Correlation between in vitro sperm preparation techniques, endometrial thickness, hormonal profile and successful pregnancy rate following IUI: retrospective and prospective study

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Abstract:

Background:
The intra uterine insemination (IUI) still an easy, simple and effective method to overcome the infertility that resulted from different causes. Although the equipments and methods of assisted reproductive technologies (ART) were highly developed, the percentage of successful IUI live-birth not exceed 20- 30%.

Objective:
The present study was aimed to elucidated the cut off values of *in vitro* sperm preparation technique, follicular number and size, endometrial thickness and hormonal profile that lead to successful pregnancy rate (PR)following IUI.

Materials and Methods:
In retrospective study, one hundred cases of infertile couples were in who were became pregnant following IUI at the Institute of Infertility Diagnosis and Assisted Reproductive Technologies, through the period from January 2007 to January 2010. Depending on the results of retrospective study, IUI was achieved for 100 infertile patients between August 2010and June 2011. The mean of significant prognostic variables were measured in both studies namely: age, type of infertility, type of sperm activation techniques, the protocol of ovulation induction medicine, the mean of hormonal status of Luteinizing Hormone (LH), Estradiol(E_2), and number with diameter of follicles, endometrial thickness a cycle day before hCG injection

Results:
The protocol of ovulation induction in IUI cycles by using clomiphene citrate(CC) and recombinant gonadotropin (rFSH) was significantly($p<0.05$) improve the IUI results compared to CC alone or CC with Pergonal®. The mean values obtained by ultrasonography namely; two follicles, and dominant follicle of 19.5mm in size with endometrium thickness of >9.5mm were gave significantly successful IUI outcome compared to other values. In prospective study similar results were obtained ,therefore the overall pregnancy rate was 37% per cycle.

Conclusion:
The calculated data of successful IUI outcome from retrospective study gave a best pregnancy rate in prospective study which was 37%.Consequently, these results can be considered as guideline for physician who interest in assisted reproduction to have a success in IUI.

Key words: IUI, *in vitro* activation, endometrium thickness, pregnancy rate

Introduction:

Infertility is defined as a year of unprotected intercourse in the fertile phase of the menstrual cycles in women under 35 years, or after 6 months in women 35 years or older without achieving pregnancy[1].The origin of infertility is similarly due to male and female factors resulted from multiple causes. Female factors account for 38% of infertility. Male factors account for 20% of infertility. Male and female factors combined cause 27% of fertility. The etiology is unknown in 11%, and other causes are identified in 4%[2,3]

Accuracy of diagnosis is the most important factor to solve the infertility problem[4]. Then the treatment can be determined by medicine and/or assisted reproductive technologies[5]

Intrauterine insemination is a method of ART recognized to be effective and inexpensive and can be offered by both secondary and tertiary fertility centers and to entail relatively few restrictions. It is not as invasive as IVF and allows fertilization to occur within the fallopian tubes and therefore is generally acceptable to most religious groups[6].

To improve the results of IUI, the selection of couples and determination of criteria before starting the IUI program until the detection of pregnancy must be noticed. Therefore, the present study is designed to find out the cut off values that led to pregnancy following IUI. Then these values will be applied in prospective study to found out the PR following IUI. Thus,this work will include retrospective and prospective studies to 1-Examine the effect of *in vitro* sperm preparation techniques on IUI outcome.2-Elucidate the correlation between endometrial thickness and hormonal profile before hCG injection and their correlation with PR following IUI.

Materials and Methods

This study was conducted at the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies at Al-Nahrain University – Baghdad through a period from July 2010 to April 2011. The proposal research is designed to evaluate two categories .

1-Retrospective study:

The study calculate one hundred cases of infertile couples who were became pregnant following IUI in the Institute ,through the period between January 2007 and January 2010. The calculated data will focus and concern on the circumstances namely; techniques of *in vitro* sperm activation (simple layer -centrifugation), hormonal profile, endometrial thickness(ET), size and number of dominant follicle(s) all in which gave best results leading to pregnancy

2-Prospective study:

The prospective experimental study included 100 infertile patients who are attending Consultant Clinic at the Institute between Augusts 2010 till June 2011.Depending on the retrospective results, IUI was done by using the criteria of successful sperm preparation technique. Optimum measurement of follicular size, number and ET by using the ultrasonography. The level of reproductive hormones (LH,FSH and Estradiol) was determined in cycle day 2-3 of menstruation and before the injection of hCG.

The IUI was done when the values of ET, Hormonal assay and follicular size before hCG are similar to the calculated data in the retrospective study to found out the PR.

Couples who were not fit for IUI were excluded from this study (such as severe oligozoospermia in men and tubal agenesis in women)

2. Initial investigation

All couples were asked to complete a self-assessment form attending their first visit. The investigation was performed to assess the normal menstruation and: hormonal assay (7). In retrospective and prospective study, hormonal tests were performed at day two of cycle these included FSH, LH, estradiol (E2).For prospective study levels serum LH and E2 were measured at cycle days 9- 11 to predict ovulation

-Ultrasonography Monitoring: In this study, it had been routinely performed a vaginal scan in the early follicular phase (Cycle day6) to excluded PCOs, previous Cyst and antral follicle. At Day 9 (Cycle day6) follow up follicle(s)diameter, and response to treatment. At cycle day12, dominant follicle size with number and endometrial thickness were assessed(9).

3. Ovulation Induction

In prospective study, the protocol used for ovulation induction was clomiphene citrate (Clomid® tablets, 50 mg, Aventis, France) two times daily for 5 days from day3 of menstrual cycle to day7(10). Also Gonal-F® from day 3 of menstrual cycle was injected and the dose was adapted depending on the ovarian response to the treatment, till maturity of follicle,(11).

All the males included in the study were examined by male infertility consultant in the Institute and standard semen analysis was performed according to the WHO guidelines(12).

4. *In vitro* activation technique:

Simple layer(Migration -sedimentation) procedure was performed for the semen samples that involved in the prospective study as described by Al-Dujaily(13).

5-Intra-uterine insemination technique

Intra-uterine insemination was performed when two ovarian follicles reach a measurement of (19.5mm) and endometrial thickness(9.5mm) were reported by ultrasonography with serum E2 level (750pg/ml) and following 36 -40 hours of human chorionic gonadotrophin (hCG, Ovitrelle

® 250 mg =6500IU) injection. About 0.5 -0.7ml of activated sperms were loaded in a syringe and then attached with IUI catheter.Insemination was performed according to Elnashar,(10). The diagnosis of pregnancy was done either by biochemical analysis (the detection of hCG in the blood after 2weeks of insemination) or by ultrasound examination,(14,15) .

6- Statistical analysis:

Statistical analysis was performed using SPSS (Statistical Package for social Science; Version (16.0) and Microsoft Office Excel 2007. Numeric data were expressed as mean ± SE. Nominal data were expressed as frequency and percent. Numeric data were analyzed using student t-test. Nominal data were analyzed using Chi-square test,(16) .

Results

1. Characteristics of the retrospective study

1.1. Assessment of *in vitro* sperm activation techniques used in the retrospective study

The number and percentage of two *in vitro* sperm activation techniques namely; The simple layer and the centrifugation techniques) that were used in IUI and resulted pregnancy in retrospective study:1-simple layer percentage (52%) 2-centrifugation (11) percentage (11%)3-by use of both methods (37)with percentage (37%).

1.2.Hormonal status of women included in the retrospective pregnant study (cycle day2)

Table (1) shows hormonal status of pregnant women involved in retrospective study.The mean of the FSH level was (4.1 +0.349) mIU/ ml with a range between 0.90 to 20.00 mIU/ml. The mean of LH level was (11.6+ 0.342)mIU/ml (ranged between 1.00 to 22.2 mIU/ml).The mean of serum estradiol level was (136.7 +2.895) pg/ml (ranged between 40 to 281.00 pg/ml).

1.3. Characteristics of the retrospective study of pregnant women, the day before hCG injection.

The endometrial thickness in the pregnant females ranges from (6-12.60) mm with a mean thickness of (9.46 +0.373) mm. The mean size of Graffian (dominant) follicle was (19.5+ 0.998)mm (ranged between 17.00 to 23.00 mm). The number of Graffian follicles of successful pregnant women was between 1-3 follicles with a mean of (2+0.062) as shown in table(2).

1.4. Ovulation induction program in the retrospective pregnant study

The ovulation induction program used by the pregnant female included in the retrospective study: 1- natural cycle (without induction) was (18) with a percent (18.%), 2-Ovulation induction by Clomid® only was (25) with a percent (25.0%) 3-Ovulation induction with Clomid® and Gonal .F® was(47) with percent(47.0%), 4-Clomid®and pergonal® was(10) with percent (10.0%).

2.Characteristics of the prospective study

2.1. *In vitro* sperm activation using simple layer technique

Table (3)showed the effect of the simple layer) migration-sedimentation technique) on human sperm parameters. for men where their spouses having the previous parameter (retrospectively). The sperm concentration was significantly (P<0.05) lower (30± 1.41) than that of before activation (55 ±0.43).The progressive motility grade A was highly significant(P<0.01)increased (45± 1.73) after *in vitro* activation compared to before activation(19.4±0.04).Progressive motility grade B (40 ±1.51) were significantly (P<0.05) increased after *in vitro* activation compared to before activation(31.01± 0.48). A high significant (P<0.01) improvement was recorded in the percentage of morphologically normal sperm following *in vitro* activation (70± 2.4) compared to that before activation(40.7± 0.4) .A significant(P<0.05) decrease in the number of round cells was recorded after activation compared to before activation (Table -3).

2.2. Levels of serum LH and Estradiol Hormones in cycle day before the hCG injection in the prospective study.

In table(4) there was a significant (P<0.05) differences in the level of LH (15.63 ± 3.63 mIU/ml) in cycle day before the injection of hCG in women were got successful pregnancy (N=37) compared to the level of LH (11.62± 1.291 mIU/ml) in the non-pregnant women group (N = 63).

Level of estradiol hormone (pg/ml) revealed a significant (p<0.05) increase before the injection of hCG in the pregnant women group (787.74 ±17.323) compared to the non-pregnant women group (549.12 +27.409).

2.3. Percentage of pregnancy in IUI in the prospective study

Intra-uterine insemination was done for 100 infertile women who have the cut off values similar to retrospective study successful results. The mean of endometrial thickness was (9.5mm), the size of dominant follicle was (19.5mm) and the number of follicles was (2),using CC and G.F only as an ovulation induction protocol. Semen samples of their spouses was prepared only by simple layer) migration -sedimentation) activation technique. The result of pregnancy rate was 37% after IUI as shown in figure 1.

Table- 1: Hormonal status of pregnant women included in retrospective study(cycle day 2)

Hormones	Minimum	Maximum	Mean	SD	SE
FSH	0.90	20.00	4.1	3.19	0.349
LH	1.00	22.20	11.6	4.60	0.342
Oestradiol	40.00	28k1.00	136.7	24.71	2.895

No. women=100

Table 2: Characteristics of endometrial thickness, size of dominant follicle, and number of dominant follicle in the retrospective pregnant study (n =100),the day before hCG injection

Characteristic	Minimum	Maximum	Mean	SD	SE
Endometrial thickness	6.00	12.60	9.5	1.05	0.373
Size of dominant follicle	17.00	23.00	19.5	1.25	0.998
Number of follicles	1	3	2	0.63	0.062

Table 3- :*In vitro* activation by using simple layer technique

In vitro activation		Sperm concentration (Million/Ml)	Sperm motility				Morphologically normal sperm (%)	Round cells (cell/HPF)
			A	B	C	D		
Before Activation	Mean	55.9	19.4	31.01	12.5	29	40.7	17.6
	SE	0.43	0.04	0.48	0.23	0.25	0.4	0.31
After Activation	Mean	30.2*	45**	40*	10	5**	70**	0.00
	SE	1.41	1.73	1.51	0.617	0.514	2.43	0.00

*P<0.05 Significant
**P<0.01 High significant
Student>s t-Test

Table 4: Comparison levels of LH and Estradiol hormones between pregnant and non pregnant women in the day before hCG injection in prospective study

Characteristics	Group 1 Successful pregnancy N = 37	Group 2 Failure of pregnancy N = 63	P	Significance
LH (prospective)	15.63+3.63	11.62+1.291	0.005	Significant*
E2 (prospective)	787.74+17.323	549.12+27.409	0.005	Significant

No women=100
Student>s t-Test

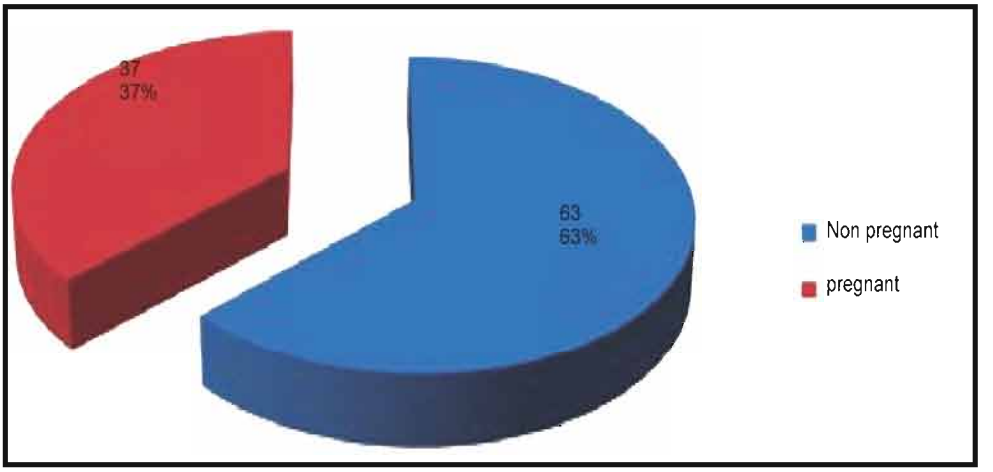

Figure - 1: pregnancy rate by Intra Uterine Insemination in prospective study

Discussion

The mean age of the whole partners shared in this study was (30.20± 4.60 years). The majority of pregnancies happened among the women their age <35 years old and in the first treatment cycle. The success of IUI also declined with increase of patient age, which is in congruity with other reports,[17]. The age-related decline in female fertility is probably due to a combination of reduction in oocyte number and quality[17] ,diminished ovarian reserve, decline in granulosa cells function, reduced endometrial receptivity,[18]

In retrospective study and then in prospective study, simple layer technique was used as the technique for *in vitro* sperm activation in which the PR was reached 37% after IUI. This procedure still remains the standard technique for patients with normozoospermia and female infertility. The technique distinguished by a very high percentage (>90%) of motile sperm, preferred enrichment of morphologically normal spermatozoa as well as the absence of other cells and debris[19].

Furthermore, The data revealed a highly significant (P<0.01) increased in the percentage of the progressive motility grade A after *in vitro* activation compared to before activation.Progressive motility grade B was significantly (P<0.05) increased after *in vitro* activation compared to before activation, this result is similar to the results of other studies,[20] It has been mention that the increase in the PR from 17.1%to 30.4% if the active sperm motility grade A exceeded 20%[20].

Measurements of FSH and E2 of women in menstrual cycle, at day 2 and day 3 combined with maternal age, are useful for predictors of pregnancy outcome. These measurements are standard practice for predicting oocyte quality and the likelihood of conception in assisted reproductive technologies (ART),[21].

The result of the present work indicated a significant increase in the level of estradiol hormone in cycle day before hCG hormone injection in pregnant women) compared to non-pregnant women. Although both groups had the same ultrasound characters (2dominant follicles measured 19.5mm), follicles are believed to contain a mature oocyte when they are 20 -24 mm and follicle size increases by 2-4 mm after the start of the LH surge, this means that hCG should be given when the largest follicle is 19.5-20 mm,[21],however, some of mature follicles in non-pregnant women didn't contain oocytes and the E_2 level was decrease [22].

The results of this prospective study identified that hCG must be administered when the serum estradiol level reaches 300- 420 pg/ml per follicle >19.5 mm in diameter, 700 -800 pg/ml per two mature follicles on the day before hCG administration. the pregnancy rate per cycle varied according to the E_2 concentration [23].It has been noticed that PR was 14.2% per cycle when E2 level was <500 pg/mL on the day of hCG administration[24], 16.2% per cycle when the E_2 was between 500 and 1000 pg/rnl, and lastly, 22.1% for over 1500 pg/rnl,[25].

It has been recorded that ultrasound should be used for timing of hCG injection to avoid complications, relies on most for the decision to increase, decrease, or` stop the gonadotropin.[26].

The present retrospective study identified that the mean of endometrial thickness was (9.5) in the day before hCG injection. There was a positive linear correlation between endometrial thickness and pregnancy rate[27, 28]. The study stated that thickness can be utilized as an indirect indicator for endometrial receptivity Ultrasonographic examination was routinely performed in ART treatments because of its accurate evaluation and being noninvasive detection. Indeed, both endometrial thickness and endometrial pattern have been regarded as prognostic parameters for successful pregnancy . It has been found that injection of hCG induced disappearance of the triple line pattern, probably representing normal physiological transformation from the proliferative to the secretory phase[29].

The current study revealed a significant relationship between the ovulation induction protocol and pregnancy rate. t has been reported that there was a significant improvement in PR(26.21%) by using CC+ rFSH protocol compared to (15%) PR following hMG protocol,[30].

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Cryopreservation of ovarian cortex and vitrification of immature oocyte

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Abstract:

Background:

Unlike sperm production in men which is continuous, women are born with all their eggs and they do not produce any more. Technique to bank eggs would allow girls and women to have the same reproductive options as men when faced with a serious disease and the long term complications of chemotherapies that threatens to destroy their eggs.

Objective:

The aim of this study is to improve the efficiency of existing ovarian cryopreservation, by following the most recent method named needle immersed vitrification (NIV) beside the slow freezing method. In this review we discussed also the historical description of the methods used, freezing and vitrification of mature and immature oocytes, this include comparison of principles, procedures and results as reported in the literature.

Materials and Methods:

A total of 285 fragments of human ovarian tissues were isolated and cryopreserved from 36 women aging 26 – 55 years old in the Reproductive Unit, Lubeck-Germany. Ovarian tissue strips of 13- mm thick of ovarian cortical biopsies obtained from patients. The collected human ovarian cortex fragments were assigned to two different cryopreservation procedures, slow-freezing and NIV. On the otherhand, a total of 80 patients (100 cycles) with polycystic ovaries (PCO) were underwent *in vitro* maturation (IVM) treatment. The number of inspirated immature oocytes was 536. vitrification of immature oocytes were carried out.

Results:

Successful freezing and storing were performed on 285 fragments of ovarian cortex tissue from 36 patients by two cryopreservation protocols as mentioned before but these tissues do not thaw and use yet. Concerning the IVM, the percentage of maturation, fertilization, survival, embryo transfer, and pregnancy rates were 55.2%, 44.5%, 96.2%, 88.1%, and 10.6%, respectively.

Conclusion:

Recent advances in freezing technology, modifications of conventional protocols used and continuing optimization of vitrification have efficiently improved the method. These results may lead to increase the possibility of preserving fertility by cryopreservation of ovarian tissue. Ovarian tissue banking can offer hope for cancer patients who want to safe guard their fertility against sterilizing chemotherapy and radiotherapy. Immature oocytes can be successfully isolated from the oophorectomy specimen regardless of the day of menstrual cycle, and undergo IVM and cryopreservation.

Keywords: Cryopreservation, immature oocytes, ovarian cortex, vitrification.

Introduction:

ART includes all fertility treatments in which both eggs and sperm are handled. In general, ART procedures involve surgically removing eggs from a woman's ovaries, combining them with sperm in the laboratory, and returning them to the woman's body or donating them to another woman. They do NOT include treatments in which only sperm are handled (i.e. intrauterine or artificial insemination) or procedures in which a woman takes drugs only to stimulate egg production without the intention of having eggs retrieved. Because ART consists of several steps over an interval of approximately 2 weeks, an ART procedure is more appropriately considered a cycle of treatment rather than a procedure at a single point in time[1]. Unlike sperm production in men which is continuous, women are born with all their eggs and they do not produce any more. The natural process of each menstrual cycle consumes approximately 5001000- eggs until the supply is exhausted (about age 51,menopause). Men have been able to cryopreserve (freeze) their sperm for decades. A woman's chances of having a pregnancy and a live birth by using ART are influenced by many factors, some of which are patient-related and outside a clinic's control e.g., the woman's age, the cause of infertility[2]. However, women have not been able to freeze their eggs reliably because the eggs are hard to retrieve and unfertilized eggs have generally not survived freezing. With the advent of IVF, oocyte, embryo and even ovarian tissue freezing became possible[2]. For all these reasons, a technique to bank eggs would allow girls and women to have the same reproductive options as men when faced with a serious disease and the long term complications of chemotherapies that threatens to destroy their eggs[3].

Cryopreservation of ovarian tissue: and the whole ovary is a new progress in ART. Transplantation of cryopreserved ovarian tissue was reported in 2004[4], in spite of the introduction of the concept of ovarian transplantation since 1906[5]. Despite its infancy to preserve fertility and lack of studies, ovarian tissue cryopreservation has several potential advantages, such as the presence of many primordial follicles with oocyte arrest in diplotene of prophase of the first meiotic division, primordial follicles being theoretically less cryosensitive than mature oocytes, and preservation of the endocrinal function of the ovary[6]. Several diseases, mainly cancers and their treatments threaten to destroy all the follicles in a womans ovaries. Other diseases rarely have a direct effect on the oocytes in the ovary. Chemotherapy or radiation used to treat cancer or some non-cancerous disorders have the unfortunate side effect of destroying the follicles in the ovary as well as the diseased cells[7]. Advances in chemotherapy and radiotherapy have increased the survival rate of cancer patients[8], amazingly up to 90% for young cancer patients[9]. Increased life span actually raises concerns on the quality of life after treatment for cancer. Having children may be difficult for cancer survivors as both radio- and chemotherapies have been proven to be gonadotoxic[10]. Recovery of ovarian function after anticancer treatment is very much affected by the loss of follicles due to chemo- or radiotherapy resulting in premature ovarian failure (POF) and consequently, infertility in many female cancer survivors. Fertility preservation before treatment for cancer is an important option nowadays to overcome infertility induced by cancer itself or by the treatment to cure cancer [6, 8, 11].

The ovary has hundreds of primordial follicles containing immature oocytes which are resist cryopreservation due to the absence of zona and cortical granules[12]. Ovarian cortex can be obtained from any female cancer patient irrespective of age and marital status with no procedural delays in cancer treatment. Amorim *et al*[13] have shown that steroidogenic and gametogenic functions are well preserved in cryopreserved ovarian tissue. All these advantages make ovarian tissue cryopreservation a better option than other means for female fertility preservation. There have been two human live births after orthotopic transplantation of cryopreserved ovarian tissue [4, 14]. Huang *et al*[15] reported that immature oocytes can be retrieved successfully from the visible antral follicles of excised ovarian tissue, matured *in vitro* and cryopreserved by vitrification. Oocyte viability rate in ovarian tissue before and after cryopreservation by vitrification was studied, first in bovine and later in humans, and a 97% survival rate of oocytes has been reported from the ovarian tissue[16]. It has been proven that slow freezing of oocytes followed by thawing would yield inferior results when compared to fresh oocytes[17,18] while, cryopreserved oocytes using vitrification will yield results comparable to fresh oocytes after thawing [15, 16, 19]. Thus, it would depend on the age of the women from whom the oocytes were harvested and frozen. On the other hand, Fasano *et al* [20] in their study showed that a significant number of immature oocytes can be collected from excised ovarian tissue whatever the menstrual cycle phases and the age of the patients, even for prepubertal girls. Gosden *et al* [21] reported the live births of young animals after autografting frozen-thawed ovarian tissue in sheep. Similar reports have been published in rats [22] and rabbits [23]. These successful attempts in animals prove that ovarian tissue cryopreservation is a feasible means of fertility preservation with a potential application for human beings.

It has been found that women with polycystic ovarian syndrome (PCOS) are characterized by abnormal endocrine parameters, anovulation, numerous antral follicles within their ovaries and frequently infertility[24]. Patients with PCOS are extremely sensitive to stimulation with exogenous gonadotrophin and are at increased risk of developing ovarian hyperstimulation syndrome (OHSS) when treated with gonadotrophins for assisted reproduction[25, 26]. Recovery of immature oocytes followed by in-vitro maturation (IVM) of these oocytes could be developed as a new method for the treatment of patients with infertility due to PCOS. Methods developed by Trounson for transvaginal ultrasound guided recovery of immature oocytes from the ovaries of patients with PCOS introduced IVM firmly into the clinical feild[27]. It has been noted that most of the follicles from patients with PCOS are not atretic and these oocytes appear to have developmental competence[28,29]. However, the maturation rate of immature oocytes retrieved from women with PCOS is lower than that of those retrieved from women with normal menstrual cycles[30,31].

Materials and Methods:

Patients and Ovarian Tissue Collection: Human ovarian tissues were collected from thirty six women at different ages ranging from 6 – 55 years old. Some of these patients [28] had undergone oophorectomy for their specific conditions, including endometrial cancer and breast

cancer. The other [8] samples were obtained from ovarian biopsy from patients who underwent ovary transposition because of cervix cancer. All the patients signed an informed consent form that was approved by Institutional Ethics Committee of German Medical Center, Department of Obestetrics and Gynecology, Medical University of Lubeck, which also approved the current study. Ovarian tissue cryopreservation begins with laproscopy. The surgeon obtained 13- mm thick, 3x10 mm strips of ovarian cortical biopsies from patients. The collected tissues was placed immediately into a sterile petri dish containing the L-15 medium supplemented with 10% FBS for the next procedure.

Human specimen preparation: The collected tissues were transferred to the laboratory, the tissues was cut into strips of 2x2 mm in size in fresh L-15 medium supplemented with 10% FBS. The total samples isolated from all patients were 285 strips. All were confirmed as showing no ovarian metastasis by an independent pathologist before data analysis.

Cryopreservation procedures: Human ovarian cortex fragments were assigned to two different cryopreservation procedures: slow-freezing and needle immersed vitrification as described above. Before cryopreservation, one or two pieces of human ovarian cortex from each patient were fixed for morphological and ultrastructure assessment of immature follicules.

Patients and *In Vitro* Maturation procedure: A total of 80 patients (100 cycle) who underwent IVM treatment. Only those patients who had polycystic ovaries were recruited. Patients ages were range between 26- 38 year. They were attended our clinic centre at Lubeck University. Oocyte recovery was performed 36 h after hCG injection. Invitro maturation were carried out according to the procedure mentioned by[40]. Mature oocytes were inseminated by ICSI and then cryopreserved at 2pn stage by vitrification method[39] followed by thawing and embryo transfer on day 2 after thawing.

Technique of Ovarian Cortex cryopreservation is summarized through the two different cryopreservation procedures used for human ovarian cortex fragments as below.

1-Slow-freezing protocol for ovarian cortex tissue cryopreservation:

A slow-freezing protocol put forward by Gosden *et al* [21]. According to the protocol, 2–3pieces of human ovarian cortex fragments place in a 1.8 ml cryovial (Nunc, Roskilde, Denmark) containing 1 ml of Leibovitz medium (L-15) supplemented with 0.1 M sucrose (Sigma-Aldrich, St Louis, MO, USA), 10% Fetal Bovine Serum (FBS) and 1.5 M dimethyl sulfoxide (DMSO, Sigma-Aldrich). After a 30 min exposure to the cryoprotectant solution at 4°C, the cryovials load in the programmable freezer (Biomed Freezer Kryo 10, Series II, Planer, UK). Slow-cooling protocol starts temperature of 4°C. The vials cool at a rate of 2°C/min to -7°C and hold for 5 min, seeding use manually and maintain at -7°C for another 10 min. Then the temperature cools to -40°C at a rate of -0.3°C/min and further cool to -140°C at -10°C/min. Finally, the vials transfer to liquid nitrogen for storage. For thawing, the cryovials remove from liquid nitrogen, hold in air for 20 s and transfer to a water bath (37°C) for 20–30 s. The contents of cryovials deplete into the L-15 medium supplemented with 0.1 M sucrose, 10% FBS and 1.0 M DMSO for 5 min and washe in a stepwise manner (1.0 M DMSO, 0.1 M sucrose, 0.5 M DMSO, 0.1 M sucrose, 0.1 M sucrose) for 5 min each. The conventional slow-cooling method certainly has resulted in the delivery of several babies[14,16, 32,33].

2-Needle Immersed Vitrification (NIV) protocol for ovarian cortex tissue cryopreservation:

The ovarian tissues dehydrated by using a two-step regimen an equilibration solution consisting of 7.5% (v/v) Ethylene Glycol (EG) and 7.5% (v/v) DMSO in Dulbeccos Phosphate Buffered Saline (DPBS) supplement with 20% FBS for 10 min at room temperature and a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose for 2 min. This vitrification solution was used successfully in recent publications on blastocyst, oocyte and mouse ovarian vitrification[34,35,36]. The long needle can hold several ovarian tissue samples in a row, and thus all the samples can be exposed to cryoprotectants and then immerse into liquid nitrogen under the same conditions. This can maximize the cooling rate and simplify the vitrification process. To vitrify human ovarian cortex fragments, 4–5 pieces of ovarian tissue strips helds in a row by a needle in the L-15 medium supplemented with 10% FBS. After two steps of dehydration procedure, the ovarian tissues carried by the needles, held by the forceps, underwent the following procedure they placed on an aseptic absorbent gauze to remove the remaining vitrification solution. Then, they plunged in liquid nitrogen directly and finally, they put into liquid nitrogen-filled cryovials and stored in liquid nitrogen. For thawing, the solid drops or the needles holding ovarian tissues take out of the vial using forceps and quickly immersed into 1 M sucrose solution, which has been pre-warmed at 37°C for 5 min. They serially transfered into 0.5 and 0.25 M sucrose solution for 5 min each and incubate in DPBS supplemented with 20% FBS for 20 min at 37°C with 5% CO2.

Technique of *in vitro* maturation and vitrification of immature oocytes: The patients received a specialized consultation, as is offered to all patients with PCOS managed by the unit. A transvaginal ultrasound and hormonal measurements [FSH, LH, estradiol (E2), prolactin, delta -4- androstenedione,17-hydroxyprogesterone] are routine quides in our centre. The first ultrasound scan was scheduled for the third day of the cycle, as well as determination of E2, LH and progesterone levels and measurement of endometrial thickness. These investigations were repeated around the sixth to eighth day of the cycle to exclude the development of a dominant follicle. The patient received 10 000 IU of hCG Chorionique Endo (Organon, France) s.c. when follicle size reached 7 mm, before selection of the largest follicle [37]. All patients received hCG according to Chian *et al.* [38, 39] who have demonstrated in IVM cycles that hCG priming increases both the percentage and rate of immature oocyte maturation.

Immature Oocyte retrieval: Oocyte recovery was performed 36 h after hCG injection. During the collection, patients received a mild i.v. sedation with propofol (Driprivanw; AstraZeneca, France). Transvaginal ultrasonographically guided oocyte collection was done using a specially designed 19-Gauge single-lumen aspiration needle (K-OPS-7035-Wood; Cook, France). The aspiration pressure was set at 7.5 kPa. Follicular aspirates containing cumulus–oocyte complexes were collected in 15 ml Nucleon (Nunc A/S, Denmark) tubes containing prewarmed 3 ml sodium heparinate 2 IU/ml (Sanofi–Synthelabo, France). Follicular aspirates were not washed on a filter but tubes were spread onto sterile polystyrene culture dishes of 60 mm diameter Nucleone (Nunc A/S). The cumulus–oocyte complexes were isolated under a stereomicroscope and then washed once in the culture medium, Universal IVF Medium (MediCult, Denmark),

warmed to 37°C in a thermostatically controlled incubator under an atmosphere enriched to 5% CO2.

In vitro maturation procedure was done as described by Chan and Tan,(39).Matured oocytes were inseminated by ICSI using the partner's spermatozoa. ICSI was performed at least 1 h after observing first polar body (PB) extrusion as suggested by Hyun *et al.*[40]. Fertilization was assessed 17–19 h after insemination for the appearance of two distinct pronuclei and two PBs. Vitrification of 2PN stage was performed as mentioned before. After thawing vitrified oocytes the zygotes were cultured in Embryo Maintenance Medium (Cooper Surgical). Embryonic development was assessed on Day 2 (41–43 h) and on Day 3 (65–67 h) after insemination according to the regularity of blastomeres, the percentage and pattern of anucleate fragments, and all dysmorphic characteristics of the embryos. The best quality embryos were transferred on Day 2 or Day 3 after ICSI.

Results:

This results showed a successful freezing and storing of some ovarian cortex tissues from 36 patients aging 6 -55 years with two different cryopreservation protocols in our reproductive unit at Lubeck Germany as mentioned before. The total number of fragments 285. These tissues do not thaw and use yet because our patients are still under treatment therapy (table 1).

On the otherhand, Table 2 represents some results which have shown that, there were no significant differences between the three cryopreservation methods compared with fresh for. Table (3) represents some author's results about ovarian cortex cryopresrevation.

Results of IVM and vitrification of immature oocytes are represented in table (4). After 100 cycle from 80 patients, the number of immatured oocytes isolated was 536. The percentage of maturation was 55.2%. The fertilization rate was 44.5%. Moreover, survival rate of vitrified oocytes, embryo transfer and pregnancy rates were 96.2%, 88.1% and 10.6% respectively.

Table I: Ovarian tissue cryopresrevation from 36 patients at different ages (6-55 years), cryopreserved biopsy numbers and methods used (Slow freezing and Vitrification).

Slow Freezing / Leibo+3M DMSO			Vitrification / NIV		
Patient #	Patient Age	Cryo Biopsy #	Patient #	Patient Age	Cryo Biopsy #
1	17	20	1	14	5
2	23	12	2	55	4
3	18	5	3	11	10
4	24	8	4	6	5
5	32	10	5	25	12
6	18	10	6	36	10
7	8	8	7	24	9
8	36	9	8	39	6
9	32	9	9	33	5
10	30	10	10	33	5
11	38	10	11	36	5
12	22	8	12	34	8
13	25	5	13	19	18
14	19	10	14	22	8
15	7	5	15	16	12
16	21	6			
17	25	2			
18	24	4			
19	25	5			
20	12	2			
21	33	5			

Total number of patients were 21 and 15 for slow freezing and NIV respectively. Mean age for the patients 6-55. The total number of biopsy were 163 and 122 for slow freezing and NIV respectively.

Table 2. Percentage of morphologically normal human primordial follicles and mouse follicles (various stages) in groups of frozen–thawed ovarian cortex and fresh tissues.

Groups	Human		Mouse	
	Primordial follicles	Primordial follicles	Primary follicles	Secondary follicles
Slow-freezing	82.93±2.31a	83.63±1.41b	74.00±1.85c1	43.5±3.29d1
Vitrification	81.34±3.72a	82.63±2.39b	73.25±2.31c2	42.75±2.12d2
NIV	83.16±2.70a	84.38±3.34b	81.75±1.83c1,c2	68.25±1.93d1,d2
Fresh	90.70±2.50	92.80±1.30	90.40±1.52	86.4±1.4

Percentage data expressed as mean+SD; NIV, needle immersed vitrification.
a,b No difference among the three groups
c1,c2 NIV versus slow-freezing and dropping vitrification group: both P, 0.001.
d1,d2 NIV versus slow-freezing and dropping vitrification group: both P, 0.001.

Table 3: Review on the historical data of Ovarian cryopreservation (diagnosis, nature of tissue, reimplantation side and outcome results).

FIRST AUTHOR, YEAR	WHERE	DIAGNOSIS	AGE at cryo	FRESH or CRYO-PRE-SERVED	SLICES or WHOLE	REIMPLANTA-TION SIDE	OUTCOME
Donnez, 2004 (4)	Belgium	Stage IV Hodg-kin's lymphoma	25	Frozen/ thawed	Slices	Orthotopic: ovarian fossa peritoneum	Spontaneous pregnancy: live birth
Donnez, 2011-a (41)	Belgium	Neuroectodermic tumour	17	Frozen/ thawed	Slices	Orthotopic: ovary	Spontaneous pregnancy: live birth
Meirow, 2005, (14) 2007 (42)	Israel	Non Hodgkin's lymphoma	28	Frozen/ thawed	Slices	Orthotopic: ovary	Mild ovarian stimulation - IVF: live birth
Demeestere, 2007(33) 2010 (43)	Belgium	Stage IV Hodg-kin's lymphoma	24	Frozen/ thawed	Slices	Orthotopic (+ heterotopic)	Spontaneous pregnancy: live birth in 2007 live birth in 2009
Andersen, 2008 (32)	Den-mark	Hodgkin's lymphoma	27	Frozen/ thawed	Slices	Orthotopic: ovary	Ovarian stimulation - IVF: live birth
Andersen, 2008 (32) Ernst, 2010(44)	Den-mark	Ewing sarcoma	27	Frozen/ thawed	Slices	Orthotopic: ovary	Mild ovarian stimulation - IVF: 1 live birth. Spontane-ous pregnancy: 1 live birth
A- Silber, 2010 (45) B-Donnez, 2011 b(46)	USA	Stage IIIb Hodg-kin's lymphoma	20	Frozen/ thawed	Slices	Orthotopic	A- Ongoing pregnancies B -live birth
Silber, 2010 (45)	USA	Premature ovar-ian failure	24	Frozen/ thawed	Slices	Orthotopic	1 live birth + 1 live birth (twins)
Piver, 2009 (47)	France	Microscopic polyangiitis	27	Frozen/ thawed	Slices	Orthotopic	IVF: live birth
Sanchez-Serrano, 2010 (48)	Spain	Breast cancer	36	Frozen/ thawed	Slices	Orthotopic	Ovarian stimulation, IVF: 2 live births (twins)
Revel, 2011 (49)	Israel	Thalassemia major	19	Frozen/ thawed	Slices	Orthotopic	IVF: live birth
Roux, 2010 (50)	France	Homozygous sicule cell anemia	20	Frozen/ thawed		Orthotopic: ovary and few strips deposited in the peritoneal window	Spontaneous pregnancy: live birth

Table 4: Outcome results from IVM cycles and vitrification of immature oocytes from PCO patients.

Patient No	Cycle No	Immature Oocyte No	Mature Oo-cyte No (%)	Fertilization Rate No (%)	Survival Rate After Thwaing No (%)	Embryo Transfer Rate No (%)	Pregnancy Rate No (%)
80	100	536	296 (55.2)	132 (44.5)	127 (96.2)	112 (88.1)	12 (10.6)

Discussions:

The recent experiments, human ovarian tissue yielded a similarly high post-warming viability using the same vitrification method. Ultrasound also revealed normal follicular growth in the grafted ovary. Most importantly, the transplanted vitrified–warmed bovine ovarian tissues were, in all the cases, histologically normal[51].There was no apparent loss of oocyte viability caused by the vitrification. Moreover, even the transplantation of this cortical tissue resulted in no histologically apparent ischaemic oocyte loss either. Histological analysis of ovarian tissue cryopreserved with the slow-cooling method has demonstrated subtle aberrations compared with fresh tissue[52].

Based on these findings in the cow, it was presumed that the cryotissue method used in these experiments could be applied to human ovarian tissue, the structure of which is similar to that in cattle. So human ovarian tissues were vitrified using the same approach. The viability of oocytes in human ovarian tissues vitrified with ultra-rapid Cryotissue method was also very high (90%). There was no significant difference between fresh and frozen tissue even though the donors of the ovaries were patients with ovarian cancer of a rather advanced age (38–40 years old). Histopathological analysis of post-vitrification human ovarian tissues demonstrated normal tissue structure after vitrification, similar to the results obtained from the latest experiments [51]. No devitrification occurred during warming, and excellent viability was demonstrated, indicating that this may now be an acceptable method for clinical trials.

Recent medical advances have dramatically increased the cure rate for cancer in young women of reproductive age, resulting in more than 90% of all young cancer patients to be cured [53, 54]. One of the adverse effects of cancer treatment, however, is infertility brought about by damage to the ovaries caused by chemo and radiotherapies. Almost all female patients with haematological cancer who undergo bone marrow transplants lose their ovarian function and become menopausal, regardless of age, and are thus rendered sterile. Approximately 1% of the total female population between 20–39 years of age in Japan are cancer survivors whose fertility has been destroyed by their treatment [55]. The solution to this dilemma would be to freeze the eggs or ovarian tissue of these patients before the initiation of cancer treatment. Vitrifying ovarian tissues by direct immersion into liquid nitrogen using a minimal volume of cryoprotectant could maximize the cooling rate and reduce toxicity of the vitrification solution with less-concentrated cryoprotectants. Moreover, the NIV method is relatively simple, convenient to manipulate and time-saving. Held by an acupunctureneedle, the ovarian tissue pieces can be exposed to cryoprotectants synchronously. So, NIV can facilitate vitrification process especially when a large number of ovarian cortex fragments require to be cryopreserved.

In the past 10 years, several groups have reported high survival rates (89.2% to 100%) and successful live births using vitrification methods. In fact, since the first report in 1999, vitrification of oocytes has resulted in a lot of live births worldwide [2, 56, 35, 57, 19].

In conclusion, immature oocytes can be successfully isolated from the oophorectomy specimen regardless of the day of menstrual cycle, and undergo IVM and cryopreservation. Patients with ovarian tumors who wish to preserve their fertility may benefit from this additional and novel possibility for fertility preservation.

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Evaluation of PSA tumor marker in some Iraqi women with polycystic ovarian syndrome.

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Abstract:

Background:

Polycystic ovarian syndrome (PCOS) is the most common cause of hyperandrogenism anovulatory infertility; it affects 5- 10% female reproductive age.The present study aims to investigate the total prostate specific antigen levels, total serum testosterone, FSH and LH in women with PCOS and compare the results with control group of normal fertile females of corresponding age group on Iraqis PCOS patients.

Materials and Methods:

Seventy patients with PCOS diagnosed depending on three criteria: Menstrual history: Oligomenorrhea, Ultrasound reveals polycystic ovaries and Biomedical and/ or clinical hyperandrogenism.Twenty normal fertile females who serve as control group in this study. Blood samples were aspirated from all individuals from 24- day of menstrual cycle to measure total prostate specific antigen(PSA), total testosterone and FSH, LH .

Results:

Patients with PCOS and controls differed significantly in PSA, Total serum testosterone ($p<0.05$). Patients with PCOS and controls have highly significant difference in LH level, the mean was (7.88± 1.83 vs 3.90± 0.73) respectively ($p<0.001$)and, highly significant difference in LH/FSH ratio and BMI parameters. ($p<0.001$). No significant differences were found in FSH ($p>0.05$). Positive correlation between PSA and testosterone, PSA and BMI.

Conclusion:

Total serum prostate specific antigen levels are higher in patient with PCOS.Total testosterone levels are higher in patient with PCOS.Serum PSA measurement might be marker of hyperandrogenism in females suffering from PCOS.

Keywords: PCOS, PSA, Tumor marker

Introduction

Polycystic ovary syndrome(PCOS) is the most common endocrine problem in women of reproductive age1. PCOS affects 5–10 percent of all women of reproductive age and is associated with anovulation/ oligoovulation, hyperandrogenism, and polycystic ovaries (PCO)[1,2]. PCOS is associated with metabolic disturbances including obesity and insulin resistance with a high risk of developing type 2 diabetes, and cardiovascular disease1. In addition, women with PCOS display reduced health related quality of life as well as symptoms of anxiety and depression[3,4]. The National Institutes of Health (NIH) 1990 preliminary consensus definition has now been replaced by a more recent definition by the Rotterdam European Society for Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) PCOS Consensus Workshop Group[5].This has suggested a broader definition for PCOS, with two of the three following criteria being diagnostic of the condition:
Polycystic ovaries (either 12 or more peripheral follicles or increased ovarian volume (greater than 10 cm[3].Oligo- or anovulation .Clinical and/or biochemical signs of hyperandrogenism.

Prostate Specific Antigen (PSA) was first discovered by Albin and Co-workers in prostatic tissue in 1970[6], and in seminal plasma in

1971 by Hara *et al*[7]. They called it gamma semino protein.Currently, PSA represents the best serum marker for prostate carcinoma, and it has the highest validity of any circulating marker for cancer today[8, 9]. PSA is a serine protease with trypsin like and a chymotrypsin –like activity[10]. It is produced almost exclusively in the cytoplasm of normal and neoplastic epithelial cells and secreted into the lumina of the prostatic duct during the formation of seminal plasma[11] .PSA is released from the normal prostate and appears at low serum concentrations in healthy men. High serum concentration can be detected in patients with advanced prostate cancer[12]. PSA is applied as a tumor marker for the clinical management of prostate cancer (PCA). PSA increased in patients with benign prostate hyperplasia (BPH), acute urinary retention and renal failure can also elevate the PSA level[13]. PSA was believed to be completely absent from all female tissues and fluids. PSA has been detected recently in some female tissues and body fluid[14]. The presence of PSA in these female tissues seems to be associated closely with steroid hormone regulation especially androgens, glucocorticoids and progestin>s[15]. Among women who have high levels of androgens, relatively high levels of serum PSA should be expected if PSA production in women is under the regulation of androgens[16].

Materials and method

Seventy women in their reproductive age (20- 40) years old, who had been diagnosed as PCOS, were recruited from infertility clinic population at the High Institute of Infertility Diagnosis and ART, Al-Nahrain University, between July 2011 and October 2011.The diagnosis of PCOS was based on the presence of polycystic ovaries on ultrasonography (10 or more follicles in each ovary, each follicle measuring 2–9 mm in diameter). One polycystic ovary is sufficient for the diagnosis with one or more of the following criteria(5):

1- Oligo-/anovulation; clinically diagnosed as oligo-/ amenorrhoea, i.e. menstrual cycles longer than 35 days, or fewer than 10 menstruations per year.

2- Hyperandrogenism; clinical or biochemical. Clinical manifestations of hyperandrogenism such as a hirsutism, acne and/or an elevated serum testosterone level. Twenty apparently healthy fertile women were served as control. Those who were matched for age and with regular menstrual cycle and normal ultrasound.

Exclusion criteria:-1- Diabetic patient.2- Patient taking medications (antiglycemic and contraceptive pill) for the previous 3 months that interfere with study results.

Blood samples were aspirated at 8:00- 12:00 am during 2nd – 4th day of menstrual cycle (early follicular phase) for those of normal cycle or progestin induced withdrawal bleeding . Blood samples were collected into plain tube and centrifuged within 30 min of collection. Serum was aspirated, and stored at -20 °c until time of assay. Serum FSH and LH level along with LH/FSH ratio testosterone and PSA were performed for those samples.Serum PSA was measured by using enzyme linked immune sorbent assay (ELISA). This kit for quantitative determination in human serum was supplied by Human Gesellschaft Biochemical. Germany.*(Normal value of PSA according to the kit is less than 4ng/ml.*

Statistical analysis:

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Numeric variables were expressed as mean ± standard deviation. Student T-test was used to compare independent two samples. Pearsons correlation coefficient was used to study correlation between two numeric variables. The differences between values were considered statistically significant at the level of ($p<0.05$) and highly significant at the level of ($p<0.001$).

Results:

The mean serum levels of total PSA in patients with PCOS was equal to (0.48± 0.38)ng/ml which was significantly elevated when compared with the normal controls group (0.25± 0.08) nag/ml. $P<0.05$(0.011) (table 1) . In the current study, the mean serum levels of total testosterone in patients with PCOS was equal to (0.33± 0.22) ng/dl which was significantly ($P<0.036$, table 1) elevated when compared with normal control group (0.22± 0.09) ng/dl. (table 1).There is positive correlation between PSA and BMI in PCO patients ($r=0.470$, $p<0.001$) (figure 1).There is positive correlation between total serum PSA and total serum testosterone ($r=0.668$, $p<0.001$, figure 2).

Table1: Comparison between control and PCOS patients

Parameter	Group 1 (control= 20) Mean±SD	Group2 (PCOS =70) Mean±SD	P value
Number	20	70	
Age	29.58±3.32	28.05±2.68	0.162 NS
PSA	0.25±0.08	0.48±0.38	0.011
FSH	5.34±0.65	5.65±1.47	0.372 NS
LH	3.90±0.73	7.88±1.83	<0.001
LH/FSH	0.73±0.12	1.43±0.36	<0.001
TESTO	0.22±0.09	0.33±0.22	0.036
BMI	24.33±1.35	27.62±1.96	<0.001

NS;not significant

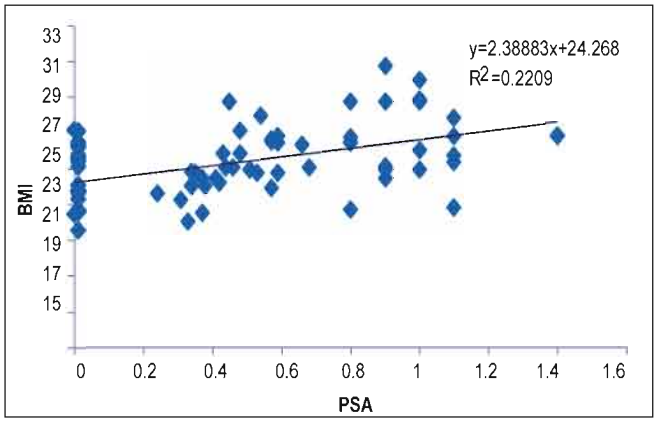

Figure (1): Persons correlation between PSA and BMI in PCOS patients ($r=0.470$,$p < 0.001$).

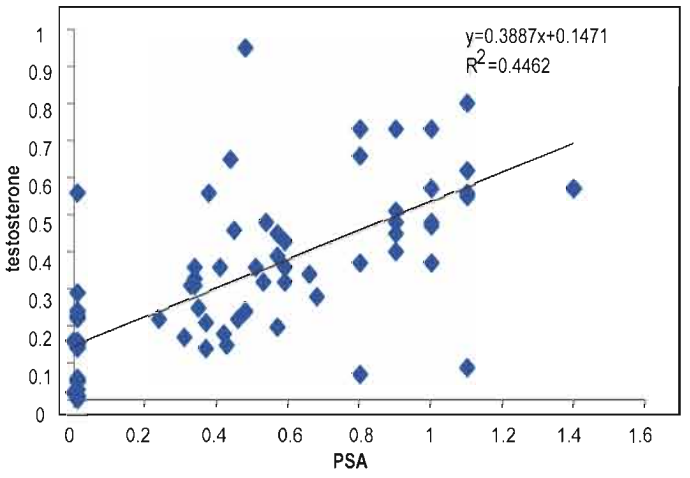

Figure (2): Persons correlation between PSA and testosterone in PCOS patients (r=0.668,p < 0.001).

Discussion

Polycystic ovary syndrome is a heterogeneous disorder, characterized by hirsute, abdominal obesity, hyperandrogenism, polycystic ovaries and insulin resistance.The syndrome is often accompanied by infertility because of anovulation[17]. PCOS develops when the ovaries are stimulated to produce excessive amounts of male hormones (androgens), particularly testosterone, either through the release of excessive LH by the anterior pituitary gland or through high levels of insulin in the blood (hyperinsulinaemia) in women whose ovaries are sensitive to this stim ulus[18]Hyperandrogenism is a key feature of PCOS with elevations of ovarian androgens, testosterone and androstenedione[19] PCOS ovaries have lower activity of aromatase enzyme[20] Aromatase is responsible for the aromatization of androgens into estrogens. It catalyzes the last steps of estrogen biosynthesis from androgens (specifically, it transforms androstenedione to estrone and testosterone to estradiol)[21,22] and this fact contribute to hyperandrogenic state in PCOS.

Until few years ago PSA was believed to be produced only in men and only in the prostate. However using high sensitive PSA assay, new concepts for the clinical use of PSA has been established[23] Determination of PSA in female tissues had become available. The expression of PSA gene is under androgenic regulation. Therefore hyperandrogenemic states, such as PCOS are expected to be presented with the higher levels of PSA[24] Different studies have already demonstrated that females with hyperandrogenism usually have elevated serum total and free PSA[24],. Also other studies have indicated that women treated with testosterone over prolonged period of time had significantly increased serum total PSA[25] .Administration of androgens or progestin>s to patients cause significant elevations of PSA in urine, serum and tissues[23,25,26] The result of the present study shows that the mean serum levels of total PSA in patients with PCOS was equal to (0.48± 0.38) ng/ml which was significantly elevated when compared with the normal controls group (0.250.08+) ng/ml. P<0.05(0.011).This finding is in agreement with Obiezu *et al* (2001)[28] who found that urinary PSA and possibly urinary Human kallikrein 2 (hk2) promising marker of hyperandrogenism in female suffering from PCOS. Rifat *et al* (2003)[24]found that serum PSA could be used as a marker for hyperandrogenemic, hirsutism or PCOS because both total and free PSA levels were found to be significantly higher in patient with PCOS than in healthy subject.Ansam A *et al* (2004) [29] found that serum total PSA can be a promising marker in patient with PCOS and hirsute in detectable level by using ELFA.Kocak (2005)[30] found that serum PSA levels in hirsute women were higher than in non-hirsute women. We conclude that total serum prostate specific antigen levels are higher in patient with PCOS,total testosterone levels are higher in patient with PCOS and serum PSA measurement might be marker of hyperandrogenism in females suffering from PCOS.

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Serum leptin level in women with polycystic ovarian syndrome

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Abstract:

Background:

Leptin, a key hormone in energy homeostasis and neuroendocrine function, has a permissive role in the pathogenesis of reproductive dysfunction.

Objective:

To assess the role of serum leptin in women with PCOS and to evaluate leptin and insulin levels in PCOS women before and after treatment with metformin.

Materials and Methods:

Sixty women of reproductive age (18- 38years) were allocated to four groups: 15 obese women with PCOS (BMI >30 kg/m^2), 15 obese controls, 15 non-obese women with PCOS (BMI 18 -30 kg/m^2), and 15 non-obese controls. Serum leptin and insulin levels were measured and compared between case and control subjects also comparison done pretreatment and after treatment with metformin.

Results:

There was a significant increase in leptin in non-obese PCOS group(8.2± 2.73) compared to non-obese control (5.64± 1.43), (P value=0.0032), insulin level was significantly higher in PCOS group (15.87 ±6.65) than control (5.47 ±1.68), (P value<0.001). There was significant decrease in BMI ,leptin and insulin levels after 12 month of metformin treatment in obese and non-obese PCOS subjects.

Conclusion:

Leptin level increased remarkably with increasing body weight and it is higher in non-obese PCOS women in comparison with non-obese healthy women.

.Key words : PCOS, Leptin, Metformin

Introduction

Polycystic ovarian syndrome is the most common endocrinopathy in women, affecting 5–10% of women of reproductive age[1] with onset manifesting as early as puberty.(1)

The principal features of PCOS are anovulation, resulting in irregular menstruation, amenorrhea, ovulation-related infertility, and polycystic ovaries; excessive amounts or effects of androgenic (masculinizing) hormones, resulting in acne and hirsutism; and insulin resistance.[2]

Because of the diversity of clinical and metabolic findings in PCOS, there has been great debate as to whether it represents a single disorder or multiple associated pathologic conditions.The current understanding is that PCOS is not only a gynecological condition but a metabolic syndrome with associated disorders such as insulin resistance and dyslipidemia[3]. Infertility is related to insulin resistance which disturbs the hormonal milieu in these women.

Approximately 50% of women with PCOS are overweight (BMI > 25 kg/m2) or obese (BMI > 30 kg/m2). Women with PCOS usually have so-called central obesity or upper-body obesity, and therefore tend to have an increased waist-hip ratio (WHR), even among subjects of normal weight[4].

Leptin, a key hormone in energy homeostasis and neuroendocrine

function, has a permissive role in the pathogenesis of reproductive dysfunction. It is the product of the ob gene secreted from adipose tissue which signals the amount of energy stores to the brain and is implicated in the regulation of food intake and energy balance.[5]

The very close association between hyperinsulinemia and hyperleptinemia suggests that expression of the (ob)gene, which codes for leptin, may be mediated by insulin both in humans and in rats[5]. In addition, it has been suggested that insulin indirectly regulates leptin secretion due to its trophic effect on the adipocytes[6].Thus, insulin, leptin, body weight, ovarian steroidogenesis and ovulation show complex interrelations. Leptin resistance was introduced in an apparent analogy with that of insulin resistance to explain why hyperleptinemia associated with obesity fails to correct the defect in energy balance and feeding behavior. [7]

Metformin is an antidiabetic drug with anorexigenic properties. It appears to affect ovarian function in a dual mode, through the alleviation of insulin resistance on ovary and through direct effect on ovary. It reduces CYP17 activity in theca cells and reduces steroidogenesis in women with PCOS[8].

The study aimed to assess the role of serum leptin in women with PCOS,to evaluate leptin levels in PCOS women before and after treatment with metformin (an insulin sensitizing agent) and to study the correlations between leptin and other hormonal parameters.

Materials and Methods

This prospective experimental study included 60 women of reproductive age (18- 38years) were allocated to four groups:15 obese women with PCOS (BMI >30 kg/m2),15 obese controls, 15 non-obese women with PCOS (BMI 1830- kg/m2), and 15 non-obese controls.

The controls were volunteers who freely agreed to participate in the study; they had regular cycle and received no treatment for a chronic illness. The diagnosis of PCOS was based on Rotterdam criteria which indicated PCOS to be present if 2 of 3 criteria are met in the female:

(1) Oligo-ovulation and/or anovulation.

(2) Excess androgen activity(clinical or biochemical).

(3) Polycystic ovaries on gynecological ultrasound (10 or more follicles in each ovary, each follicle measuring 2–9 mm in diameter).

The serum of patient was collected after an overnight fast of 10 -12 h, separated after centrifugation into two samples; the first sample was analyzed immediately for FSH ,LH ,Prolactin , Estradiol ,Testosterone and lipid profile. The second sample was stored at -20 c until a time of analysis of leptin and insulin using ELISA technique.

The PCOS patients received metformin tablet 500 mg twice daily (Glucophage® MERK-SERONA) and the above measures were repeated after 12 weeks (serum ,leptin, and insulin) and comparison done between the results before and after treatment with metformin.

Results

Figure (1) shows no significant increase in leptin level in obese PCOS (22.04 ±7.79 ng/ml) in comparison with age and BMI matched obese control group (24.29± 10.06 ng/ml), (P value =0.49), while a significant increase in insulin level noticed in obese PCOS (21.53± 13.52 mIU/ml) compared to obese control (8.79 ±3.21 mIU/ml), (p value =0.0014) as shown infigure (2).

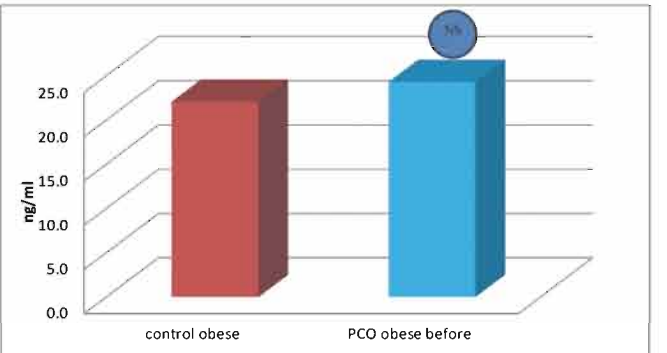

Fig. (1): Comparison of serum leptin concentration between control obese and PCO obese before treatment groups

NS:p value >0.05

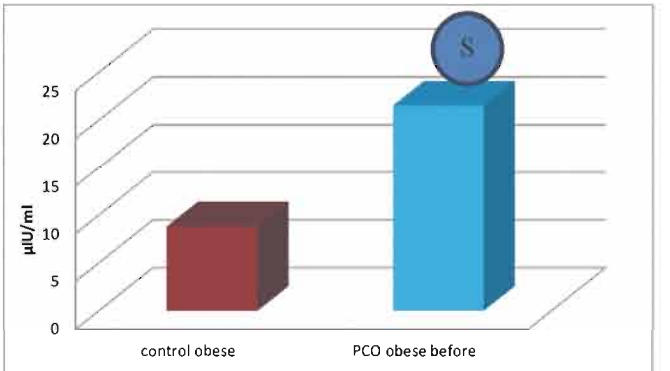

Fig. (2): Comparison of serum insulin concentration between control obese and PCO obese before treatment groups

Figure (3) displays significant increase in leptin level in non-obese PCOS group (8.2 ±2.73) in comparison with non-obese control (5.64 ±1.43), (P value =0.0032). Insulin level was significantly higher in PCOS (15.87± 6.65) than control (5.47 ±1.68),(P value <0.001) as demonstrated in figure (4).

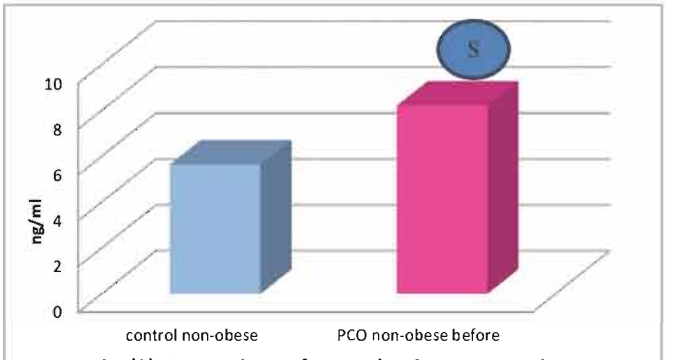

Fig. (3): Comparison of serum leptin concentration between control non-obese and PCO non-obese before treatment groups

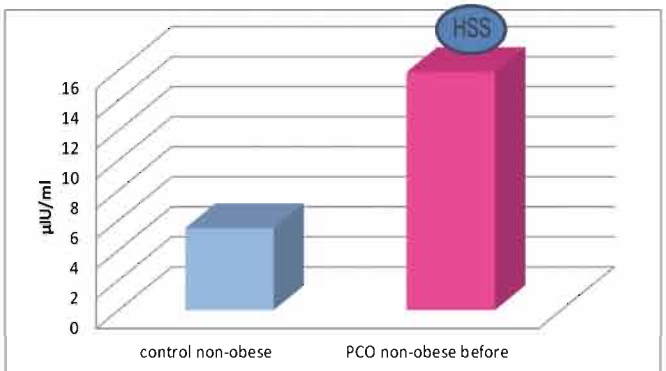

Fig. (4): Comparison of serum insulin concentration between control non-obese and PCO non-obese before treatment groups

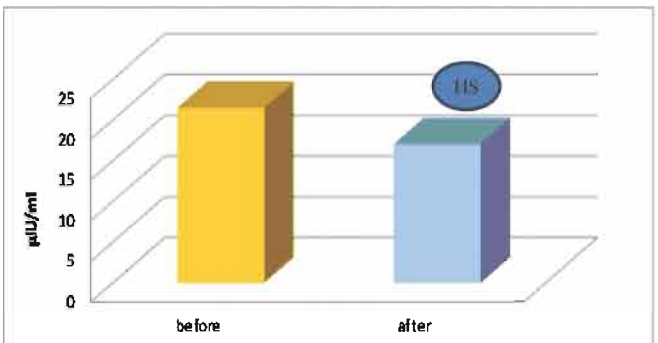

Fig.(7): Comparison of serum insulin concentration between PCO obese before treatment and after treatment groups

There was a significant decrease in BMI after 12 months metformin treatment in obese (33.32 ±2.66) compared to BMI before treatment (35.41± 3.47), P value =0.0032 as shown in figure. (5).

There was a significant decrease in leptin level in obese PCOS after treatment (20.7± 8.56 ng/ml) in comparison to that before treatment (24.29± 10.06 ng/ml) (p value< 0.05) as demonstrated in figure (6). Figure (7) shows a highly significant decrease in insulin levels in obese PCOS subjects after treatment (17.09 ±10.36uIU/ml) compared to that before treatment (21.53± 13.52 mIU/ml),(P value <0.001).

There was a highly significant decrease in BMI after 12 month metformin treatment in non-obese (21.952.05±Kg/m2) in comparison to BMI before treatment (23.572.02±Kg/m2), (P value<0.001) as noticed in figure (8).

There was a highly significant decrease in leptin level(6.272.17±ng/ml) observed in non-obese PCOS subjects after treatment compared to that before treatment (8.22.73±ng/ml)(p value <0.001) as shown in figure (9). Figure (10) demonstrates a highly significant decrease in insulin level in non-obese PCOS subjects after treatment (10.946.03± mIU/ml) compared to that before treatment (15.876.65±uIU/ml), (p value <0.001).

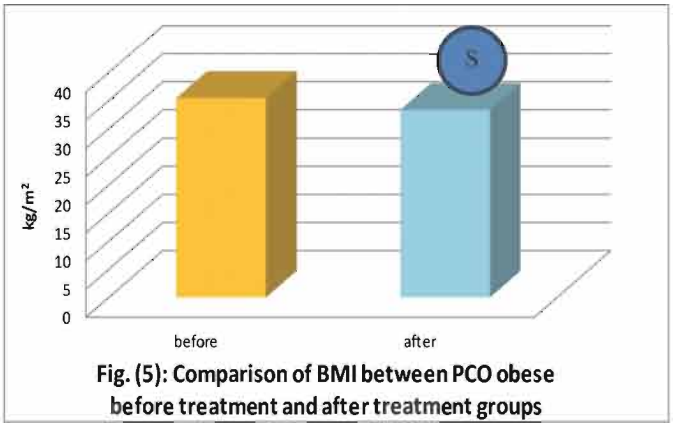

Fig. (5): Comparison of BMI between PCO obese before treatment and after treatment groups

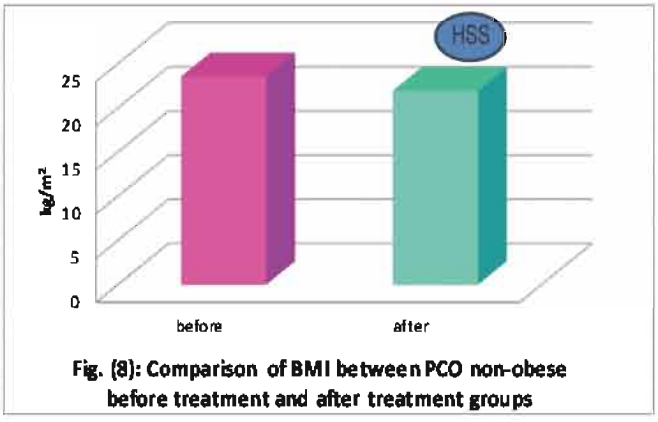

Fig. (8): Comparison of BMI between PCO non-obese before treatment and after treatment groups

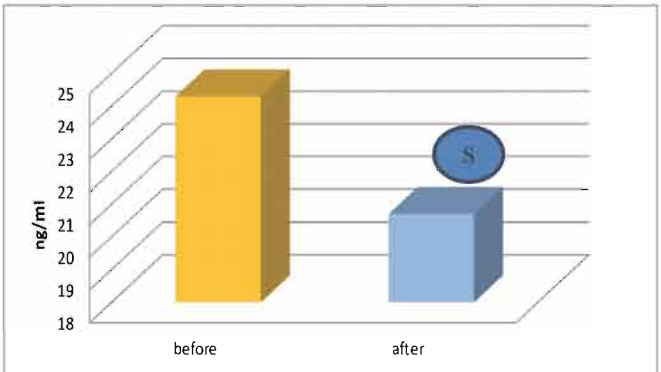

Fig. (6): Comparison of serum leptin concentration between PCO obese before treatment and after treatment groups

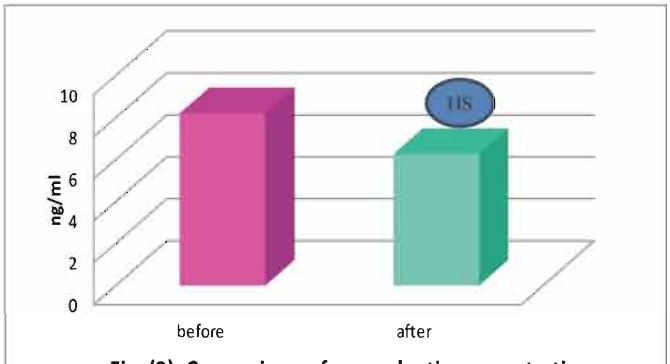

Fig. (9): Comparison of serum leptin concentration between PCO non-obese before treatment and after treatment groups

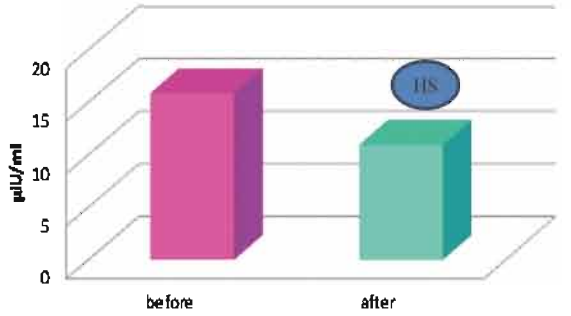

Fig. (10): Comparison of serum insulin concentration between PCO non-obese before treatment and after treatment groups

NS (Non-Significant): P value>0.05
S (significant):P value<0.05
HS (Highly Significant):P value<0.001

Discussion

Recent studies have shown that PCOS is not only a gynecological condition affecting women of reproductive age but also a comprehensive syndrome with a variety of associated metabolic disorders, such as insulin resistance and dyslipidemia.[9] [3] [10]

In the present study, BMI was significantly higher in non-obese PCOS subjects compared to non-obese control .Women suffering from PCOS shown to have higher amount of body fat compared to healthy women even when they are of normal weight [10]. BMI correlate with body fat and the obesity contributes to the manifestations of PCOS by increasing the magnitude of hyperandrogenism and the rates of anovulatory cycles.

Leptin levels are increased in obesity and may play a role in the development of insulin resistance and type 2 diabetes mellitus[11]. In the present study, leptin levels increased significantly with in both obese PCOS women and obese controls. The difference in mean leptin levels in non-obese and obese patients was highly significant.

In the current study there was no significant increase in leptin levels in obese PCOS when compared with obese control subjects, while a significant increase in leptin was found in non-obese PCOS subjects in comparison with Non-obese control.

Metformin treatment tends to restore the secretory capacity of neuropeptide Y(NPY) in obese women with PCOS offer a potential mechanism for the weight-reducing effect of metformin through normalization of appetite regulation in PCOS women.[12]

Metformin treatment resulted a significant decrease of serum leptin concentrations at three months of treatment, this is in accordance with previous studies[12] [13] [14]. Messenger RNA for leptin receptors has been found in both ovarian granulosa and theca cells[15], suggesting a possible direct role of leptin in ovarian function, as metformin may inhibit lipolysis in adipose tissue and thus play some role in the metabolism of fat cells[16], a direct effect of metformin on the secretion of leptin in fat tissue cannot be excluded.

During metformin treatment fasting serum insulin concentration decreased significantly and insulin sensitivity improved in obese and non-obese PCOS women, suggesting that the alleviation of hyperandrogenism brought about by metformin may be mediated by a decreased insulin action. Insulin stimulates leptin production *in vitro*[17] and in vivo[18].

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Role of leptin in infertile men before and after treatment with clomiphene citrate and vitamin E

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Abstract:

Background:

Leptin is an adipocyte –secreted protein that participates in the regulation of energy homeostasis. It prevents the body from storing fat, controls hunger and cravings, regulates food intake and energy expenditure, provides the body with an index of nutritional status and controls the whole body fat metabolism. Leptin is a product of ob gene created by adipocytes. It seems to signal metabolic information to the reproductive system.

Objective:

The aim of this study is to evaluate the relationship between serum leptin and infertility in oligozoospermic men.

Materials and Methods:

Seventy men were investigated; fertile normozoospermic as a control (n = 35) and infertile oligozoospermic (n =35). The patients underwent estimation of body weight (kg), height (cm), body mass index (BMI), semen analysis, serum FSH,LH, testosterone and leptin, lipid profile and serum malondialdehyde (MDA). The infertile group was given a treatment course of clomiphene citrate (50 mg) and vitamin E (400mg) and then all the previous parameters were reevaluated after 3 months .

Results:

Mean body weight was significantly higher in infertile oligozoospermia compared to controls.The height showed no significant difference between the two groups.Hormonal profile revealed significant (P=0.0001) difference in FSH between control group and oligozoospermic group (7.04± 4.20 versus 3.35± 1.47 mIU/ml), but after treatment with clomiphene citrate and vitamin E there is a slight non-significant (P=0.231) decrease in the level of FSH in oligozoospermic group.The level of testosterone showed a statistically significant (P=0.0001) difference between fertile normozoospermic (5.52 ±1.29 ng/ml) and infertile oligozoospermic (2.40 ±0.96 ng/rnl).This hormone showed a significant (P=0.0001) increase in its level after treatment with clomiphene citrate and vitamin E (3.48± 1.56 ng/ml). Other hormone showed non significant difference between the two groups pre and post treatment. Lipid profile showed no significant difference between the two groups . There was no significant (P=0.477) difference in serum MDA between the control and the infertile groups (4.69± 1.32 vs. 5.18± 1.19 µmol/L) respectively,but after treatment the results showed a mild significant (P=0.026) decrease (4.30± 0.67 µmol/L) in comparison to the level in the control group and also a significant (P=0.0001) decrease in comparison to the level before treatment. Infertile oligozoospermic had significantly (P<0.0001) higher serum leptin level (8.03± 1.22 ng/ml) than control (3.62 ± 1.00 ng rnl).

Conclusion:

Serum leptin demonstrated a significant positive correlation with age, body weight, BMI and a significant inverse correlation with serum testosterone. It had nonsignificant correlation with the height and sperm concentration. These results are suggestive of a link between the adipocyte derived hormone leptin and male reproduction.

Key words: Leptin, Male infertility, FSH, LH.

Introduction

In our life millions of men and women use condoms, diaphragms, or any way of contraception to avoid pregnancy, meanwhile about (9- 14)% of couple can not conceive, to them these seem tragic, sadness which could lead to disruption of marriage[1]. Infertility is a term used to define the in ability to conceive despite regular unprotected sexual intercourse over a specific period of time, usually either 1or 2 years; in fact, around 10- 15% of couples have to wait more than 12 months, the time proposed by the WHO as the maximum normal limit before achieving pregnancy[2]. There is anincrease in the percentage of infertility problem in the last years, but this may be due to recent advances in infertiltiy treatment and access of patients to such information have led to early presentation of these patients and their request for treatment.This may give a false impression of an increasing infertility problem. However, there is concern that male fertility is declining due to environmental factor.Male factors alone constitute (25 -30)% of infertility[3]. and they contribute to another 30% in combination with female factor. Known etiology of male infertility can be divided according to affecting factor into pretesticular, testicular and post testicular factors. Primary testicular disorders are most commonly responsible include cryptorchidism, testicular tortion or trauma, genetic defect, varicocele, gonadal dysgenesis, infection as mumps[4]. The most common pretesticular causes, hypogonadisim, estrogen excess, hyperprolactinemia, medical cause as liver disease[5], and post testicular causes due to obstruction of reproduction channel either congenital or acquired. Other causes are anti-sperm anti-bodies and obesity [6] .

Leptin is an adipocyte –secreted protein that participates in the regulation of energy homeostasis It prevents the body from storing fat, controls hunger and cravings, regulates food intake and energy expenditure, provides the body with an index of nutritional status and controls the whole body fat metabolism[7]. Leptin is a product of ob gene created by adipocytes. It seems to signal metabolic information to the reproductive system

Male ob/ob mice treated with leptin have elevated serum level of FSH, increased testicular and seminal vesicle weight and elevated sperm count, leptin has been shown to stimulate GnRH as well as LH and FSH secretion. *In vitro* results suggest that testosterone may be important regulator of leptin secretion[8]. A strong association between serum level of testosterone and leptin was reported in untreated and testosterone treated hypogonadal men[9].The aim of the present study was to assess the potential contribution of leptin to the male infertility by studying serum leptin, gonadotrpins, testosterone, lipid profile, malondialdehyde and the effect of medical treatment (clomiphene citrate and vitamin E) on the above mentioned parameters

Materials and Methods

Sample collection

This prospective study enrolled a total of 70 males attending outpatient clinics at High Institute of Infertility Diagnosis and Assisted Reproductive Technology, Alnahrain University. The mean age of the fertile men was 34.37 ±6.73 (range 23 -45 years) and for the infertile is 33.86 ±5.99 (range 21- 45 years). All of them had been married for more than 2 years. They were divided into: fertile normozoospermic (n = 35), infertile oligozoospermic (n = 35) groups. Individuals with chronic disease e.g. diabetes, liver or renal diseases, patient with infection as history of mumps, orchitis or any previous operation, obstructive azoospermic, highly obese subjects cases were excluded. A detailed medical history, physical examination, estimation of body weight (kg) and height (cm) were carried out. Body mass index (BMI) was calculated by dividing the weight (kg) by square of the height (meters).

Semen samples collection:

Ejaculates were obtained in the morning (7.00 hours and 9.30 hours) after 5 days of sexual abstinence. The samples were examined immediately after liquefaction according to WHO guidelines.

Blood Samples:

About 10 ml of blood was withdrawn from the patient and control subjects. Samples were collected between 8 to 10 a.m. after 10 -12 hours fasting, the blood was allowed to clot at 37°C in an incubator. Serum was separated after centrifugation at 3000 rpm for 10 min. and kept in polypropylene tubes at - 20°C until time of estimation.

Biochemical analysis :

Serum leptin, FSH, LH, testosterone and prolactin, lipid profile and malondialdehyde level were measured in the control and infertile patients prior to treatment. All infertile groups received clomiphene citrate (50 mg) and vitamin E(400 mg) and then all of the aforementioned parameters (serum leptin, LH, FSH, prolactin, testosterone and MDA and lipid profile) were measured .

Blood samples were obtained from the patients and control subjects. prepared by centrifugation (3000 rpm for 10 minutes). Hormones were examined by MiniVIDAS® (bioMerieux, France)

Serum total cholesterol, triglyceride,HDL and LDL were determined by enzymatic colorimetric method using commercially available kit (biomaghreb).The concentration of serum MDA was determined using spectrophotometric method. Serum leptin was measured by ELISA.

Results

The mean sperm concentration of fertile control group was (69.86 ± 18.27x10^6/ml) (range 35–120x10^6/ml)and of oligozoospermic group was (12.68 ± 6.00x10^6/ml)(range 2–19x10^6/ml).After treatment with clomiphene citrate and vitamin E,there is a slight increase in sperm concentration (15.28 ± 8.18x10^6/ml)(range 225-x10^6/ml)(Table1).The mean body weight was significantly ($P<0.017$) higher in oligozoospermic group than in the control. The body mass index(BMI) was significantly ($P<0.020$) higher in oligozoospermic group than in the control .Serum FSH is much higher in oligozoospermic group than in the control and there is a slight decrease in its value after treatment with clomiphene citrate

and vitamin E as shown in Table 1. LH and prolactin concentrations and lipid profile showed no significant difference between the two groups and even after treatment with clomiphene citrate and vitamin E.

Serum testosterone showed a significant (P<0.0001) difference between the studied groups. It is much lower in oligozoospermic group than in the control. Treatment with clomiphene citrate and vitamin E caused a significant (P<0.0001) increase in its value. Serum leptin is much higher in infertile oligozoospermic group than in the control but after treatment with clomiphene citrate and vitamin E there is a significant decrease (Table 1). Serum MDA shows no difference between the two groups but after treatment with clomiphene citrate and vitamin E there is a significant (P<0.001) decrease in comparison to its pretreatment value. There is a significant inverse correlation between leptin and testosterone.

Table 1. Age,height,weight,BMI,sperm concentration,hormones,lipid profile and serum leptin in normozoospermic and oligozoospermic men after treatment with clomiphene citrate and vitamin E .

Parameters	Control normozoospermic men	Infertile oligozoospermic men (pretreatment)	Infertile oligozoospermic men (post treatment)
No.	35	35	25
Age (years)	34.37 ±6.73	33.86 ±5.99	-
Height (cm)	173.43 ±8.66	175.29± 8.43	-
Weight (kg	75.36± 8.19	81.09± 10.35	-
BMI (kg/m2)	25.15 ±2.09	26.31± 1.99	-
Sperm concentration ($x10^6$/ml)	69.86± 18.27	12.68± 6.00 P=0.0001*	15.28 ±8.18 P=0.0001*
FSH (mIU/ml)	3.35± 1.47	6.79± 4.20 P=0.0001*	6.04± 2.87 P=0.0001*
LH (mIU/ml)	2.97± 1.36	2.88± 1.63 P=0.936	3.25± 1.40 P=0.439
Prolactin (ng/ml)	10.45 ±3.98	12.32 ±3.96 P=0.015*	11.35± 4.36 P=0.372
Testosterone (ng/ml)	5.52± 1.29	2.91 ±0.96 P=0.0001*	3.84± 1.56 P=0.0001*
Cholesterol (mmol/L)	4.20± 0.74	4.31 ±0.99 P=0.969	4.16± 0.74 P=0.823
Triglyceride (mmol/L)	1.92 ±0.74	1.71± 0.70 P=0.127	1.66± 0.53 P=0.144
HDL (mmol/L)	0.81 ±0.25	0.84± 0.25 P=0.962	0.91± 0.11 P=0.083
LDL (mmol/L)	2.96± 0.89	3.02± 1.02 P=0.631	2.90 ±0.87 P= 0.812
Leptin (ng/ml)	3.62± 1.00	8.08± 1.22 P= 0.0001*	3.11 ±1.56 P=0.128
MDA (umol/L)	4.96± 1.32 P=0.477	5.35± 1.19 P=0.001*	4.30± 0.67

Discussion

In the present study the infertile oligozoospermic group demonstrated higher serum leptin levels compared with the fertile normozoospermic groups. Similar results were reported previously by Steinman *et al.*[10] and von Sobbe *et al.*[11].

After the course of treatment of Oligozoospermic men with clomiphene citrate and vitamin E, a significant decrease in serum leptin was found. The cause of these changes in leptin level is not yet known because the real mechanism of how the Leptin act in the regulation of the reproduction is not yet well established.

Conflicting reports on whether circulating leptin level change with age.The current study showed that serum leptin had linear correlation with patients age, explained by the increase in body fat mass and/ or decrease serum total testosterone with increasing age. Isidori *et al.*[12] reported that adult humans of different body weights showed gradual decline of serum leptin levels during ageing higher, while Koistinen *et al.*[13] showed that fasting serum leptin levels were similar in different age groups in males. In contrast, Robert *et al.*[14] found no effect of age on the relationship between circulating leptin and body fat mass. Ostlund *et al.*[15] reported that leptin was inversely related to age even after adjustment for percent body fat and gender. Therefor, age and weight also be an important regulator of plasma leptin. In rats leptin gene expression increase with age, independently of increasing adiposity[16].

There was a significant positive correlation between human BMI and serum leptin, in the present study there is a significant difference between oligozoospermic and control. weight and BMI are higher in oligozoospermic, even in control patient there is positive correlation between BMI and leptin .Considine *et al.*[17] and Ostlund *et al.*[15] explained these relations by the increased release of leptin from large than small fat cells(Lonnqvist *et al.*)[18] On average, leptin release per gram adipose tissue is two times greater in obese than in lean subjects. In addition, an increased number of fat cells, particularly in extreme obesity, contribute to increase in serum leptin as the fat mass is the main regulator of leptin levels. Campostano *et al.*[19] and Reseland *et al.*[20] demonstrated that the long-term changes in lifestyle through decreased intake of dietary fat and increased physical activity could reduce plasma leptin in humans beyond the reduction expected as a result of changes in fat mass.

In the current study there is a significant difference between FSH in control and infertile and this is because of the negative feedback mechanism in steriodogenesis and spermatogenesis. After treatment with clomiphene citrate and vitamin E, there is a mild decrease in serum FSH. This agreed with Wang *et al.*[21] and Check *et al.*[22] who found positive effect of the treatment, while MicicDotlic[23] and Sokol *et al.*[24] found no efficacy over placebo in use this treatment with oligospermic men .

The results of the present study revealed a significant inverse correlation between leptin and testosterone. This inverse correlation matched the lower serum leptin in males than in females due to the higher serum testosterone[25]. This correlation was explained by binding of testosterone to androgen binding receptors on the adipocytes with subsequent increase in lipolysis or direct suppressive effect on ob gene expression[26]. Also, leptin possibly has a direct inhibitory effect on testosterone production by binding to Leydig cells[27] and it appears to act as a direct inhibitory signal for testicular steroidogenesis [28].It has been observed in obese men that the peripheral leptin receptors in the testis are directly exposed to high-leptin concentrations with possible negative effects on gonadal functions[29]. Although Soyupek *et al.*[30]. The suggested effect of leptin on reproductive functions originated from the systemic effect.

In this study we found no significant difference in lipid profile between the normal control and the infertile oligozoospermic men pre and post treatment. This agreed with Yamamoto *et al*[31]and Brinsko *et al*[32] who observed that the concentrations of serum lipids are not related with quality of semen parameters in infertile men. Recently, it has been reported that increased VLDL and triglyceride as well as decreased serum testosterone were significantly correlated with decreased sperm motion characteristics[33]. Other semen parameters (e.g. sperm concentration, normal morphology) did not show any correlation with serum lipids

Malondialdehyde (MDA) is a marker of lipid peroxidation and increases in oxidative stress states[34] In this current study the result of MDA measurment in fertile normozoospermic and infertile oligozoospermic pretreatment shows the same level with no significant difference between the two means. But after treatment the measurement shows a significant decrease in comparison to the level in pretreatment group and also a mild significant decrease in comparison to the level in control group.This is in good agreement with Nweke *et al.*[35] who studied the effect of modest supplement of Vitamin E on lipid peroxidation. Vitamin E is an important lipid soluble antioxidant molecule in the cell mernbrane. It is thought to interrupt lipid peroxidation and enhance the activity of various antioxidants that scavenge free radicals generated during the univalent reduction of molecular oxygen and during normal activity of oxidative enzymes[36]. The results of *in vitro* experiments suggest that vitamin E may protect spermatozoa from oxidative damage and loss of motility .Recent randomised control trials have reported vitamin E to be effective in treating infertile males with high-ROS levels[37]. Vitamin E treatment decreased malondialdehyde (MDA) concentrations. The same observation was made by Veniza *et al.*(38) after vitamin E treatment also found decreased MDA value[38].

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Measurement of serum Inhibin B as a predictive evaluation of ovarian response following ovulation induction program in intrauterin insemination

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Abstract:

Background:

The evaluation of ovarian reserve has been, and still, the focus of substantial clinical research. The assessment of ovarian reserve is valuable for determining stimulation protocols, and because of limited predictive value of age alone, or other passive hormonal analysis in estimating response to the exogenous stimulation, Dynamic research was done on serum inhibin B.

Objective:

Measurement of serum inhibin B as a predictive evaluation of early ovarian response following ovulation induction program in intrauterine insemination(IUI).

Materials and Methods:

Thirty couples were involved in IUI program. They divided into two groups: group (1) 15 women were treated by Gonadotropin(Gn) recombinant follicle -stimulating hormone (rFSH). While group (2), 15 women were treated by Clomiphene citrate (CC). Treatment started at day 3 of the menstrual cycle. Serial hormonal profile tests namely; inhibin B, Estadiol (E2), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH) were done at days 3, 7 of the menstrual cycle and the day of human chorionic gonadotropin hormone (hCG) injection with recurrent ultrasonograghy (U/S) to confirm the number and development of the follicles.

Results:

There was a significant difference in the level of inhibin B ($P< 0.05$) between day3 and 7 of the cycle with the mean of the follicle number 1.67± 0.18 in group(1), while in group (2) there was a non significant difference ($P>0.05$) in the level of inhibin B between day 3 and 7 of the cycle with the mean of the follicle number 2.14 ±0.17. Percentage of pregnancy rate in IUI in this study was 19.23%.Percentage of pregnancy rate in group (1) was 25% ,while in group (2) was 14.28% ,but there is a significant difference ($P<0.05$) in the pregnancy rate between the two groups.

Conclusion:

It was concluded that serum inhibin B obtained at day 7(day 5 of (Gn) therapy) offers an early and accurate prediction of ovarian response to (Gn) stimulation while there was no significant change in the level of inhibin B between day 3 and 7 of the cycle in patients treated by CC .

Key Words: Inhibin B, Gonadotropin, Clomiphene Citrate, IUI.

Introduction

The hormonal control of ovarian function by gonadotropins plays a key role in the physiologic process of follicular growth and differentiation, that is selection of a single dominant follicle followed by a mono ovulation[1]. A patient's ovarian response to stimulation medicine is mainly determined by her ovarian reserve, which comprises the quantity and functional capacity of follicles. Pre-evaluation of ovarian reserve and prediction of ovarian response would provide a valuable means of assisting clinicians in selecting appropriate dose of therapeutic agents for each patient[2]. Traditional criteria used to predict ovarian response to ovarian stimulation drug include the patient's age ,baseline serum concentration of hormones such as (FSH), (LH), Estradiol (E_2), FSH to LH ratio[3]. Inhibin B was suggested as an early sensitive predictor of ovarian reserve [4].

Inhibins are dimeric polypeptides produced by granulosa cells and composed of an α-subunit along with a βA-subunit (inhibin A) or a βB-subunit (inhibin B). Inhibin B concentrations rise across the luteal-follicular transition and peak in the mid follicular phase, suggesting that they are secreted by the developing cohort of follicles, and may mark the number or quality of developing follicles at the baseline [5]. With the better understanding of the control, synthesis and secretion of the inhibins and their potential endocrine role in the menstrual cycle, attention has turned to the possibility of this family of peptides to provide a more direct index of ovarian reserve[6,7].

Therefore, this study was designed to identify prospectively the values of basal and dynamic measurements of inhibin B following ovarian stimulation therapy (recombinant FSH or CC) in predicting early ovarian response and pregnancy outcome in IUI.

Materials and Methods

All the females age involved in this study were (19 -39) years old with regular menstrual cycle excluding poly cystic ovarian syndrome (PCOS), FSH<15(mIU/mL), and normal prolactin level. They are examined generally and gynecological after thorough history taking[8] . Assessment of male infertility by the physician is based on a patient's history, physical examination, and semen analysis[9].

Group (1) treated by Gonal- F(R) 75 IU on alternate day[10], while group (2) treated by CC tablet containing 50 mg/ (1×2)/ daily from day 3 to day7 of the menstrual cycle[11]. The ultrasonography study was performed at day 3 of the cycle to detect antral follicle number and diameter in the two groups, and any uterine abnormalities[12].

Diameter of the follicle (s) was monitored by serial examinations of vaginal ultrasound . At least one or two mature (dominant) follicles were recognized about 17 – 20 mm , supplemented by the ovulatory hormone Ovitrelle(R) (Merck/Serono) (hCG) 250 µg, 6500IU was used to induce ovulation. *In vitro* Activation Technique: Simple layer procedure was performed for the semen samples preparation that involved in this study. Then IUI was done by IUI catheter after 34 -36hr[13]

Hormonal assays, serum levels of Inhibin B (pg/mL), FSH (mIU/mL), LH (mIU/mL) and E_2 (pg/mL)were measured by using ELISA(enzyme-linked immunosorbent assay) at days (3,7 of the cycle and the day of hCG injection).

Results

The basal level of inhibin B in group (1) at day 3 of the menstrual cycle mean ±SE was 40.94± 1.99, at day 7 of the cycle, the mean ±SE of inhibin B was 50.88± 1.87. At day of hCG injection, the mean ±SE was40.35 +3.11. There is statistically significant difference (P<0.05) among the three values as shown in Figure (1).

The basal level of inhibin B mean±SE in group (2) at day 3 of the menstrual cycle was 39.69 ±3.41, at day 7 of the cycle ,it was 36.71± 3.61, while at day of hCG injection , the mean±SE was 31± 4.25. There is statistically no significant difference(P>0.05) among the three values as shown in figure (2).

The basal level of E2 in group (1) at day 3 of the menstrual cycle mean±SE was 42.53 ±3.20 , at day 7 of the cycle, the mean ±SE was 97.18 ±13.90. At day of hCG injection , the mean ± SE was 203.52 ±28.47. There is statistically highly significant difference P<0.001among the three values as shown in figure (3)

The level of E2 in group (2) at day 3 of the menstrual cycle mean ±SE was 50.493.97±. At day 7 of the cycle, the mean±SE was 118.24± 15.45. While at day of hCG injection, the mean±SE was 342.46± 64.30. There is statistically highly significant difference P<0.001among the three values as shown in figure (4).

Regarding the clinical characteristic of patients in two groups there were significant deference in follicle number as shown inTable (1).

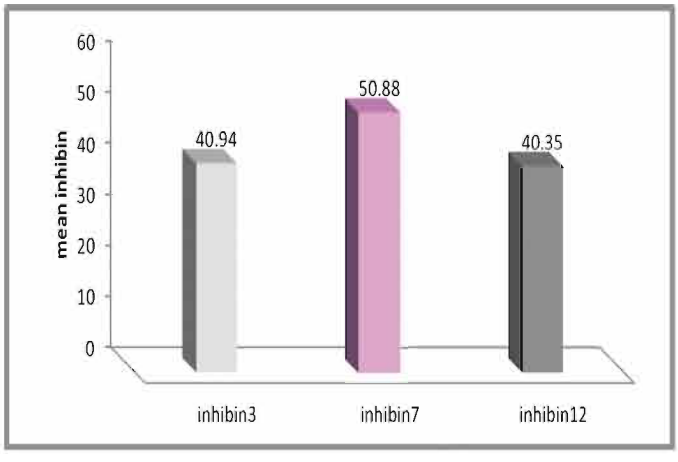

Figure (1)inhibin B level changes in group (1) after treatment by rFSH

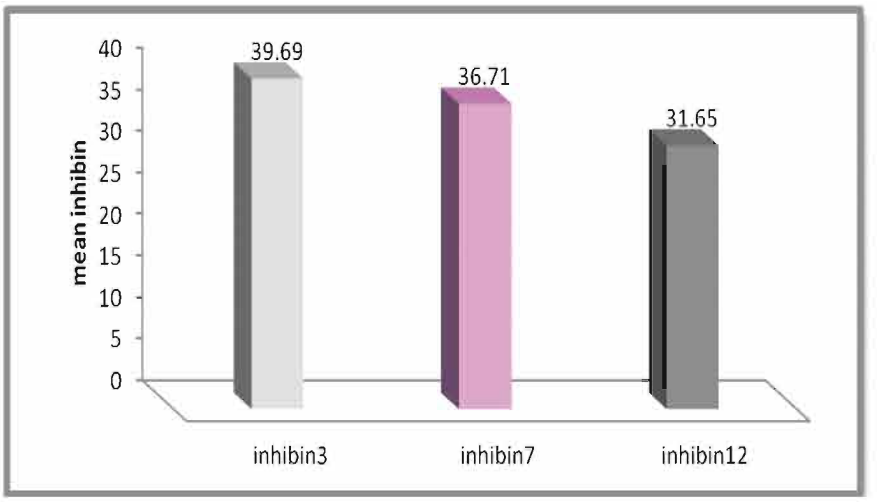

Figure (2)inhibin B level changes in group (2) after treatment by (cc)

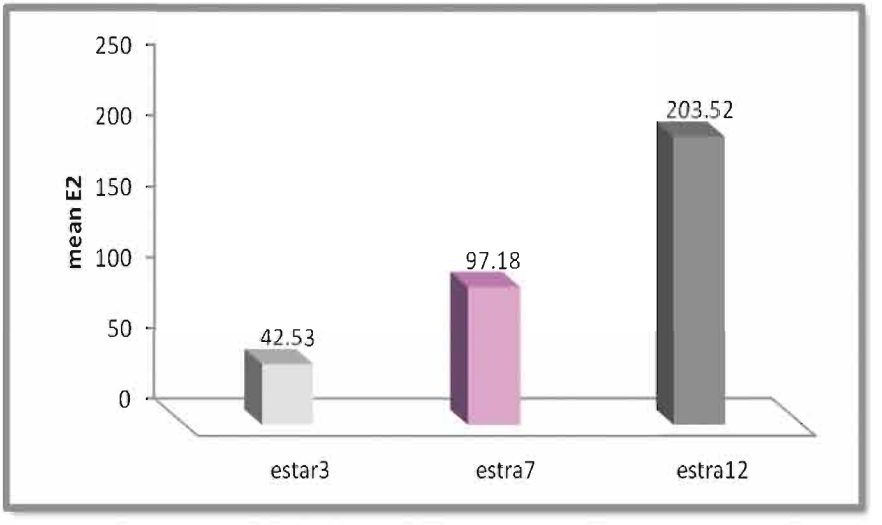

Figure (3) E2 level changes in group (1) after treatment by rFSH

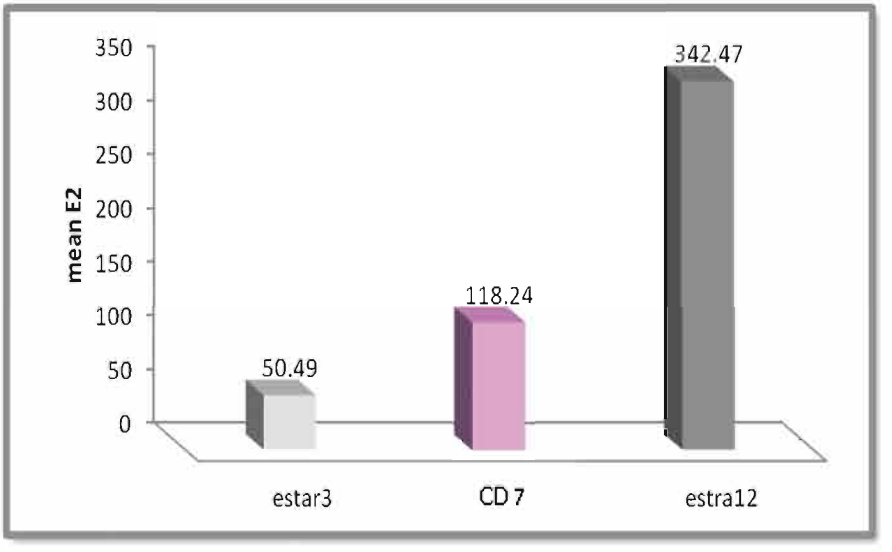

Figure (4) E2 level changes in group (2) After treatment by (CC)

Table (1) Comparison clinical characteristic of patients in the two groups(1) patients treated by rFSH ,(2) patients treated by CC.

Characteristics	Group 1	Group 2		LSD	P value
Age	29.50 ±1.64A	29.07± 1.78A		6.89	0.129 NS
BMI	27.02± 1.00A	28.95± 0.53A		4.37	0.161NS
Antral follicle number	7.83± 0.53A	9.07 ±0.70A		3.37	0.198 NS
Follicle number	1.67+ 0.18B	2.14± 0.17B		2.45	0.007*
Endometrial thickness	8.99± 0.17A	8.81 ±0.18A		3.24	0.201NS

NS-non significant *-significant (P<0.05)

Table(2): Comparison between the pregnancy outcome and (hormonal levels of inhibin B, E_2 , and various variables) in group(1) .

Parameter	Pregnancy	No	Mean	SEM	P value
Age	Yes	3	26.00	3.51	0.236
	No	9	30.67	1.80	
Antral follicle	Yes	3	9.33	1.33	0.107
	No	9	7.33	0.50	
Follicle number	Yes	3	2.00	0.57	0.329
	No	9	1.56	0.17	
Endometrial thickness	yes	3	8.9333	0.26	0.856
	No	9	9.0111	0.22	
inhibinCD3	Yes	3	47.9933	1.87	0.006*
	No	9	38.5911	1.40	
inhibinCDV	Yes	3	55.8967	1.37	0.127
	No	9	49.2078	2.21	
Inhibin B day hCG injection	Yes	3	42.7000	4.13	0.685
	No	9	39.5767	4.00	
E2-CD3	Yes	3	47.6533	5.19	0.382
	No	9	40.8322	3.88	
E2-CD7	Yes	3	96.4733	32.99	0.978
	No	9	97.4233	16.23	
E2-Day of hCG injection	Yes	3	244.73	71.31	0.430
	No	9	189.79	31.11	

Group(1)- patients treated by rFSH

- P<0.05 significant difference

Table(3): Comparison between the pregnancy outcome and (hormonal levels of inhibin B , E_2 , and various variables) in group(2)

Parameter	Pregnancy	N	Mean	SEM	P value
Age	Yes	2	34.50	2.50	0.227
	No	12	28.17	1.93	
Duration of infertility	Yes	2	5.00	1.00	0.761
	No	12	4.58	0.51	
Antral follicle	Yes	2	10.00	2.00	0.612
	No	12	8.92	0.78	
Follicle number	Yes	2	2.50	0.50	0.433
	No	12	2.08	0.19	
Endometrial thickness	Yes	2	9.25	0.55	0.345
	No	12	8.74	0.19	
Inhibin B- CD3	Yes	2	36.69	5.76	0.735
	No	12	40.19	3.92	
InhibinB- CD7	Yes	2	34.24	3.34	0.792
	No	12	37.12	4.20	
InhibinB-day hCG	Yes	2	24.58	10.67	0.519
	No	12	32.82	4.72	
E2-CD3	Yes	2	53.53	3.97	0.769
	No	12	49.99	4.61	
E2-CD7	Yes	2	1.82.07	68.21	0.092
	No	12	107.60	13.66	
E2-Day hCG	Yes	2	704.64	326.36	0.014*
	No	12	282.10	42.06	

Group(2)- patients treated by CC.
* - P<0.05 significant difference

Discussion

In group (1),

There was a significant difference (P< 0.05) in the level of inhibin B among day 3 , 7 of the cycle and day of hCG injection. This result is in agreement with the those authors[5,14] reported that inhibin B is the predominant inhibin in the small antral follicles rising in the early to mid follicular phase , then falling throughout the late follicular phase and remaining low for the duration of the luteal phase.

In this study, it was detected that E_2 at day of hCG injection is more sensitive index of ovarian response to Gn stimulation, this result agrees with the other authors[15] who reported that , during ART cycle, plasma E2 measurements are routinely used to calibrate the Gn doses in conjunction with data obtained by ultrasound, and its directly related to follicular size and that the contribution of mature follicles to E2 out put may be estimated at about 200pg/ml. While there is a better correlation of inhibin B during the mid-follicular phase, when granulosa cell function is strongly dependent on FSH[16].

There was significant difference (P<0.05) of LH level among day 3, day 7 and the day of hCG injection, this is due to the fact that sign of LH measurements during Gn stimulation are usually restricted to the detection of the endogenous LH surge, specially required for women undergoing IUI[15].

2-Group(2) received clomiphene citrate

There was no significant difference (P>0.05) in the level of inhibin

B among day 3, day 7 of the cycle and the day of hCG injection, also no significant difference (P>0.05) in the level of FSH among day 3, 7 and the day of hCG injection due to the fact that clomiphene citrates long retension within tissues, so physiologic response of the developing follicles is assumed to overcome the influence of CC on the hypothalamic-pituitary axis and reduce FSH levels to normal range by cycle day 10 as shown in figure (2 -4)[17]. Our results agree with the facts[18] reported that the rate of follicular growth and number of follicles in patient who received CC from cycle days 5- 9 was significantly better than the group who received CC from cycle day 2 -6.

Pregnancy outcome in IUI

There was only significant correlation between basal inhibin B level at day 3 of the cycle and pregnancy rate in group (1), this result agrees with that found by those authers[4], reported that inhibin B serve as a better and direct measure of ovarian reserve. There was a significant correlation between the pregnancy outcome and serum E_2 level at day of hCG injection in group(2), this is in agreement with those authers[15] reported that E_2 is more sensitive index of ovarian response to Gn stimulation during ART cycle.

Percentage of pregnancy rate in IUI in this study was 19.23%. Percentage of pregnancy rate in group (1) was 25% ,while in group (2) was 14.28% with the fact that using swim up *in vitro* activation techniques of the semen samples, gets safely separation of spermatozoa based on their motility and morphology and gained significantly higher percentage of motility[19], but there is significant difference (P<0.05) in the pregnancy rate between the two groups, this is agree with those authors[20] found that in IUI with the use of rFSH get higher pregnancy rate.

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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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Study the effects of glutathione on fertility potential *in vitro* of male diabetic induced mice

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Abstract:

Background:

Diabetes has been found to induce various subtle molecular changes important for sperm quality and function: The mammalian spermatozoa are rich in polyun- saturated fatty acids, making them particularly sensitive to the deleterious effects of lipid peroxidation, which may result in irreversible loss of motility and a low level of fertilizing capacity. An antioxidant, glutathione (GSH) a polypeptide is important in biological oxidation-reduction reactions and improves sperm motility.

Objective:

The study was aimed to investigate the effects of GSH injection on healthy and diabetic male mice fertilization potential *in vitro* following *in vitro* fertilization (IVF)

Materials and Methods:

Inducing diabetes in mice through intra peritoneal alloxan injection. The treatment with GSH divided into two periods 10 and 20 days, studying the *in vitro* fertilization outcomes.

Results:

Glutathione daily IV injected in a period of 10 to 20 days for the diabetic induced male mice significantly increase the sperm concentration and motility and decrease the abnormal and dead sperms percentages in treated group compared to untreated group. The Fertilization rate was significantly ($P<0.05$)increased in mice treated with GSH compared to untreated one.

Conclusion:

Glutathione injections may decrease the oxidative stress caused by diabetes and enhance the *in vitro* fertilization potentials in male mice.

Keywords: Glutathione, diabetic mice, *in vitro* fertilization

Introduction

Diabetics have high levels of oxidative stress, which basically means too many free radicals and not enough antioxidants to neutralize them[(1)]. Diabetics also have low levels of intracellular glutathione. The high levels of oxidative stress and the low GSH levels further complicates the diabetic state which leads to even higher levels of oxidative stress and even lower levels of GSH[(2)]. Glutathione, being the master antioxidant, would naturally be the best choice as an antioxidant. Furthermore, inflammation leads to and contributes to insulin resistance. Glutathione, on top of being the most potent antioxidant, is also a powerful ant-inflammatory[(3)].

It has long been known that spermatozoa produce reactive oxygen species (ROS), mitochondria being the main source of the ROS production. Furthermore, leukocyte infiltration of semen is associated with decreased fertility due to leukocyte-produced ROS[(4)]. Thus oxidative stress is a major factor in the aetiology of male infertility. At the level of the isolated spermatozoon, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of these cells and their ability to support normal embryonic development. [(5)]. A large number of independent clinical studies have demonstrated a correlative relationship between male

infertility and evidence of oxidative stress in the ejaculate[6]. In the suppression of antioxidant enzyme expression, a concomitant increase in peroxidative damage, the disruption of spermatogenesis and an increase in germ cell apoptosis[7]. However, spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of¬ male infertility[8]. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress[9].

In this study the results of IVF of ova by epididymal sperm obtained from diabetic induced mice treated with GSH will be elucidate.

Materials and methods

Experimental Animals

Male albino mice *(Mus musculus)* of age six months were kept in aluminum and plastic cages the dimensions of which were 260mm x 200mm x 140mm. They were supplied with mice food and water *ad libitum*. Mice were provided by ("Mario Negri" Institute for Pharmacological Research, Milano, Italy) and were bred at Charles River Italia (Calco, Lecco, Italy). They were housed at a temperature of 21 ± 1°C with relative humidity of 55 ± 10% and 12 hours light/dark cycle. Procedures involving animals and their care were conducted according international laws and policies[10]. The animals were grouped into four groups; each group consisted of six mice. Group 1: negative control: A group, Group 2: diabetic group + GSH: B10 and B20, Group 3: diabetic group: C10 and C20, Group 4: positive group + GSH: D10 and D20. Mice were sacrificed by cervical dislocation.

Diabetes mice induction[11]

Inductions of diabetes in mice were as follows:

1. A 48- Hour fasting.
2. IP Alloxan injection in (150) mg /kg body weight.
3. After 30 min. of injection the starvation period was ended.
4. After 10 days of injection the blood sugar level was checked by Accu check portable device.

Treatment with reduced glutathione[12]

The doses were prepared, depending on human doses(10 mg/ kg); therefore the mice dose account as 0.35 mg/ mice of normal antioxidant dose. The effective dose was counted as ten times dose used to get the 11.37 mg/mice in 0.5 normal saline 0.9 % daily for B and D group IV through the tail in two periods: 10 days and 20 days.

In vitro **fertilization (IVF) protocols**

Super ovulation program (SOP) and oocyte collection were done as described by Al- Dujaly and Al-Saadi[13]

1. The adult female mice (11 weeks) age were injected IP with 10 IU pregnant mare chorionic gonadotropin (PMCG)
2. After 48 hr. of the 1st. injection, were injected IP with (10 IU) human chorionic gonadotropin (hCG).
3. After (1416-) hrs. female mice were sacrificed and the whole reproductive system was carefully obtained in Petri-dish; thoroughly washed by normal saline 0.09 %.
4. Under a microscope, a slash was made in the ampulla with a fine needle and dragged out the ovulated oocytes to the medium as shown in figure (1).

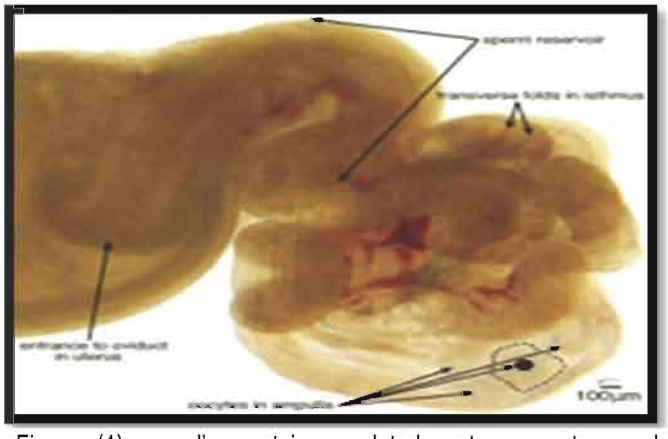

Figure (1) ampulla contains ovulated mature oocytes scale magnification 100x light microscope.

5. By using glass pasture pipette, the oocytes were collected to a new drop of cultured medium (RPMI-1640) + drop of HCG, incubated for (2- 4) hrs. at 37°C in Co2 5% humidity incubator *for in vitro* maturation (IVM) .

2. *In vitro* **fertilization (IVF) protocol and sperms collection**

1. Sperms from adult male mice were collected after sacrificed by cervical dislocation and getting the testis with the epididymus out the animal and washed thoroughly by normal saline 0.09 %.
2. The sperms were collected in the Earls salt medium drop. Sperm counts were made on epididymal sperm released from a single epididymis of each animal figure (2).

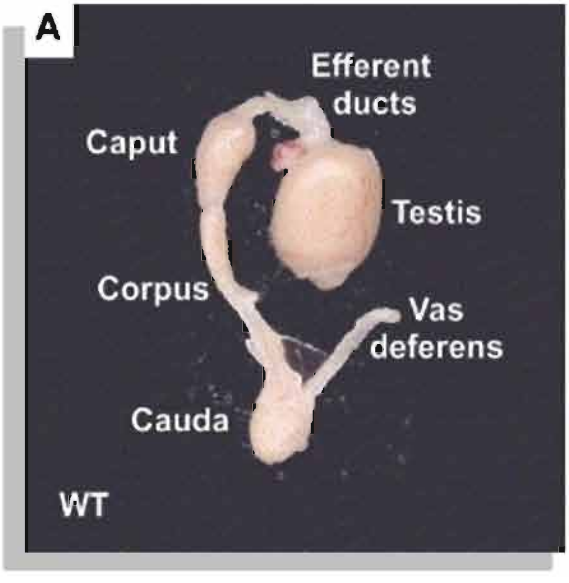

Figure (2) male mice reproductive apparatus,(14)

The tissue was minced with scissors, and then incubated in 0.5 ml of sperm motility buffer (135 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 30 mM HEPES pH 7.4, 10 mM sodium lactate, 1 mM sodium pyruvate, 20 mg/ ml bovine serum albumin, 25 mM NaHCO3). Sperms were allowed to swim up for 30 min at room temperature. Sperm motility was visually monitored under a phase-contrast microscope. Numbers of epididymal sperm were determined by hemocytometer counts either undiluted or diluted 10-fold.

All counts were made in duplicate and averaged, morphology for the sperm>s head and tail. Viability dead/live % (eosin red staining (sigma), were used). The temperature had to be controlled in range of 37◦C in normal humidified environment.

3. Assisted reproduction techniques according to (13): drop of warm 37◦C RPMI-1640 medium in a fertility dish +drop of 5 oocytes from IVM medium + 2

4. µl (250 x 105) of sperms preparation, moderate humidity.

5. Drops of paraffin oil (0.2 ml) were added around the fertility drop of medium to keep the fertility medium out of dryness.

6. The prepared dishes were incubated in 37◦C humidified CO2 5 % for overnight for fertilization.

Statistical Analysis

The data were statistically Analyzed by using analysis of variance (ANOVA) test to show the effect of different factors in the studied parameters. The least significant difference (LSD) was used to compare between means (15)

Results

Sperm evaluation and *in vitro* fertilization

The data in table (1) revealed that the sperm evaluation according to sperm concentration per one micro liter recorded the lowest significant value to the diabetic groups (B and C). The (B) group did not change significantly in comparison with the (A) control group.

The morphologically abnormal and dead sperms per one micro liter showed the significantly lowest value for (B20), the diabetic treated mice. (D10), which showed a significantly higher sperm concentration per one microlitre and (D20) with a significantly higher sperm motility in the culture medium. But, with group (C20), the 20 days of diabetes mice showed the significantly higher abnormal sperm morphology and dead per one micro liter percentage values recorded between all the groups.

Table (1): The sperm characters according to the experimental groups

Groups of mice	Sperm concentration m/ml	Abnormal morphology percentage	Dead sperms/1 µ percentage	Sperm motility percentage
A	1.833.333 ± 12.5	6.60 ± 0.09	18.3 ± 1.03	88.30 ± 4.25
B10	4.400 ± 0.45 a	15.00 ± 0.45	22.00 ± 1.56	87.50 ± 4.25
B20	15.813 ± 0.62	3.60 ± 0.07 a	4.66 ± 0.07 a	80.00 ± 3.79
C10	4.500 ± 0.76	6.50 ± 0.03	7.82 ± 0.26	68.33 ± 3.53
C20	8.462 ± 3.47	40.83± 2.13 b	30.86 ± 1.89 b	31.66 ± 1.73 a
D10	4.786.186 ± 42.69 b	7.23 ± 0.17	14.00 ± 0.78	81.66 ± 4.07
D20	3.436.185 ± 31.56	3.70 ± 0.07	7.90 ± 0.62	97.00 ± 4.91 b

Values: mean± SEM, significant level: P>0.05,
No. mice in each group =6 males
a: the lowest significant value, b: the highest significant value, A: control group, B10: diabetic induced mice treated with GSH for 10 days, B20: diabetic induced mice treated with GSH for 20 days, C10: diabetic induced mice after 10 days diabetes, C20: diabetic induced mice after 20 days of diabetes, D10: healthy mice treated 10 days with GSH, D20: healthy mice treated 20 days with GSH.

Figure (3) shows a images of vital(a and b) and abnormal sperms forms (c) with (d) which is a cross section of testis under an electron microscope showed the normal developing spermatozoon and (e) is the fully developed normal mice sperm under an electron microscope.

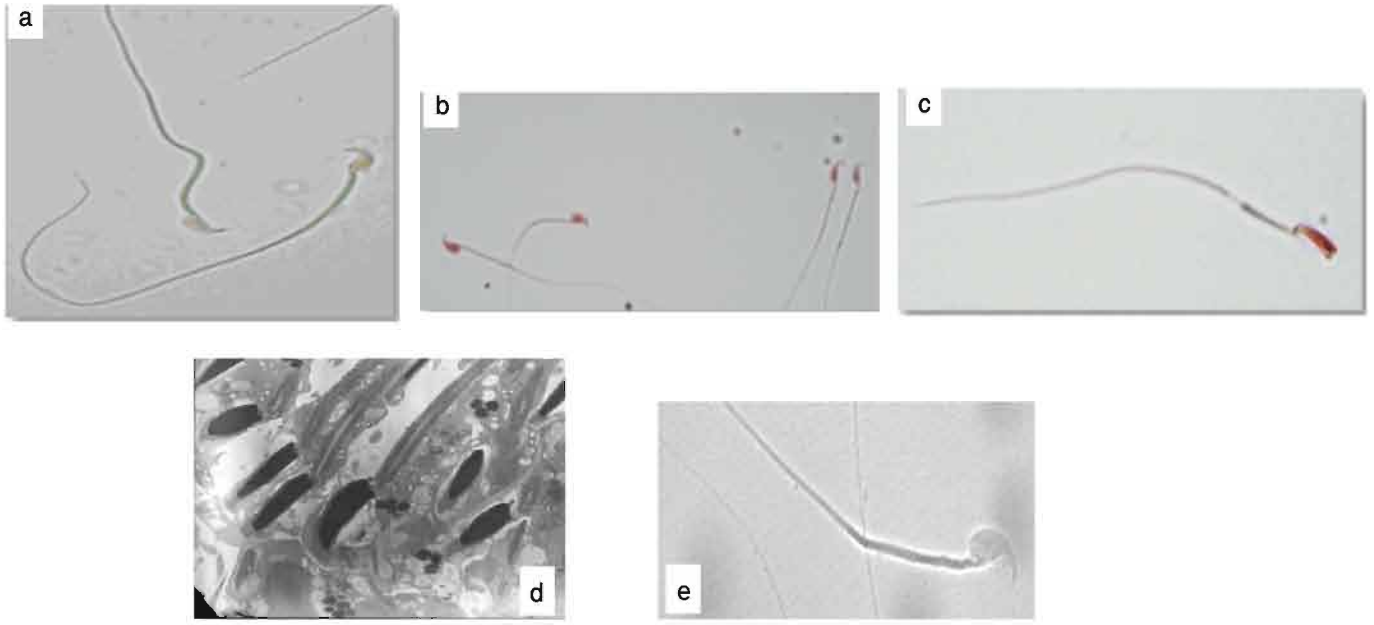

Figure (3) mice sperm, (a:a live sperms, b: dead sperm, c: morphologically abnormal sperms under light microscope power 40x (a: vital sperms, b: dead sperms, c: abnormal sperms) stained by eosin), (D: Sperms in testis cross section, e: normal mouse sperm (under electron microscope) scale bar 5 µm), (captured by the author).

Figure (4) (f) shows the healthy unfertilized oocyte surrounded by cumulus cells super ovulated from female mice 11 weeks of age collected from ampulla under light microscope 40X. Figure (4) (g) shows magnification of one of the ovulated oocytes. All the oocytes in this study have the same criteria, as shown in figure (4),

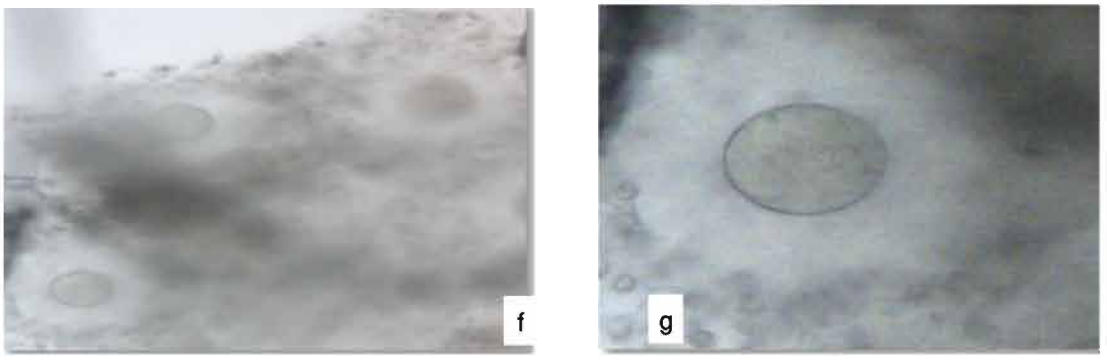

Figure (4) ovulated oocytes, F: ovulated mouse oocytes from ampula surrounded by cumulus cells under 40x power light microscope, g: mature mouse oocyte surrounded by cumulus cells under 40x power light microscope magnified in Microsoft office picture manager, (Captured by the author).

figure (5), which shows that (H): normal one day mouse embryo, (I): normal 2 days embryo (under 40x power dissecting microscope).

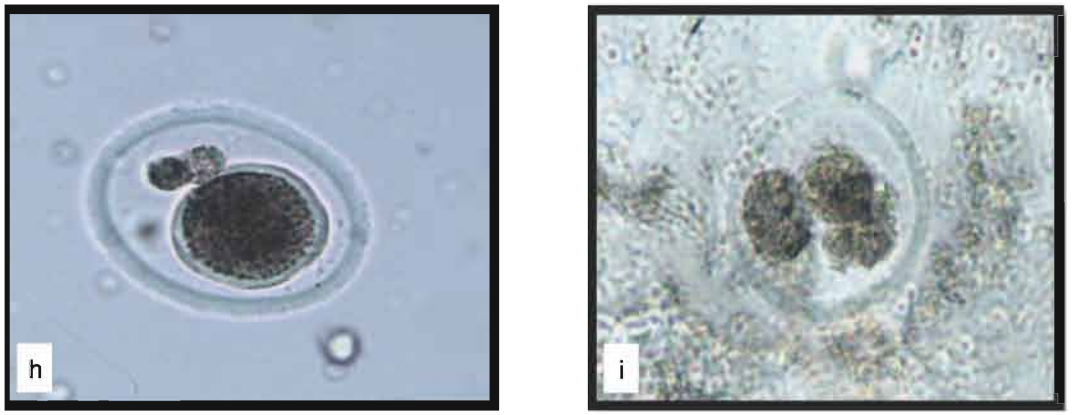

Figure (5) fertilized oocytes evaluation under dissecting microscope,H: normal one day mouse embryo, I: normal 2 days embryo (under 40x power), (Captured by the author).

According to these evaluations, table (2) recorded the percentages of the normal and abnormal fertilized oocytes. (Figwes

Table (2): The *in vitro* fertilization percentage evaluation differences according to the experimental groups:

Groups of mice	Fertilization percentage	Abnormal embryo development percentage
A	78.66 ± 3.67	0.00 ± 0.00 a
B10	38.00 ± 1.89	0.00 ± 0.00 a
B20	63.00 ± 2.65	0.00 ± 0.00 a
C10	16.83 ± 1.57a	70.30 ± 2.89 b
C20	14.70 ± 0.86 a	46.00 ± 1.74
D10	81.16 ± 3.58	0.00 ± 0.00 a
D20	83.30 ± 4.08 b	1.60 ± 0.64

Values are mean± SEM, significant level: P≤0.05,
No. mice in each group= 6 males,

A: control group, B10: diabetic induced mice treated with GSH for 10 days, B20: diabetic induced mice treated with GSH for 20 days, C10: diabetic induced mice after 10 days diabetes, C20: diabetic induced mice after 20 days of diabetes, D10: healthy mice treated 10 days with GSH, D20: healthy mice treated 20 days with GSH.

Discussion

The data in table (1) agreed with[16] and[17] in the highest significant sperm concentration for (D) group, the healthy treated group due to the effect of antioxidants on the testis performance in sperm production.

While the study by Desai, *et al.*,[18] showed no negative or positive effects on sperm concentration. And that disagree with the results which significantly affected by oxidative stress caused by diabetes, the significant affects on the sperm concentration of the (B) group than on the (C) group, which gives a basic role for the antioxidant GSH effect on enhancing the testis function producing more normal healthy viable sperms; even if it does not reach the control healthy level, it is significantly higher than the diabetic group. [19;20] in their study they pointed out that sperm motility is affected by the oxidative stress, and that agreed with the results recorded in table (1), This shows that the role of antioxidant GSH had not exceeded the normal standards in sperm movements but had therapeutic possibilities in diabetic males under an oxidative stress condition. The morphologically abnormal and dead sperms per one micro liter showed the significantly lowest value for (B20), the diabetic treated mice. This result agreed with a suggestion made by (6) that antioxidants protect sperms from oxidative stress and keep it vital. This suggestion agreed with (D_{10}), which showed a significantly higher sperm concentration per one microlitre and (D_{20}) with a significantly higher sperm motility in the culture medium. But, with group (C_{20}), the 20 days of diabetes mice showed the significantly higher abnormal sperm morphology and dead per one micro liter percentage values recorded between all the groups. The significantly lowest sperm movement, according to[21], shows that diabetes oxidative stress leads to an increase in the number of abnormal dead sperms. The negative correlation between the motility percentages and the dead or abnormal sperm percentages per one microlitre in a manner that the less movement the greater abnormal dead sperm percentages[22] agreed with this correlation.

Dead sperms lose the membrane selective permeability and let the stain come inside which turn sperms to pink under a light microscope while the a live sperms were not stained, so sperms look transparent or green under microscope. All the oocytes in this study have the same criteria, as shown in figure(4), to detect all the abnormality fertility outcomes that belong to sperm factors as suggested by[23]. The fertility results, with the sperm evaluation reveded strong evidence on *in vitro* fertilization outcomes, good sperm quality probably gives good *in vitro* fertilization outcomes[24]. It showed significant improvement for the percentage of increasing normal fertilization *in vitro* and decreasing abnormal embryo development. It has been Estimated that this could mean that lowering the ROS activity could protect the sperms morphology and viability, hence fertility performance.

The results of this study agree with a conclusion drawn by Vernet[26], who, assumed that GSH antioxidants led the normal healthy sperms to good fertility outcomes.

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Effect of penicillin on uterus in mice

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Abstract:

Background:

Background: The reproductive failure is a significant public health concern. Although relatively little is known about factors affecting fertility, there is sufficient evidence to hypothesize that antibiotic may influence the fertility.

Objective:

This study was established to explore the individual impact of different doses of penicillin on some reproductive parameters.

Materials and Methods:

Forty adult female albino mice (12 -18 weeks) and weight (25- 28) gm divided into four groups. Control group (G1) was treated daily with normal saline, and other three groups that treated with different doses of penicillin G2 (2mg/kg/B.wt), G3 (3mg/kg/B.wt), G4 (4mg/kg/B.wt)for four cycles twice daily (IM) injection at proestrus phase and sacrificed at estrus phase. Parameters were assessed include, thickness of endometrium, length of epithelial cells, number and diameter of uterine gland using histological section and measurement by (motic image plus).

Results:

The results of this study demonstrate that there is significant decrease ($P<0.05$) in thickness of endometrium, height of epithelial cell, number and diameter of uterine glands of female mature mice after treatment with high doses (3mg/kg/B.wt), while, there is no significant decrease ($P>0.05$) after treatment with (2mg/kg/B.wt); (3mg/kg/B.wt) compare to control group.

Conclusion:

The result showed higher doses for long period of penicillin has impact of some reproductive parameters of mature female mice.

Key word: penicillin , reproduction , mice

Introduction

Fertility is the capacity of the women to conceive at the reproductive age , whereas, fecundity is the probability of achieving live birth in a single menstrual cycle[1].On the other hand, infertility is defined as the inability to become pregnant after 12 months of unprotected intercourse[2].The impact of lifestyle on reproductive performance may vary depending on individual etiology and circumstances. Lifestyle factors have had a dramatic impact on general health and the capacity to reproduce, including; age, weight, smoking, diet, exercise, psychological stress, caffeine consumption, alcohol consumption and exposure to environmental pollutants and antibiotics that have a role in fertility are included[3]. Antibiotic can be divided in to two groups:

1. Bactericidal drug: which kill bacteria with an efficiency of >99.9%.
2. Bacteriostatic drugs, which merely inhibit bacterial growth[4].

Antibacterial drug target interactions are predominantly fall into three classes: inhibition of DNA replication and repair,

inhibition of protein synthesis, and inhibition of cell-wall turnover[5]. The bactericidal antibiotic killing mechanisms are currently attributed to the class specific drug-target interactions[6];[7];[8];[9].

β- Lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam nucleus in their molecular structures. This includes penicillin, cephalosporin, monobactams, and carbapenems[10]. Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics[11]. Because the penicillins may have toxic effect in fertility, therefore, the present study was designated to investigate the effect of different doses of penicillin on the Histological changes in uterus of mice.

Materials and Methods

All experiments were performed on healthy mature females (BALB/C strain), their ages ranged between 8 -10 weeks with a body weight (B.wt.) ranged between 25 -28 g. Mice were obtained from the colony of the Animal House unit of the High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University. Forty mature female mice were divided into major control (10 mice) and three treated groups. Treated groups were divided according to different doses of penicillin namely (2mg/kg/ B.wt, 3mg/kg/B.wt, and 4mg/kg/B.wt) for four cycles.

Preparation and administration of Procaine benzyl penicillin

Penicillin solution was prepared by dissolving completely of benzyl penicillin powder in 10 mL of normal saline to optian different doses of penicillin (2,3,4 mg/kg/B.wt.). Each dose administrated to a limited group of mice. The female mice were divided into 4 groups, one as control (injected with normal saline) and 3 treated groups (injected with different doses of penicillin). All females were intramuscular route of administration was used for treatment with penicillin 16 days period when the mice reach estrus cycle. After that each of them was sacrificed and the abdominal cavity was opened then uterus was taken out and the histological examination was done as described Bancroft and Stevens[12][13] to measure histological changes in uterus for each group.

Statistical analysis

Statistical analysis was performed by using SPSS (Statistical Package of Social Science; version 17). Crude data analysis was done using student's t-test so called paired sample t-test for tables with mean and standard error of mean (S.E.M.) to compare between pre-and post treatment for all groups. As well as, ANOVA test was applied to compare among mean groups of different penicillin concentration doses in the experimental study. Significance level was set at ($P<0.05$)[14].

Results

1. The effect of procaine benzyl penicillin on endometrial thickness

Figure (1) presented the result of endometrial thickness after 16 days of treatment for the control and treated groups. Non significant differences ($P>0.05$) in the endometrial thickness were assessed after 16 days treatment with low dose penicillin (2 mg/Kg/B.wt.) and (3 mg/Kg/B.wt.) when compared to the control group. Significant reduction ($P<0.05$) in the endometrial thickness were assessed after 16 days treatment with high doses penicillin (4mg/Kg/B.wt.) when compared to the control group. Furthermore, non significant differences ($P>0.05$) in the diameter of endometrium thickness were assessed after 16 days treatment among different groups treated with penicillin doses.

2. The effect of procaine benzyl penicillin in the number and diameter of uterine gland

Non significant differences ($P>0.05$) in the number of uterine glands were assessed after 16 days treatment with low dose penicillin (2 mg/Kg/B.wt.) and (3 mg/Kg/B.wt.) when compared to the control group. Significant decrease ($P<0.05$) in the number of uterine glands were assessed after 16 days treatment with high doses penicillin (4 mg/Kg/B.wt.) when compared to the control group. Also, non significant differences ($P>0.05$) in the number of uterine glands were assessed after 16 days of treatment with different penicillin doses as presented in the figure (2).

Non significant differences ($P>0.05$) in the diameter of uterine glands were assessed after 16 days treatment with low dose penicillin (2 mg/Kg/B.wt.) and (3 mg/Kg/B.wt.) when compared to the control group. However, significant decreased ($P<0.05$) in the diameter of uterine glands were assessed after 16 days treatment with high doses penicillin (4 mg/Kg/B.wt.) when compared to the control group. Also, non significant differences ($P>0.05$) in the diameter of uterine glands were assessed after 16 days of treatment with different penicillin doses as presented in the figure (3).

3. The effect of procaine benzyl penicillin on height of epithelial cell layer

Figure (4) showed the result of height of epithelial cell layer at 16 days treatment for control and treated groups. Non significant differences ($P>0.05$) in the height of epithelial cell layer were assessed after 16 day of treatment with low dose penicillin (2mg/Kg/B.wt.) and (3 mg/Kg/B.wt.) when compared to the control group. Significant decrease ($P<0.05$) in the height of epithelial cell layer were recorded after 16 day of treatment with high doses penicillin (4 mg/Kg/B.wt.) when compared to the control group.

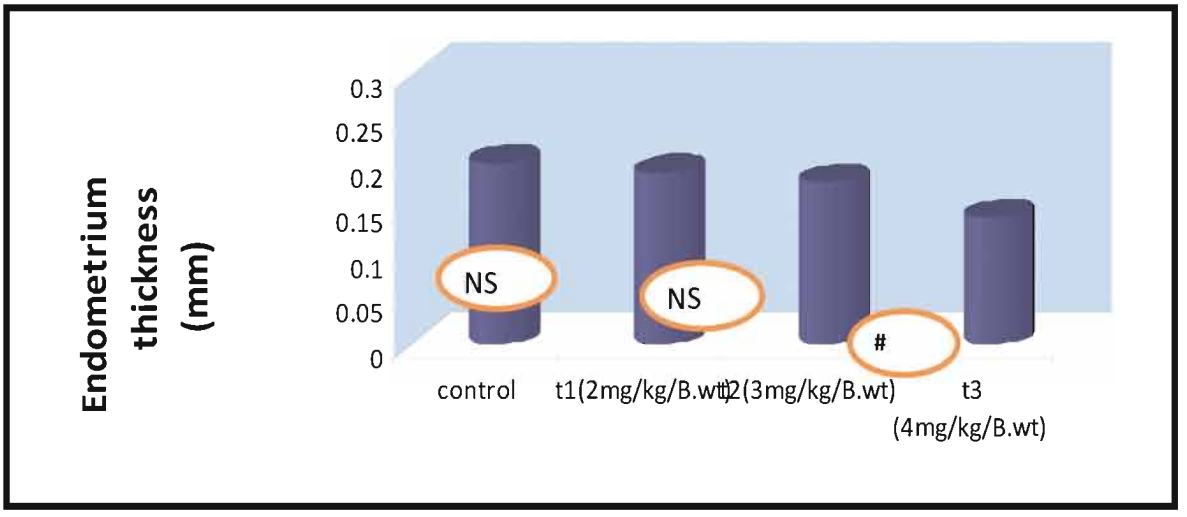

Figure (1): The effect of different doses of penicillin injected for 16 days on thickness of endometrium (mm) of mature female mice.

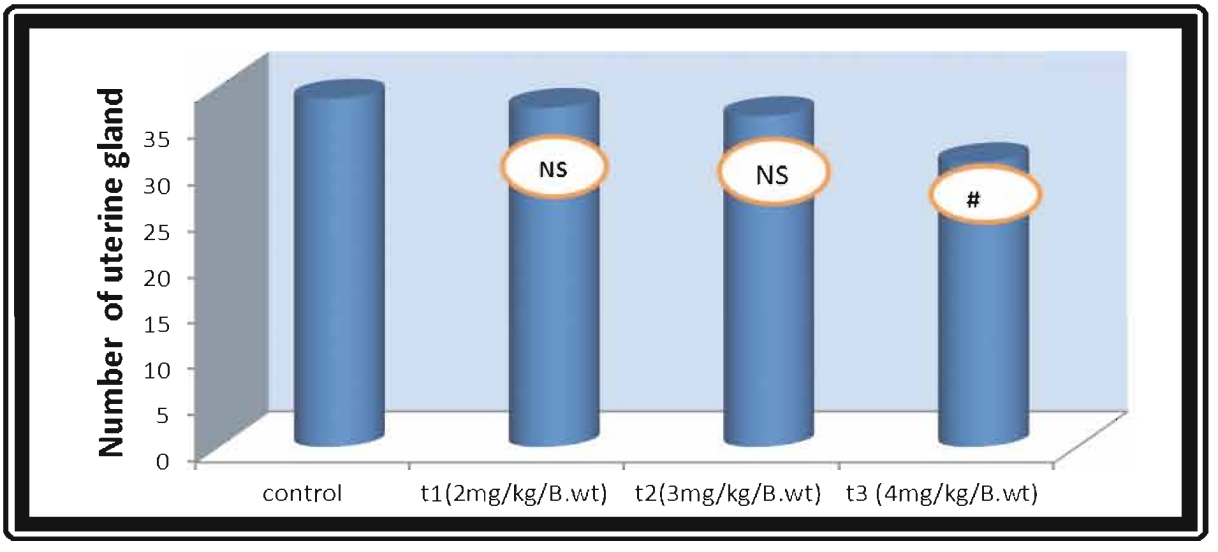

Figure (2): The effect of different doses of penicillin injected for 16 days on diameter of uterine glands of mature female mice.

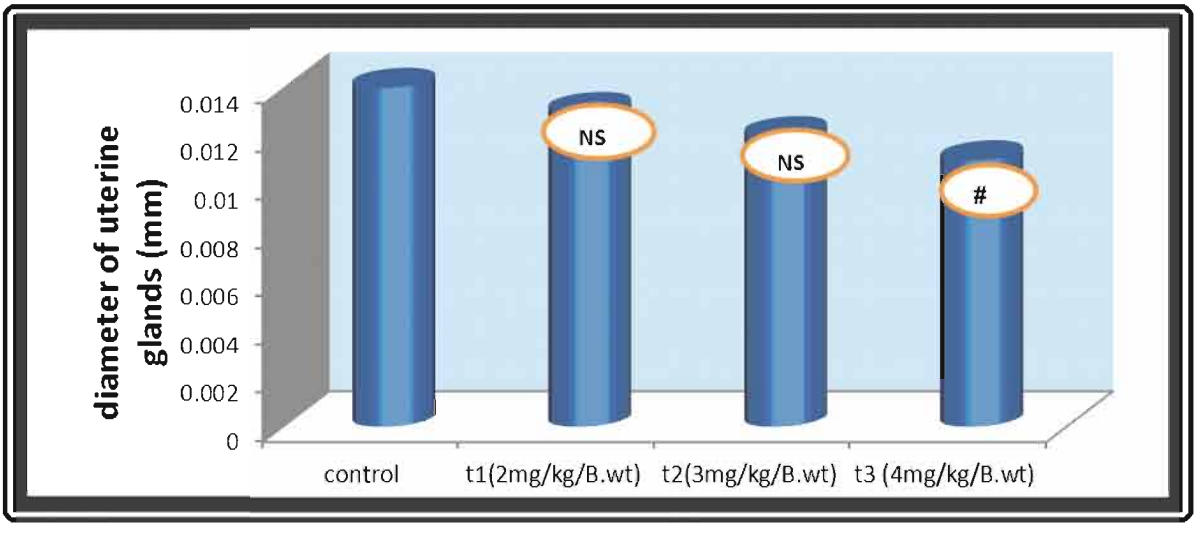

Figure (3): The effect of different doses of penicillin injected for 16 days on diameter of uterine glands of mature female mice.

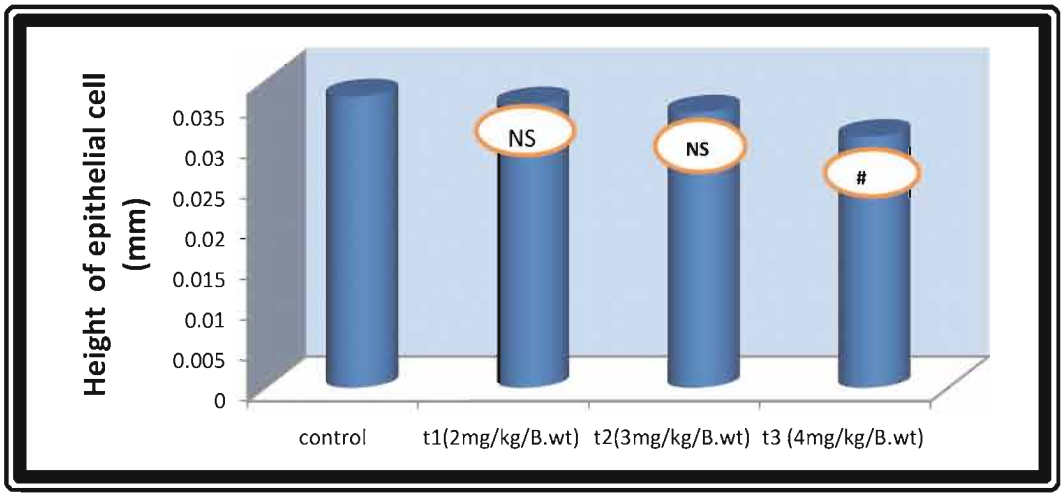

Figure (4): The effect of different doses of penicillin injected for 16 days on height of epithelial cell layer (mm) of mature female mice.
Ns: Non significant differences s (P>0.05).
≠: Significant decreased (P>0.05).

Discussion

The endometrium is the site of embryo implantation and its preparation for embryo reception is dependent on the estrogen and progesterone. These hormones cause some molecular and cellular events during uterine receptivity and the balance between them is important for cyclical changes of endometrium[15].

One major aspect of estrogen action on the uterus is the influence on proliferative processes. It is well known that estrogen strongly increases proliferative activity in all uterine tissues[16][17]. Another important result of (especially chronic) estrogen action on the uterus is morphogenetic alterations that include changes in the types of luminal and glandular epithelia, the number and shape of glands, the glandular to stromal ratio, and the morphology of epithelial cells[18].

The results of this study showed that there is a significant decrease (P0.05) in the thickness of endometrium, height of epithelial cell, number and diameter of uterine glands after administration of high dose penicillin (4mg/kg/B.wt), which may be attributed to the decrease in the levels of those ovarian sex hormones. Whereas, there is no significant decrease in these parameters after administration of (2mg/kg/B.wt.) and (3mg/kg/B.wt.) doses of penicillin, which may be due to no effect of penicillin as well as normal

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