medium	27.8± 3.35	
Progressive motility (A+B)%	CC-free medium	18.5± 5.12	0.003*
	With CC medium	35.5± 4.56	
Morphologically Normal sperms (%)	CC-free medium	68.0± 4.42	0.029*
	With CC medium	69.6± 4.26	

*Significant difference in rates using Paired-t-test at

Table 2: Effect of CC on *In vitro* Fertilization rate after 24 hours of insemination.

Parameters	CC-free medium group (Control)	CC medium group (Treated)
Collected oocytes	343	345
Fertilized oocytes after 24 hours	173	212
Fertilization rate	50.4%	61.5%
P value	0.004*	

*Significant difference in rates using Pearson Chi-square test

3-Embryonic Development:

3.1. Embryonic Development after 24 hours:

After 24 hours of insemination and incubation ,the treated group showed 119 embryos at the two cells stage out of 212 developed embryo, and the rest 93 embryos at the three-four cell developmental cleavage stage. Whereas, the control group showed 89 embryos at two cells stage out of 173 embryo and the remaining 84 embryos were at the three to four cells stage,(Table 3).

There was a significant (P<0.001) improvement in the total number of embryos in treated group compared to the control group. There was no significant (P>0.05)difference in the total number of two cells, three-four cells stage of embryo in treated group compared to control group as shown in Table 3.

Table 3:Effect of CC on embryo grading score after 24 hours of insemination by *in vitro* fertilization process.

Embryos		No	Grade A		Grade B		Grade C		Grade D		p value
2-Cells stage	CC-free medium	89	18	20.2	21	3.6	23	25.9	27	30.3	0.0001*
	With CC medium	119	41	34.5	48	40.3	17	14.3	13	10.9	
3 4 - -C e l l s stage	CC-free medium	84	11	13.1	16	19.1	25	29.7	32	38.1	0.0001*
	With CC medium	93	39	41.9	34	36.6	14	15.1	6	6.4	
	P value without CC					0.435					
	P value with CC					0.541					

*Significant difference in rates using Pearson Chi-square test

3.2. Embryonic Development after 48 hours:

After 48 hours post insemination with *in vitro* fertilization program, the treated group showed 34 embryos at two cells stage, 115 embryos at three - four cells stage and 63 embryos at five - eight cells stage out of 212 developed embryos . On the other hand , the control group showed 38 embryos at two cells stage ,94 embryos at three - four cells stage and 41embryos at five - eight cells stage out of 173 developed embryos. There were no significant (P>0.05) differences at the total number of two cells stage, three-four cells and five –eight cells stage of embryos after 48 hour as shown in Table 4.

Table 4: Comparison in embryo grading score between treated medium with 10%CC- Ham's F-12 medium and CC-free ham's F-12 medium after 48 hours of insemination by *in vitro* fertilization process.

Embryos		No	Grade A		Grade B		Grade C		Grade D		p value
2-Cells stage	CC-free medium	38	7	18.4	10	26.3	13	24.2	8	30.3	0.179
	With CC medium	34	10	29.5	14	41.2	6	17.5	4	10.9	
3- 4-Cells stage	CC-free medium	94	5	5.3	22	23.4	32	34.1	35	38.1	0.0001*
	With CC medium	115	31	26.9	36	31.3	18	15.7	30	26.1	
5- 8-Cells stage	CC-free medium	41	4	9.8	9	22.0	11	26.8	17	41.1	0.199
	With CC medium	63	16	25.4	15	23.8	14	22.2	18	28.6	
	P value CC -free medium						0.206				
	P value withCC medium						0.411				
*Significant difference in rates using Pearson Chi-square test											

Discussion

1- *in vitro* ***sperm direct activation***: The present study showed an enhancement effect in certain sperm function parameters following 30 minutes incubation compared to before activation status. This effect may firstly result from the direct activation technique with PBS. The activation technique may prove positive result with regard to sperm motility and grade activity than of simple layer and centrifugation techniques[11]. It also sustain the epididymal sperms to get ride from the decapacitating factors in the seminal plasma and make the sperm ready for successful fertilization *in vitro*[12]. On the other hand, the PBS medium provides the necessary salts which required for activation of intact sperms leading to swim up more number of sperm. Secondly , adding of CC. to the PBS medium enhances different sperm function parameters following 30 minutes of activation, mainly sperm concentration, total sperm motility percentage and grade activity of forward progressive movement as a result of CC components e.g. carbohydrates, amino acids and vitamins. However, the difference in sperm concentration between the treated medium and the control medium may be attributed to direct activation technique with PBS medium, when only the sperm with active motility swim up to the upper layer of the medium [11]. In addition, components of CC powder may explain the booster effect on epididymal tissue that allow the sperm to move out and released. Moreover, incubation of epididymal semen sample with 10% CC-PBS medium for 30 minutes before insemination results in a significant increase in the percentage of sperm motility and grade activity of forward movement (grade A and grade B) of semen sample. This finding may emphasize to CC effect.

Citrullus colocynthis is one of the plants which has an estrogenic activity due to the presence of flavonoids which is known to be a phytoestrogenic and has the ability to bind to human estrogen receptor[13]. Estrogen is known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyper active motility[14].

2-**Fertilization rate**: In this study, there was a significant increase in the FR after 24 hours of insemination.This result might be attributed to many causes which participate with this observation; firstly is the sperm concentration, which has been recorded to have the largest reliability coefficient for conception, followed by motility and morphology and has an influence on fertilization if patient treated with IVF[12].

The other factor is the sperm motility which have a great role during the fertilization process and take part in determining the rate of assisted reproduction treatment .This study present an increase in FR correlated with an increasing sperm motility and grade activity of forward movement.

This finding is in agreement with Battin ,*et al.*[15] who recognized that the sperm motility after swim-up method was associated with the rate of fertilization. Sperm morphology is the other important factor ,which was known to be the best predictive factor in natural fertilization, intrauterine insemination and ordinary *in vitro* fertilization. There was apositive significant relationship between the percentage of fertilization rate and the percentage of morphologically normal spermatozoa [16].

The CC extract contain a large amount of active compound that provide a nourishment and protection to the oocytes and early cleaved embryos. The CC contain enzymatic antioxidants such as catalase, super oxide dismutase, glutathaione reductase and glutathaione-S-transferase and non enzymatic antioxidant (ascorbic acid, α-tocoperol, reduced glutathaione, total carotenoids and flavonoids. Flavonoids and phenolic compounds are widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities[17]. All these components showed antioxidant capacity and have a great role in scavenging process to the ROS particles[18].

The fruits of CC. also contain certain minerals like: Ca++, Mg, Mn, K, P, Fe and Zn[19]. Calcium is a universal secondary messenger in cells controlling diverse biological processes. It plays a major role at fertilization and is thus already involved at the very beginning of life. Sperm not only delivers its genetic material but also triggers rises in intracellular

calcium concentration and consequently awakens the oocyte which is blocked at the metaphase of the second meiotic division (MII arrest). For all species an increase in $Ca2^{++}$ is necessary and sufficient for the completion of oocyte activation and initiation of embryonic development[20].

3-Embryonic development: The present study demonstrated a significant improvement in ED and embryo quality after 24 and 48 hours of insemination and incubation with CC –Ham's F-12 medium. At the same time, there was an increase in the number of grade A and grade B of 2-Cell stage, three-four cell stage embryos. This improvement in ED and E quality can be attributed to the addition CC within the medium, since nearly all variables during the insemination and culture procedure were fixed and controlled. The enhancement effect of the CC that exhibited in this study may be attributed to the positive influence of CC active compounds like: protein and amino acid, carbohydrate, vitamins and minerals all these ingredients have different effect on sperms, oocytes and early embryonic development[21].

Colocynthis –Amino acid demonstrated a wide range of beneficial effect on the preimplantation development. Exogenously adding amino acids can improve the development of mammalian *in vitro* produced embryos to the blastocyst stage, increase the total cell numbers[22], and improve embryo quality and through decreasing the lipid droplet size, protecting the cellular inner structure, and maintaining metabolism[23].

Most culture media contain carbohydrate, lactate, pyruvate, and glucose. The addition of 10% CC extract to the Ham's F-12 medium aid to sustain the embryonic development potential and embryo grading score compared with the control group. This result may be attributed to the quality of carbohydrate in the CC extract which is considered a great energy source for the embryonic growth[24].

Furthermore, CC aqueous extract contain certain minerals such as: Calcium, Magnesium and Potassium[19].These minerals participate in the first cleavage and development of early embryos. Calcium is essential for embryos to undergo compaction *in vitro*[25].While high potassium level in CC- culture media may have a beneficial effect on sperm capacitation and *in vitro* embryo development [26].

Moreover, the vitamins are the other substances found in CC powder extract that may interfere with the enhancement of ED in this part of study. Vitamins are the key components of cellular metabolism, have been shown to have significant effects on embryo quality during the culture of rabbit, mouse, and hamster embryos[27].

The extract of CC contains a wide range of antioxidant compound that have a great role during the scavenging process ,such as amino acid and metal ion which have demonstrated that the adding of metal ion agents to the culture media may decrease the production of oxidants and help in successful embryo development and pregnancy[28]. In addition to these active antioxidant ingredients, CC contains enzymatic antioxidant like (catalase, super oxide dismutase, glutathaione reductase and glutathaione-S-transferase) and non-enzymatic antioxidant like (ascorbic acid, α-tocoperol, reduced glutathaione, total carotenoids and flavonoids)[18] .It is concluded that CC extract can be added in the medium used for IVF programs more studies are req prove its cytogentic safety on the sperms, ova and embryos .

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