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Editorial-Iraq.J.Emb.Inf.Res

The advent of new technologies is one of the features of the scientific research in the twenty first century. The progress of science is now taking place in the direction of new applications and new techniques, rather than a «break through» theoretical progress. In the field of assisted reproductive technologies, the issue of new techniques is clear. In fact, this horizon of science is based on technical advancement and progress. Every day, new possibilities are coming true, and what was just a research topic changes into every day practice.

Such phenomenon is not unique for assisted reproductive technologies, other disciplines are also sharing the same features, for example dentistry in medical field.

However, the applications of techniques in assisted reproductive technologies have a unique feature, that is, it possesses an ethical context and legal dimension.

For instance, sex selection, is now practical in all infertility and *in vitro* fertilization centers. The techniques of selection of particular gender of sperms are now very handy and can be done routinely and according to scientific standards. Moreover, selection of gender-specific embryos can be accomplished by many techniques including in situ hybridization, and sex selection prior to embryo transfer can done.

All the above mentioned techniques can practically be done in the laboratory with success and ease. But, what about the ethical and legal aspect of them, are they ethically acceptable? Is there law or regulations that controls their application What about the authority of the parents in this aspect, and the responsibility of medical side in this issue?.

We urge all the workers in the field of assisted reproductive technologies to bear in mind the ethical and legal aspects of these technologies and to work together with social and governmental activities to put forward guidelines for the application of new technologies, without such collaborate efforts, the field of human reproduction will fell into chaos. Human being are not laboratory animals or experiments, humanity is one of greatest values mankind possess.

Professor Dr. Anam R. Al-Salihi
Dean, High Institute for Infertility Diagnosis an Assisted Reproductive Technology
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Invitation

The Iraqi Journal of Embryos and Infertility Researches is a new and specific scientific journal designed for specialist in the reproduction field. Interestingly ,this journal is the first one in Iraq that geared and concerned in the researches of infertility, assisted reproductive technologies , and embryology.

The objective of this journal is :1-to provide clinical and laboratory knowledge of the reproduction and embryology 2-to focus on the difficulties and solutions of fertilization and embryonic development.

Our goal has been emphasized to built a cornerstone of this special science and eliminate barriers of difficulties that most researchers and specialist may faced through publishing their research.

It is our pleasure to invite all the scientists and researchers in the fields of reproductive physiology, embryology, endocrinology, biochemistry , gynecology, urology, histology and cytology with others in the area of reproduction to submit their manuscripts to publish in this journal.

We will intend to become a worldwide journal through better scientific approaches and technical advances to publish the journal in the way it should be.

Professor Dr. Saad S. Al-Dujaily
Vice Dean for Scientific Affairs and Postgraduate Studies
High Institute for Infertility Diagnosis and ART
Editor-in- Chief of IJEIR

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IJEIR FORMAT
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Iraqi journal of Embryos and Infertility Researches is a Peer -reviewed journal published annually by the Institute of Embryo researches and Infertility Treatment of Al-Nahrain University. It publishes manu- scripts in the fields of reproductive medicine especially those related to the Fertility and

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Effect of cryopreservation on some sperm parameters of infertile patients

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Abstract:

Background:
All men have the natural desire to have their own biological children. Cryopreservation of male gametes is an important aspect of human fertility preservation. With the advancement in assisted reproductive technology, indication for sperm cryopreservation is expanding.

Objectives:
To evaluate the effect of cryopreservation by using Sperm Freeze TM kit on some sperm parameters for infertile patients.

Materials and methods:
Ninety five semen samples were taken from male patients. The seminal fluid analysis was performed according to the standard criteria of WHO (1999). The patients were divided into four groups depending on their sperm concentration and motility: Normozoospermic, oligozoospermic, asthenozoospermic and oligoasthenozoospermic patients. Cryopreservation in liquid nitrogen was done by using Sperm Freeze TM kit for the samples and thawing was performed after three months.

Results:
In general, the post-thawing study revealed a considerable highly significant ($P < 0.001$) decrease in the sperm parameters which include the means of sperm concentration, sperm motility, progressive motility and normal sperm morphology as compared to that of pre-cryopreservation. Whereas the percentage of immotile sperm was highly significant ($p<0.05$) increase as compared to pre-cryopreservation in all groups except for oligozoospermic patients in which there was a significant ($p<0.05$) increase.

Conclusions:
After semen cryopreservation, all parameters were significantly decreased.

Key words: Cryopreservation, Infertility sperm parameters, WHO.

Introduction

Infertility can affect one or both reproductive partners equally(1). Cryopreservation of male gametes is an important aspect of human fertility preservation. With the advancement in assisted reproductive technology, indication for sperm cryopreservation is expanding. The exciting developments that have occurred over the years in this field have resulted in frozen sperm being as good as fresh sperm in fertilizing oocytes (2).

Although the term cryopreservation may sound as though this is one of the recent advancements in medicine, the concept is not modern (3). In fact, as early as the eighteenth century, the effect of freezing on arresting human sperm motility was observed (4). The work of Polge et al. (5) laid the foundation for cryopreservation of human sperm. In 1953, Sherman and Bunge froze human sperm equilibrated with 10% glycerol on dry ice (-75°C) with a 67% survival rate (6). The introduction of a method for freezing human semen in liquid nitrogen vapor and its storage at -196°C in 1963 was followed by reports of normal births with its use (7).

The objective of any sperm freezing technique is to stop all cellular activity and to maintain the integrity of the sperm so that after thawing the motility and fertilization potential can be restored (8). A variety of cryopreservation protocols are now used with different cryoprotectants and freezing procedures (9).However, during cryoprzeservation,spermatozoa are exposed to physical and chemical stress that results in adverse changes in membrane lipid composition, sperm motility, viability and acrosome status. All these changes reduce the fertilizing ability of human spermatozoa after cryopreservation (10).

Compared with other cell types, spermatozoa seem to be less sensitive to cryopreservation damage because of the high fluidity of the membrane and the low water content (about 50%). The primary cause of cellular damage during cryopreservation is the formation of intracellular or extracellular ice crystals (11). During cryopreservation the release of the reactive oxygen species (ROS) which induce peroxidative damage and diminishing the antioxidant activity of the spermatozoa making them more susceptible to ROS damage which lead to cell apoptosis. The release of apoptosis-inducing factors from the mitochondria leads to DNA fragmentation (12).

It has been shown that banked sperm can survive and retain their ability to fertilize after many years of storage. For example two pregnancies were reported after successful IUI following sperm cryopreservation for 28 years(13). Similarly a live birth was reported following ICSI treatment using sperm that has been cryopreserved for 21 years (14). Therefore, the objective of the present study was to evaluate the effect of cryopreservation on semen parameters.

Materials and Methods

Ninety five samples were taken from male patients aged (24-51years) during their attendance at the Higher Institute of Infertility Diagnosis and Assisted Reproducti Technologies Al-Nahrain Universit/ Baghdad, during the period from February to November 2011. The patients subjected to complete physical examination both systemic and local in infertility clinic. Semen samples were collected by masturbation at wide open, clean, dry and sterile disposable Petri dish containers, after a period of 3 to 5 days of sexual abstinence. The semen specimens were kept warm in an air incubator at 37°C to allow normal liquefaction. The standard seminal fluid analysis involving macroscopic and microscopic examinations were performed according to the standard criteria of WHO (1999). Macroscopic parameters were semen appearance, volume, liquefaction time, viscosity and acidity (pH). Microscopic parameters of spermatozoa were sperm concentration, sperm motility (%) and normal sperm morphology (%). The patients were divided into four groups depending on their sperm concentration and motility:

1. Normozoospermic patients (the sperm concentration >20x10^6/mL).
2. Oligozoospermic patients (the sperm concentration <20x10^6/mL).
3. Asthenozoospermic patients (total progressive motility < 50%).
4. Oligoasthenozoospermic patients (the sperm concentration <20x10^6/mL and total progressive motility < 50%).

All subjects in the present study had normal sperm morphology according to the WHO criteria (1999) which should be >30%. The exclusion criteria include sample with normal sperm morphology <30%.

Freezing was made by using Sperm Freeze TM kit (FertiPro N.V., Industrie Park Noord 32, 8730 Beernem, Belgium). Sperm Freeze is a 15% glycerol based cryoprotectant in HEPES buffer. It contains 0.4 % human serum albumin (HIV and Hepatitis negative) to protect the sperm from damage due to the freezing procedure.

Each sample, after liquefaction, (1ml) of semen was mixed with (0.7ml) of Sperm Freeze in the freezing vial. Adding the medium was in drops while gently swirling. Sperm freeze should be at room temperature to avoid cold-shock. Then the mixture was left for 10 minutes at room temperature for equilibration. Each vial was labeled with code number; refer to the name of patient and his wife, and date of cryopreservation. Freeze vertically for 15 minutes in the vapour of the liquid nitrogen. The freezing vials were put in the aluminum cane and stored in liquid nitrogen. Thawing was done for each sample after three months of cryopreservation in liquid nitrogen.

1. Vials were removed from the liquid nitrogen.
2. Vials were placed under tap water for 5 minutes.
3. Semen analysis assessment to the thawed samples according to WHO (1999) criteria.

Statistical analysis : Data were collected and analyzed by using SPSS (Statistical Package for Social Studies, Version 17) for descriptive statistics involving means and

standard error of mean (SEM) and to compare means of semen parameters pre- and post-cryopreservation using paired t– test to detect the significant differences. P value less than 0.05 considered as statistically significant.

Results

The male subjects were classified according to their age into four groups. The age group between 30- 39 years which include 54 subjects (56.8%) was highest one and the lowest one was in age group >50 years which include 1 subject (1.1%) as shown in figure (1).

The normal sperm morphology percentage for all male subjects involved in the present study were within normal ranges (according to WHO 1999; Normal morphology >30%). The patients were classified according to the semen parameters into 29 subjects (30.5%) as normozoospermic males and 66 patients (69.5%) as infertile patients as shown in figure (2). The subjects were classified as normozoospermic when they were having the normal values of semen parameters according to WHO 1999. When the semen parameters were not within normal value, the subjects were considered as infertile.

The infertile patients were subdivided into oligozoospermic, asthenozoospermic and oligoasthenozoospermic patients. They were 2 patients (3%), 51 patients (77.3%) and 13 patients (19.7%); respectively as presented in the figure (3).

In the present study sperm concentration, sperm motility (%), progressive motility (%), non-progressive motility (%), immotile sperm (%) and normal morphology (%) were studied regarding pre- and post-cryopreservation.

In general for all subjects, normozoospermic, asthenozoospermic and oligoasthenozoospermic patients, the means of sperm concentration of samples were highly significant (P<0.001) decreased post-cryopreservation. Both means of sperm motility (%) and progressive motility (%) were decreased significantly (P<0.001) in post- cryopreservation as compared to that of pre-cryopreservation. Regarding the mean of immotile sperm (%) which was significantly decreased (p<0.001) in all subjects while in normozoospermic patients the difference was not significant after cryopreservation (Tables 1, 2, 3, 4). A highly significant decrease (P<0.001) was observed after cryopreservation in the mean of immotile sperm (%) in asthenozoospermic and in oligoasthenozoospermic (Tables 3 and 4). Furthermore, the percentage of normal sperm morphology was highly significant decreased (P<0.001) compared with before cryopreservation . For oligozoospermic subjects after cryopreservation, the means of sperm concentration, progressive motility (%) and normal sperm morphology percentage were decreased but this decrease was statistically not significant (P>0.05). In contrast, there was a significant decrease (P<0.05) in the percentage of motility, while the percentage of non-progressive motility was not significantly increased, whereas the percentage of immotile sperm was significantly (p<0.05) increased as compared to pre-cryopreservation (Table 5).

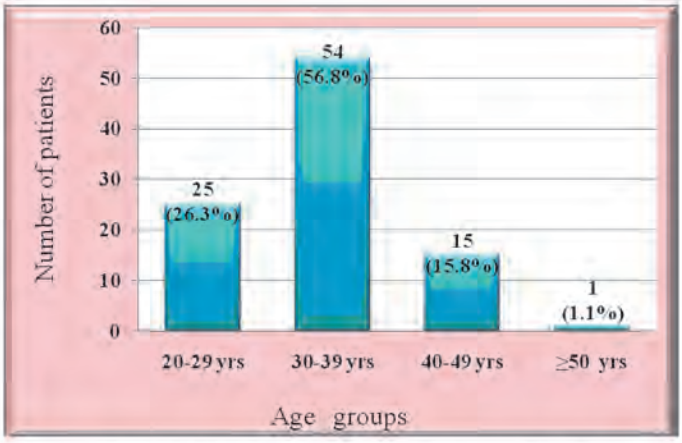


Figure (1): Distribution of patients involved in the present study and classified according to age group. N= 95

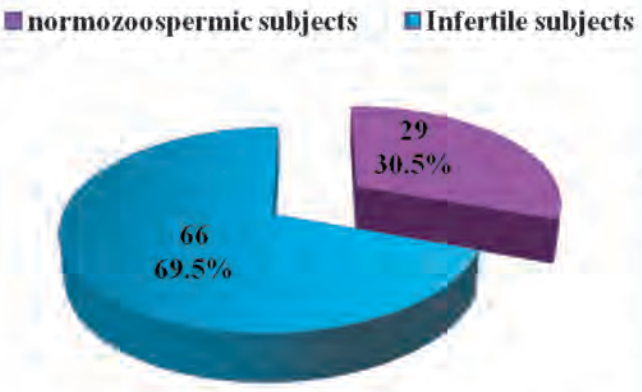


Figure (2): Distribution of subjects involved in the present study classified into normozoospermic and infertile males according to semen parameter. N = 95

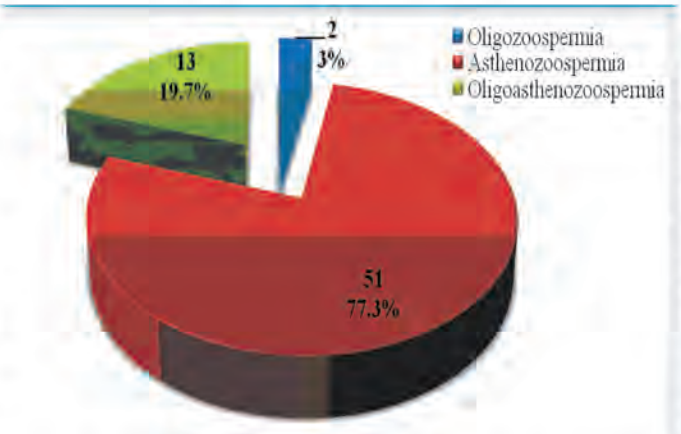


Figure (3): Distribution of infertile patients involved in the present study classified according to semen parameters. N = 66

Table (1): Sperm parameters pre- and post- cryopreservation for subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	52.93± 2.25	43.06± 2.25	Highly sig.	>20
Sperm motility (%)	58.87± 1.36	40.42± 1.96	Highly sig.	>50%
Progressive sperm motility (%)	37.54± 1.54	22.95± 1.65	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	21.33± 0.83	17.47± 0.92	Sig.	
Immotile sperm (%)	41.13± 1.36	59.58± 1.96	Highly sig.	
Normal sperm morphology (%)	33.29± 0.40	27.96± 0.49	Highly sig.	>30

N = 95

Statistical significant difference P<0.05

Highly significant P< 0.001

Table (2): Sperm parameters pre- and post- cryopreservation for normozoospermic subjects involved in the study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	67.24± 3.07	56.10± 2.80	Highly sig.	>20
Sperm motility (%)	71.28± 1.50	52.93± 3.15	Highly sig.	>50%
Progressive motility (%)	54.76± 1.40	32.93± 3.24	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	16.52± 1.09	20.00± 1.62	Non sig.	
Immotile sperm (%)	28.72± 1.50	47.07± 3.15	Highly sig.	
Normal sperm morphology (%)	33.76± 0.82	27.41± 0.70	Highly sig.	>30

N= 29

Statistical significant difference P<0.05

Highly significant P< 0.001

No statistical significant difference P>0.05

Table (3): Sperm parameters pre- and post- cryopreservation for asthenozoospermic subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre-	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	55.47± 2.11	44.69± 2.75	Highly sig.	>20
Sperm motility (%)	55.29± 1.47	39.31± 2.15	Highly sig.	>50%
Progressive motility (%)	30.84± 1.30	21.47± 1.75	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	24.45± 1.14	17.84± 1.19	Highly sig.	
Immotile sperm (%)	44.71± 1.47	60.69± 2.15	Highly sig.	
Normal morphology (%)	33.18± 0.54	29.16± 0.73	Highly sig.	>30

N=51

Highly significant P< 0.001

Table (4): Sperm parameters pre- and post-cryopreservation for koligoasthenozoospermic patients involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	16.46± 0.65	12.15± 1.19	Highly sig.	>20
Sperm motility (%)	44.69± 3.03	16.92± 4.29	Highly sig.	>50%
Progressive motility (%)	23.46± 3.12	6.92± 3.17	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	21.23± 1.87	10.00± 2.33	Highly sig.	
Immotile sperm (%)	55.31± 3.03	83.08± 4.29	Highly sig.	
Normal sperm morphology (%)	32.85± 0.98	24.69± 1.04	Highly sig.	>30

N =13

Highly significant P< 0.001

Table (5): Sperm parameters pre- and post- cryopreservation for oligozoospermic subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	17.50± 0.50	13.50± 1.50	Non sig.	>20
Sperm motility (%)	63.25± 3.75	32.50± 7.50	Sig.	>50%
Progressive motility (%)	51.00± 1.05	15.25± 5.02	Non sig.	Grades A+B > 50
Non-progressive motility (%)	12.25± 2.75	17.25± 2.50	Non sig.	
Immotile sperm (%)	36.75± 3.75	67.50± 2.50	Sig.	
Normal sperm morphology (%)	32.50± 2.50	26.50± 1.50	Non sig.	>30

N= 2

Statistical significant difference P<0.05

No statistical significant difference P>0.05.

Discussion

The evaluation of infertility is initiated typically after 1 year of failure to conceive. The cornerstone of the evaluation of the man remains semen analysis; and the semen parameters given most importance have been the sperm concentration, progressive sperm motility, and normal sperm morphology in the ejaculate (125).

The major age group of infertile males was between (30 -39) years which constitutes (56.8%) of total number of infertile males involved in this study, while the smallest group (1.1%) was within ≥50 years age as shown in figure (1). This result was in agreement with the results obtained by Al-Ani (17) and Muhammad (18) and this may explain that the youngest age groups are seeking more medical advices and are more directed to fathering a child; in addition they are facing more psychological pressure and stress about their families (15). It was assessed that semen quality, frequency of ejaculation, and sperm functions gradually decrease with highly developed age and starts to decrease after 35 years (19). The pollutions, stress, and economic difficulties that our country faced might be the main factors of decline in semen quality leading to infertility which affect this active age group where leading to infertility which affect this active age group (20).

The subjects were classified in the present work according to the semen parameters into 29 normozoospermic males (30.5%) and 66 infertile patients (69.5%) as shown in figure (2). There were significantly lower main seminal fluid parameters, the traditional fertility parameters, of infertile patients when compared with healthy fertile men; this result goes with the findings of Ibrahim (21) and Sakkas (22).

In the present study, asthenozoospermic patients were the largest group forming about (77.3%) of the infertile patients, while the oligozoospermic and oligoasthenozoospermic patients were constitute (3%) and (19.7%) respectively (Figure 3). The findings of Milardi (23) agreed with this result. These results may explain the causes in which the motility is very important and delicate characteristic of sperms that can be affected by any minor effectors as mentioned by Gaur (24) and Shaaban (25).

In general, the results in this study about semen cryopreservation were highly significant decreased ($P<0.001$) in the means of sperm concentration, sperm motility (%) and progressive sperm motility (%) post-thawing procedure compared to pre-cryopreservation. There was a significant decrease ($P<0.05$) in the mean of non-progressive sperm motility percentage, while the mean of the immotile sperm percentage was highly significant increased ($P<0.001$) after cryopreservation.

However, detailed examination showed that the proportion of fully functional sperm in a freeze–thawed sample is considerably reduced (26). The explanation may be the sperm motility is the function must vulnerable to cryoinjury (149,150). Post-thaw motilities are routinely only 50% of pre-freeze values as mentioned by other studies Nijis (29) and Anger (30). The quantitative motility assessments showed reductions in progressive motility (25 -75%) (31), this functional impairment is due to structural damage in the flagella caused by alteration in permeability and membrane fluidity (32). Furthermore, the percentage of normal sperm morphology was highly significant decreased ($P<0.001$) compared with before cryopreservation. This result goes with that of Connell (33) and Ozkavukcu (34). Further damage has been reported by Dan Yu and Luke Simon (35) as reduction in intact acrosomal caps. There is also an increase in gross morphological abnormalities; particularly in amorphous sperm heads, midpiece anomalies and cytoplasmic droplet. The ultimate cryoinjury, which occur in up to 30% of sperm, is the fatal loss of membrane integrity (30).

The degree of sperm cryodamage from infertile men has been reported to be significantly higher than in sperm from normozoospermic men (31, 32). Since many of abnormal sperm have retained cytoplasm, major source of free radicals, the amount of ROS produced during cryopreservation of such sperm may be higher than that of normozoospermic men, which may be the cause of this increase of damage especially the degree of DNA damage. This result was observed by Kalthur (36).

Normozoospermic semen samples appear to be more resistant to damage induced by freezing and thawing compared with oligozoospermic or asthenozoospermic samples. It has been reported that motile spermatozoa can be recovered after five refreezing and thawing cycles in normozoospermic samples, but only after two cycles in cases of oligozoospermia (37). Spermatozoa of infertile men were also found to be less resistant to damage during cryopreservation compared with spermatozoa from fertile men (31).

Poor quality semen may be more prone to DNA damage and cell death after cryopreservation than normal semen samples and thus have lower fertilizing capacity (38). Ibrahim observed that spermatozoa from infertile men possess significantly more DNA damage than do those of healthy fertile controls and there was a significant correlation between sperm chromatin integrity of infertile patients, their sperm concentration and sperm normal morphology; but not with their sperm progressive motility (21). This may come with the context that the genome of human spermatozoon appears to be more susceptible than the plasma membrane to oxidative damage.

Therefore, from the results of the present study it was concluded that all parameters of semen were significantly decreased after cryopreservation.

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Evaluation of Different Cryopreservation Protocols of the Testis Using 8-Hydroxy 2-Deoxyguanosine as Marker of DNA Damage.

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Abstract:

Background:
Chemotherapy and radiotherapy can destroy or severely reduce spermatogenesis and thereby jeopardize fertility in the long term. There is still no medical treatment that guarantees fertility preservation after chemo- and radiotherapy. Now with recent improvement of assisted reproductive technologies (ART) and possibility of using testicular spermatozoa or epididymal spermatozoa at *in vitro* fertilization (IVF) by intracytoplasmic sperm injection (ICSI), cryopreservation of testicular tissue is an option in fertility preservation for males who will lose spermatogenic cells as a result of chemo- and radiotherapy and for males with azoospermia.

Objectivs:
This study is an attempt to evaluate oxidative DNA damage in the mice testicular tissue after cryopreservation/thawing cycle when using different types of cryoprotectants as well as to investigate the changes that occur in mice testicular tissue after cryopreservation/thawing cycle as a model for human being.

Methods:
Fifty mature fertile male mice between 8- 12 weeks old were used in the current study. For each mouse, one testis was evaluated histologically and immunohistochemically (using 8-Hydroxy 2>-Deoxyguanosine as marker of oxidative DNA damage) without cryopreservation (control group). The other testis was evaluated histologically and immunohistochemically (using 8-Hydroxy 2>-Deoxyguanosine as marker of oxidative DNA damage) after six weeks of cryopreservation using different types of cryoprotectants (cryopreserved group).

Results:
The microscopically observation of slides obtained from cryopreserved testis differs according to the type of cryoprotectant (glycerol, 1,2 propanediol and dimethylsulfoxide), in the dimethylsulfoxide group, tissue sections showed no major differences versus control group, but in the histological sections obtained from tissue cryopreserved by glycerol as cryoprotectant showed moderate morphological and structural changes as compared with the control group. While, the tissue samples subjected to 1,2 propanediol as cryoprotectant displayed the severest morphological and structural changes as compared with the control group.
Immunohistochemical results of the present study showed a highly significant ($P<0.001$) increase in oxidative DNA damage in the cryopreserved testis (46.78%, 63.45% and 32.59% represent cryoprotectant glycerol, 1, 2 propanediol and dimethylsulfoxide, respectively) compared with control group (19.27%) after six weeks of cryopreservation.

Conclusion:
From the results of the current study, it was concluded that there was alteration in testis histology after cryopreservation/thawing cycle and increase the level of oxidative DNA damage after cryopreservation/ thawing cycle. As well as dimethylsulfoxide cryoprotectant provide good protection for testis histology and DNA.

Keywords: Cryopreservation, Oxidative DNA damage, Reactive oxygen species, 8-OHdG.

Introduction

In contemporary, improvements in treating cancer have enabled many younger persons with cancer to survive (1). However, treatment of these cancers is often highly detrimental to both male and female reproductive function. In male, testis is highly susceptible to the toxic effects of radiation and chemotherapy at all stages of life. The most common strategy to preserve fertility is cryopreservation of sperm before the treatment for later use, but with the introduction of intracytoplasmic sperm injection (ICSI) in 1992 had revolutionized the treatment of severe male factor infertility. Not only ejaculated spermatozoa could be used successfully with ICSI, also epididymal and testicular spermatozoa could be used with great success, so that cryopreservation of testicular tissue became another option for fertility preservation (2 -3).

Despite of cryopreservation used extensively in an *in vitro* fertilization (IVF) programs, but it was known as a cell damaging procedure and may lead to deleterious changes in the cell structure {e.g., membrane, mitochondria and deoxyribose nucleic acid (DNA)} due to temperature variations and increase the levels of the reactive oxygen species (ROS) generation in spermatozoa (45-). Cryopreservation of testis in mice using different types of cryoprotectants agent (CPA) include: {Propanediol (1,2PrOH), Glycerol and Dimethylsulfoxide (DMSO)}, evaluate oxidative damage to testicular tissue DNA using 8-OHdG level, and put forwards recommendations regarding the optimum type of cryoprotectant which inflects the least DNA damage of testicular tissue during cryopreservation.

Materials and Methods:

- Mice management: Fifty healthy mature fertile male mice of Mus Musculus between 8- 12 weeks old and 25- 35 gm of weight were obtained from the Animal House at the National Center for Drug Control and Research. Room temperature was between 22 -24 °C, the air of the room was changed continuously using ventilating fan. Photoperiod was automatically controlled of 13± 2 hours light from 6 A.M. to 7 P.M. daily regimen.

- Study Design: Mice were scarified by cervical dislocation and testes were excised and rinsed in culture media. For each mouse, one testis was evaluated histologically and immunohistochemically without cryopreservation (control group). The other testis was evaluated histologically and immunohistochemically after six weeks of cryopreservation using different types of CPA (cryopreserved group).

-Freezing media and protocol (6,7): Cryomedium consisted of Hams F-12 culture medium (Sigma-ALdrich) supplemented with three different types of cryoprotactent (DMSO, Glycerol and 1,2PrOH). These CPA was added to the culture media at concentration 15% glycerol (BDH), 15% DMSO (BDH) and 15% 1,2PrOH (Merck). When using DMSO or 1,2PrOH as cryoprotectant, the 0.1M/l of sucrose (BDH) was added to the solution.

The cryopreservation of samples was done at the sperm activation and freezing laboratory of Assistant Reproductive Technology (ART) unite in the High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University.

After sacrificed male mice by cervical dislocation, the testis was dissected under dim yellow light and it was washed by using culture media then it was transported to the 1.8 cryovial that contains 1.5 ml of freezing media supply with one type of CPA. After that the cryovial was placed in the cryovial holder (NUNC) and remained for about 10 minute in the refrigerator when using DMSO as CPA and at room temperature when using 1,2PrOH and glycerol as CPA for equilibration. Finally the cryovial holder that contains cryovial was suspended in the liquid nitrogen vapor for 30 minutes before plunged in the liquid nitrogen and then plunged in liquid nitrogen.

After six weeks of cryopreservation, the thawing processes to samples were done through rapidly transfer the cryovials from liquid nitrogen to the water bath (Memmert) at 37 ºC until melting ice, for at least 5 minutes, and then the CPAs were removed from sample by descending concentration of CPA gradually as fallowing:

a- 10%, 5% and finally washed by culture media only when use glycerol as CPA for about 5 minute for each concentration.

b- 10% with 0.05M sucrose, 5% with 0.025M sucrose and finally washed by culture media only when use DMSO or PrOH as CPA for about 5 minute for each concentration.

- Histological and Immunohistochemical evaluation of mice testis (8, 9): The samples were fixed with 10% neutral buffered formalin (BDH) for overnight (24 hours). After that, the tissues were embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 5 µm. Some sections were stained with hematoxylin and eosin, and the other sections were used for immunohistochemical assay. For immunostaining of 8-OHdG, the slides were placed in a drying oven (hot air oven) at 65°C for one hour. Then the formalin-fixed sections were deparaffinized in washes in xylene through ethanol to distilled water. To block endogenous peroxidase activity, 0.3% hydrogen peroxide was applied to the specimens for 10 min. All slides then were incubated with protein block for 5 min to block nonspecific binding. Primary antibodies were added to the slides and incubated for two hours in a humidified chamber at room temperature. Secondary antibody (biotinylated link) was then applied and incubated for 10 min at room temperature. Enough drops of streptavidin reagent were applied covering the whole section and incubated at room temperature for 10 minutes in humid chamber. Then Substrate-chromogen solution were applied on each section covering the whole specimen and incubated in darkness at room temperature for 10 minutes. After that enough drops of the haematoxylin (as a nuclear counter stain) solution were applied covering the whole section and incubated at room temperature for 1 minute. Finally enough drops of an aqueous-base mounting

medium were applied onto the tissue sections and covered with coverslips and left to dry.

- Assessment of Immunohistochemical Staining: For assessment of Immunohistochemical Staining, image were captured by using Microns microscope contain TV-Based computer with a 40X objective and at least ten images were captured for each sample. Actual assessments of immunohistochemical staining were done by using Aperio positive pixel count algorithms to analyze digital slides. The Aperio positive pixel count algorithm can be used to quantify the amount of a specific stain present in a slide image. This algorithm has a set of default input parameters when first selected. These inputs have been pre- configured for Brown color quantification in the three intensity ranges (weak, positive, and strong). The algorithm is applied to an image by using Image Scope software v10 (Aperio).

Statistical Analysis: Statistical analysis was performed with SPSS V. 17 (statistical package for social sciences) and also Microsoft Excel Work Sheet 2010 programs. Paired sample t- test was applied by use SPSS statistical program to compare between the result of cryopreserved groups and control group. Data were expressed as mean ± standard error of mean. And the values were considered statistically significant when p-value 0.05.

Results

-Results of histological evaluation

In control group, the paraffin section of testis stained with Hematoxylin & Eosin showed normal tissue morphological and structural and showed normal components of germinal epithelium that composed of seminiferous tubules with normal sperrmatogenic lineage cells (spermatogonia, spermatocyte and spermatid) and supporting (Sertoli) cells. Between the seminiferous tubules were seen the interstitial cells (Leydig cells), they are large polyhedral cells that have spherical nucleus (Figure 1).

In cryopreserved groups, different types of CPAs (glycerol. I,2PfOH and DMSO) were used. After six weeks of cryopreservation the paraffin sections of testis stained with H&E stain showed morphological and structural alteration in seminiferous tubule component and interstitial tissue. But these alterations differ according to the type of CPA. In the cryopreserved group, the tissue samples cryopreserved by DMSO as CPA showed no major differences versus control group. These samples contain well-preserved testicular cords and the main prominent histological changes observed after cryopreservation by DMSO as CPA was mild disruption of interstitial tissue and mild increase in the intertubular space. In addition to that, the testicular cords showed mild detachment of cells in the seminiferous tubules from the basement membrane and contained necrotic cells confined to the center of the testicular cords that was not seen in the control group (Figure 2). While in the paraffin sections obtained from tissue cryopreserved by glycerol as CPA showed moderate morphological and structural changes. These changes characterized by moderate disruption of interstitial tissue and moderate increase in the intertubular space. Also, detachment of spermatogonia from the basement membrane of the testicular cords and necrotic cells in the lumen seminiferous tubules were observed (Figure 3).

The tissue samples subjected to 1,2PrOH as CPA displayed the severest morphological and structural changes. The most typical damage was rupture of the stroma and the cell-cell connections in the interstitial tissue and inside the seminiferous tubules, mainly in the basal compartment. The lamina propria was influenced and showed swelling after cryopreservation with 1,2PrOH. Also, destruction of the basement membrane in addition to sever detachment of spermatogonia from the basement membrane and necrotic cells in the lumen seminiferous tubules were observed (Figure 4).

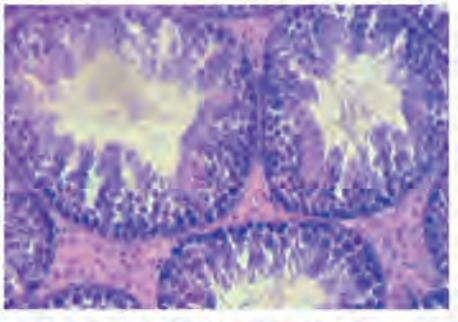


Figure 1: Paraffin section from mice testis of control group show normal tissue structure, this figure showed normal components of germinal epithelium that composed of seminiferous tubules with normal spcrmatogenic lineage cells (spermatogonia, spermatocyte and spermatid) and supporting (Sertoli) cells. Between the seminiferous tubules were seen the interstitial cells (Leydig cells), they are large polyhedral cells that have spherical nucleus. Stained by H&E (40X).

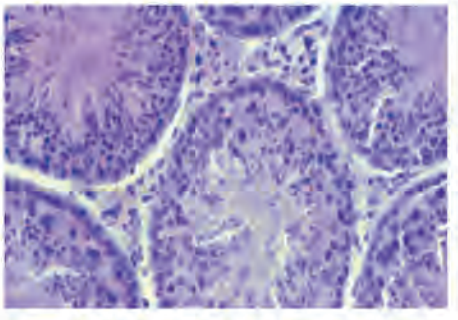


Figure 2: Section from cryopreserved mice testis by DMSO as CPA showing mild disruption of interstitial tissue and mild increase in the intertubular space in addition to mild detachment of spermatogonia from the basement membrane and necrotic cells in the lumen seminiferous tubules, stained with H&E (40X).

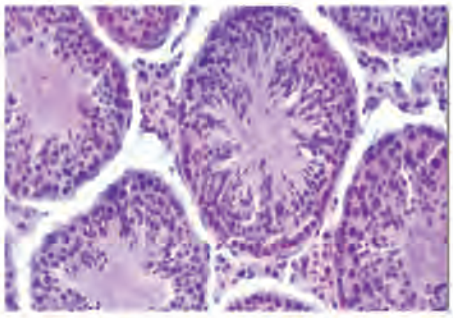


Figure 3: Section from cryopreserved mice testis by glycerol as CPA showing moderate disruption of interstitial tissue and moderate increase in the intertubular space, also detachment of spermatogonia from the basement membrane of the testicular cords and necrotic cells in the lumen seminiferous tubules, stained with H&E (40X).

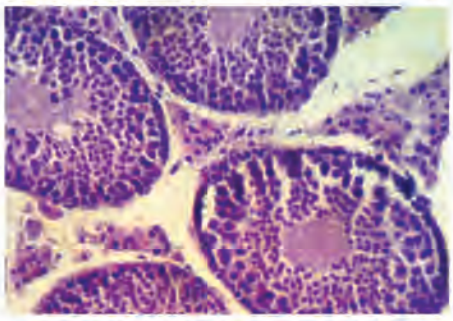


Figure4: Section from cryopreserved mice testis by I,2PrOH as CPA showing rupture of the stroma and the cell-cell connections in the interstitial tissue and inside the seminiferous tubules in addition to swelling of the lamina propria and detachment of the cells (spermatogonia) in the seminiferous tubules from the basement membrane. Also, destruction of the basement membrane and necrotic cells in the lumen seminiferous tubules,stained with H&E (40X).

-Result of immunohistochemical assay:

In this study, we considered the cell positively stained for 8-OHdG when the nucleus was being stained; the pattern of stain is diffuse and color of stain is brown. The cells labeled by the antibody display a staining almost entirely confined to the nucleus and with a diffuse pattern, the color of stain is brown. Rarely cytoplasmic staining is observed, which may represent cytoplasmic synthesis or breakdown.

Testicular sections of mice normal and cryoprescrved testes were stained with anti-8-OHdG antibody. Both control and cryopreserved sections showed variable distribution of 8-OHdG, but with the cryopreserved group displaying significantly higher staining compared to control group. In control group, tissue sections of the testes showed normal tissue morphology and normal components of seminiferous tubule and there are weak positive signals for anti-8-OHdG antibody were detected in the testicular sections (Figure 5-A). Digital image for the testicular sections of control group were captured at 40x objective and analyzed by computer software (Aperio ImageScop program) for quantification of 8-OHdG staining by converting the digital image into pseudo-color markup image as shown in (Figure 5-B). In this figure, the testicular section of control group showed positive signals for anti- 8-OHdG antibody in variable intensity. The weak intensity of positive signals appeared in the center of the seminiferous tubule and around numerous germ cells. But these positive signals increased in intensity in the interstitial tissue of the testis while there are no positive signals were observed in nucleus of the germ cells. In cryopreserved group, tissue sections stained immunohistochemically displaying significantly higher staining intensity compared to control group vary according to the type of the CPA that used. Tissue sections obtained from the samples cryopreserved by DMSO as CPA showed varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and the interstitial tissue of the testis (Figure 5-C). The strong positive signals appeared in the nucleus of the germ cells especially in the spermatogonia, but the weak positive signals appeared around the germ cells and in the interstitial tissue of the testis (Figure 5-D).

Whereas in the tissue samples cryopreserved by glycerol as CPA, the intensity of the positive signals increased in both seminiferous tubules and the interstitial tissue of the testes more than in control and DMSO group (Figure 5-E). These positive signals appeared clearly in (figure 5-F) after performing computer image analysis.

While the tissue samples subjected to 1, 2 PrOH as CPA displayed the stronger positive signals compared with the other groups (Figure 5-F). These strong positive signals concentrated mainly in the nucleus of the germ cells and in the interstitial tissue of the testes. In addition to that, weak positive signals distributed among the germ cells and in the lumen of the seminiferous tubules (Figure 5-G).

Finally, 8-OHdG immunohistochemistry staining in the testicular sections of control and cryopreserved groups were quantified as the percentage of the positive pixels for 8-OHdG in the testicular sections. The Mean + Standard Error (SE) of the percentage of the positive pixels for 8-OHdG in the testicular sections of control group was 0.193+ 0.011 while in cryopreserved groups Mean + Standard Error (SE) of the percentage of the positive pixels for 8-OHdG in the testicular sections were 0.326 ±0.01 , 0.468± 0.011 and 0.635± 0.013 represented CPAs DMSO, glycerol and 1,2PrOH respectively (Figure 6). Statistical analysis showed highly significant increase ($p<0.00l$) in mean of percentage of the positive pixels for 8-OHdG in cryopreserved groups as compared with control group as shown in the table (1).

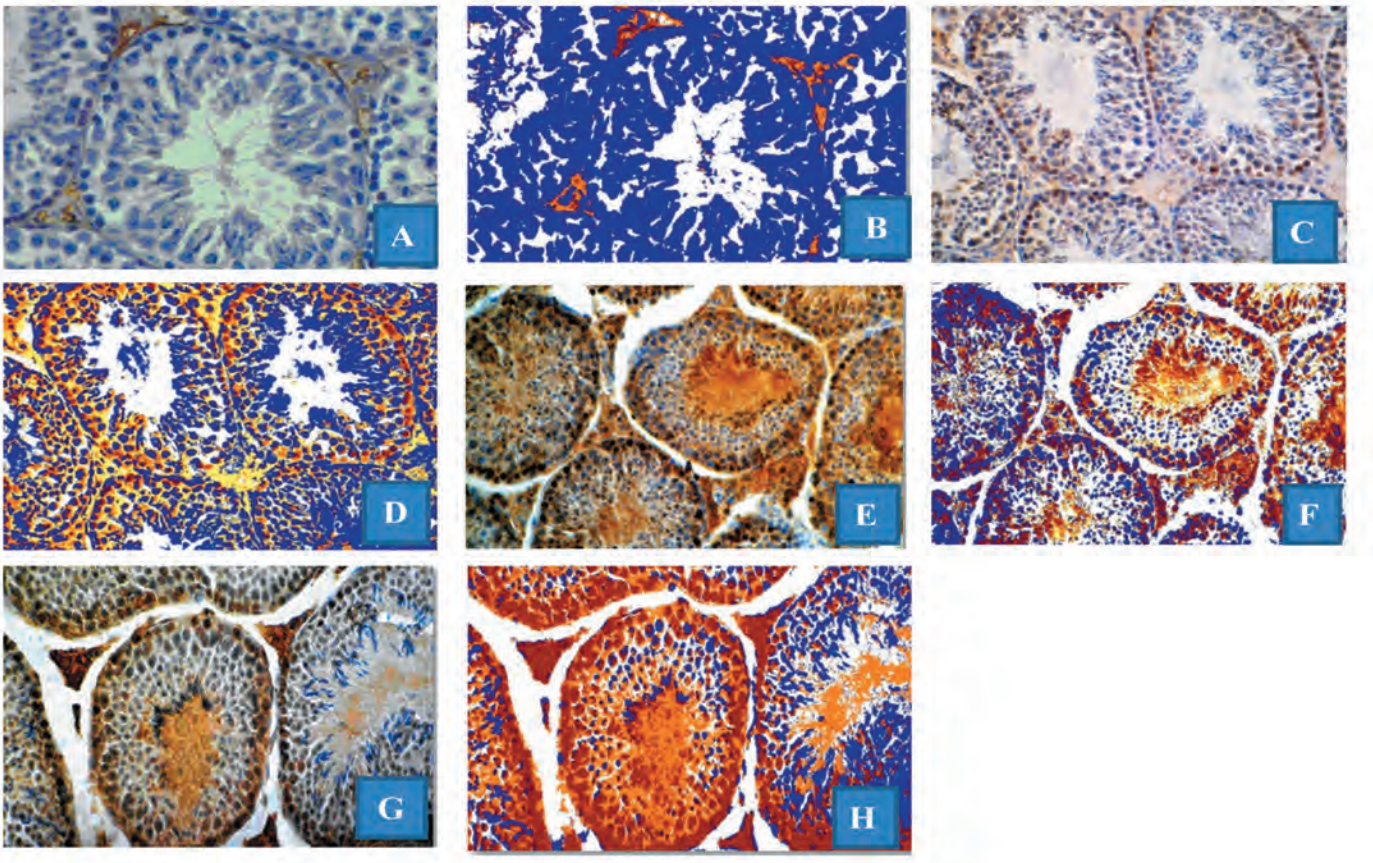


Figure 5: A- Showed the testicular section of control group stained immunohistochemically, a weak positive signal for 8-OHdG appeared in the interstitial tissue of the testis (40X). B- Pseudo-color markup image of testicular section of control group as a result of image analysis. This figure showed positive signals for 8-OHdG in variable intensity (40X). C- Section from cryopreserved mice testis by DMSO as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). D- Pseudo-color markup image after image analysis of testicular section cryopreserved by DMSO as CPA. This figure showed positive signals for 8-OHdG in variable intensity (40X). E- Section from cryopreserved mice testis by glycerol as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). F- Pseudo-color markup image after image analysis of testicular section cryopreserved by glycerol as CPA. This figure showed positive signals for 8OHdG in variable intensity (40X). G- Section from cryopreserved mice testis by I,2PrOH as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). H- Pseudo-color markup image after image analysis of testicular section cryopreserved by I,2PrOH as CPA. This figure showed positive signals for 8-OHdG in variable intensity (40X).

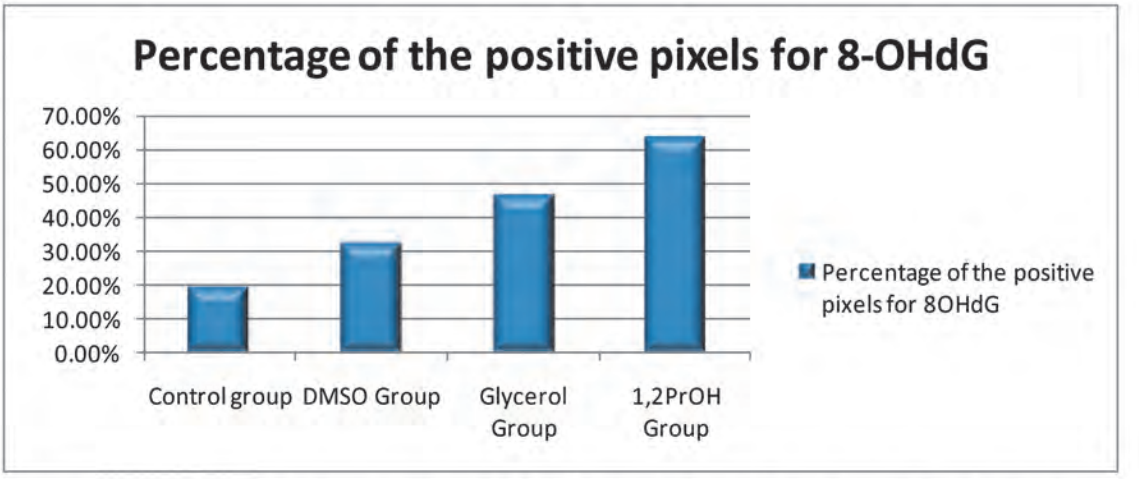


Figure 6: Percentage of the positive pixels for 8OHdG in the testicular sections of control group and cryopreserved groups.

Table 1: Percentage of the positive pixels for 8OHdG in the testicular sections of control group and cryopreserved groups

Groups	Mean + S.E.	S.D	P value	T test
Control	0.193+0.011	0.137	---	
DMSO	0.326+0.010*	0.122	≤0.001	8.267
Glycerol	0.468+0.011*	0.130	≤0.001	16.131
1,2PrOH	0.635+0.013*	0.153	≤0.001	29.238

Paired sample t test: comparison between glycerol, 1,2PrOH and DMSO groups with control group.
* Reveals highly significant differences as compared with control group (10).

Discussion

In the present study, histological paraffin sections showed certain histological and morphological changes that happened after cryopreservation/thawing cycle and these changes differ according to the type of CPA. These changes might be mainly due to ice crystals formation, increase salt concentration, and thermal and osmotic shock. In addition, CPA toxicity might play a role in these changes and finally the cryopreservation process result in increase in level of the ROS which will damage the cells and DNA. One of important pathway leading to that damage occurs by lipid peroxidation.

In the current study, cryopreservation of testicular tissue with DMSO as CPA showed no major differences as compared with control group. This result in agreement with study done by Keros *et al.*(11) they are showed no clear structural changes were observed between fresh and cryopreserved testicular tissue after using the protocol developed for adult testicular tissue containing DMSO as CPA.

Also, a study done by Goossens *et al.*(12) showed that cryopreservation of testicular tissue using protocol containing DMSO as CPA provide the best morphology of the basal compartment as compared with another protocol containing ethylene glycol as CPA.

Although glycerol has been used as a reliable agent in lowering the freezing point of intracellular water, and widely used for cryopreservation of spermatozoa and suspensions of testicular cells, but its use for tissue preservation has been limited by its poor penetration qualities (13). In the present study, cryopreservation of testicular tissue using glycerol as CPA showed moderate morphological and structural changes as compared with control group. These results are in agreement with study done by Keros, *et al.*(14) they showed that the testicular tissue samples subjected to glycerol as CPA displayed changes in basal compartment of the tubules. These changes characterized by rupture of the stroma and the cell–cell connections in the interstitial tissue and inside the seminiferous tubules, mainly in the basal compartment, in addition to the detachment of spermatogonia from the basement membrane were observed, and these results supported this study results.

Whereas, cryopreservation of testicular tissue with 1,2PrOH as CPA showed the severest morphological and structural changes. This result in agreement with study done by Travers *et al.*,(15) in which they tried to evaluate several protocols for cryopreservation of rat immature testicular tissue by using DMSO and 1,2PrOH. They found that the cords damages appeared to be less frequent with freezing protocol using DMSO than 1,2PROH in terms of morphological alterations. These damages are characterized by the gap formations and epithelium detachment from the basement membrane, and these results supported this study results.

Also, the results were obtained by Keros *et al.*(14) under light microscopic include complete disruption in the interstitial tissue, difficult to recognize between cells inside the seminiferous tubules and detachment of cells from the basement membrane after cryopreservation/thawing cycle by 1,2 PrOH and sucrose for cryopreservation of testicular tissue, it goes with result of this study.

The immunohistochemical assay used in this study is adequate to detect significant differences in the oxidative DNA damage between cryopreserved groups and control group by using the 8-OHdG as a biomarker of oxidative DNA damage.

Clear advantages associated with immunohistochemical analysis include the facts that this method is relatively inexpensive, quick, highly sensitive and easy to perform,

uses light microscopy, and is a well-established routine procedure in clinical laboratories (16). As well as, this method is appropriate for a remarkably wide range of applications. Any cell- or tissue-bound immunogenic molecule can, theoretically, be detected in situ using this technique. Immunohistochemical techniques detect antigens in tissues or in cells. Proteins (including immunoglobulins), carbohydrates, nucleic acids, lipids and other compounds can act as antigens (17).

In the present study, percentage of oxidative DNA damage (19.27%) as expressed by immunohistchemical assay was reported among healthy control mice. This is possibly due to the normal existence of 8-OHdG in the nuclear DNA. This fact is supported by study done by Magnus *et al.*, (18) showed that the 8-OHdG "which is a chemical modification of DNA caused by ROS in the cell" seems to exist normally in the nuclear DNA at a level of -0.11- per 105 deoxyguanosines (dGs). Also, a study done by Dai *et al.*, (19) revealed that the level of the 8-OHdG positively correlated with age. They stated that the nuclear 8-OHdG levels in the testis were high (more than two lesions per 106 dGs) from three to 52 weeks of age. In addition, 8-OHdG has been shown to be present in mitochondrial DNA at a level ≈ 16-fold higher than that present in nuclear DNA, an observation consistent with the high levels of ROS generated in this organelle (20).

In the current study, the central issue in the results of immunohistochemical assay showed highly significant increase in the percentage of the oxidative DNA damage ($P<0.001$) in cryopreserved group (46.78%, 63.45% and 32.59% representing CPAs glycerol, 1, 2 PrOH and DMSO; respectively) after six weeks of cryopreservation when compared with control group (19.27%). This study suggests that cryopreservation provoke oxidative stress (generation of ROS overwhelms these antioxidant defenses); this aspect is in agreement with many researchers (21, 22). However, other groups demonstrated an increase in ROS following cryopreservation / thawing cycle due to slow recovery of antioxidant activity (23).

In normal condition excessive production of ROS is limited by various regulatory systems, some enzymatic (catalase, superoxide dismutase, and glutathione reductase) and others, nonenzymatic (vitamin E, vitamin C, taurine, hypotaurine, and pyruvate) (24). It has been postulated that the effects produced by ROS depend on their nature, quantity, length of exposure and on the time length of exposure (25).

As well as attack from ROS on DNA is considered a major source of spontaneous damage to the DNA as well as other macromolecules such as proteins and lipids. There are various intra- and extracellular sources of oxygen radicals. The major intracellular source is believed to be electron leakage from the metabolism process in mitochondria, but the main extracellular source ionizing and ultraviolet radiation (26).

Excessive production of ROS is potentially toxic to sperm quality and function (27). This is because ROS are highly reactive oxidizing agents, among which are included H_2O_2, $O_2^{\cdot}$ and $OH^{\cdot}$ (28). Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa (29). Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements (30).

Despite the importance of CPAs in the cryopreservation of tissues and cells, but they differ in their suitability and protection for the DNA, as the study showed 63.45% when using 1, 2 PrOH compared to 46.78% and 32.59% when using glycerol and DMSO respectively. DMSO provided superior cryoprotection compared to 1,2PrOH and glycerol. This observation is in agreement with study done by Keros *et al.*, (14) they reported better testicular tissue cryopreservation with DMSO as CPA. Moreover, Loskutoff *et al.*, (31) reported that epididymal sperm had better post-thaw motility when frozen with DMSO rather than with glycerol, ethylene glycol or propylene glycol. These results may be due to the low molecular weight and penetration ability of DMSO (14). In addition to that, the DMSO has ability to scavenge the hydroxyl radicals ($OH^{\cdot}$), one of the most reactive and damaging free radicals (32).

Although the glycerol is a natural metabolite and it is considered a safe option for the cells; metabolically and genetically, but showed 46.78% of oxidative DNA damage when using it as a CPA. This is in agreement with study done by Keros *et al.*, (14) they reported that glycerol led to a lower protection than DMSO for adult testicular fragments. These results could perhaps be attributed to slowly movements of glycerol across cell membranes and the cells may be damaged by osmotic shock during its removal. Therefore, the purported toxic effect of glycerol may be caused by osmotic shock (33). Furthermore, study done by Guidet and Shah (34) showed that glycerol increase in H_2O_2 generation, which is one of the reactive free radicals. Whereas, cryopreservation of testicular tissue with 1,2PrOH showed highly percentage of oxidative DNA damage (63.45%). This high percentage might be due to the presence of hydroxyl groups in their structure (35), which are highly reactive species and a powerful initiator of lipid peroxidation (36). In addition to that, 1,2PrOH might be responsible for the development of intermediate toxic metabolites, as is the case for the closely related ethylene glycol, whose catabolism leads to formation of toxic aldehydes (37). Hovatta *et al.* and Hreinsson *et al.*, (3839-) supported the results of this study when referred that cryopreservation method optimized for human ovarian tissue using 1,2PrOH and sucrose not to be optimal for cryopreservation of testicular tissue. In addition, Szurek *et al.* (40) showed that DMSO and ethylene glycol are safer to use for oocyte cryopreservation than 1,2PrOH.

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Effect of cryopreservation on DNA normality of mice epididymal sperms following *in vitro* preparation with pentoxifylline and *Glycyrrhiza glabra*

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Abstract:

Background:

Many studies conducted on the pentoxifylline (PX) and *Glycyrrhiza glabra (G.glabra)* as motility stimulants showed positive effect on sperm preparation *in vitro* improving the progressive forward movement. However, it was not known the impact of these stimulants on the nature of genetic material, especially after cryopreservation.

Objective:

The experiment was designed to find out any harmful effect of a medium containing a mixture of PX and aqueous extract of *G.glabra* on the sperm DNA before and after cryopreservation.

Materials and Methods:

Fifty mature fertile male mice 812- weeks old were used. Epididymal sperms were obtained from caudal region and prepared *in vitro* by direct activation technique using four media namely;PX alone, *G.glabra* alone, a mixture of PX with *G.glabra* and Ham's F-12 (as control medium). Certain sperm function characteristics were examined as well as evaluation of DNA integrity by acridine orange test and comet assay before and after cryopreservation.

Results:

The results showed a highly significant ($P<0.001$) improvement in the sperm forward movement, with a highly significant ($P<0.001$) decrease in DNA abnormality using acridine orange test and comet assay, following *in vitro* activation by the four media compared to before activation. Following *in vitro* activation and cryopreservation, the results of epididymal sperm showed a highly significant ($P<0.001$) decrease in DNA abnormality by using PX medium alone, *G.glabra* medium alone and the mixture of PX and *G.glabra* compared to control medium and the results before cryopreservation.

Conclusion:

According to the results of present study, it has been found no toxic effect of medium containing PX and/or *G.glabra* on the epididymal sperms DNA after activation *in vitro* and cryopreservation using acridine orange test and comet assay. The best result obtained when using a medium containing a mixture of PX+ *G.glabra* together. These data could be applied for preparation and activation of epididymal and testicular sperms obtained from obstructive azoospermic men.

Keyword: Pentoxifylline, *Glycyrrhiza glabra*, **DNA integrity, cytogenetic tests, Cryopreservation .**

Introduction:

Assisted reproductive technologies with cryopreservation were widely used to overcome the male infertility in addition to preserve the fertility e.g. before cytotoxic chemotherapy, radiotherapy or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction(1). Freezing and thawing involve damage to the plasma membrane and acrosome of human spermatozoa as evidenced by significant ultra-structural changes demonstrated by electron microscopy (2) . This damage was exacerbated during the cryopreservation process by a decrease in the lipid content of the membranes, which contain a high proportion of polyunsaturated fatty acids; the decrease in lipid content indicates lipid peroxidation in the presence of reactive oxygen species (3). It was cleared that abnormalities in the male genome characterized by damaged DNA may be indicative of male subfertility regardless of the routine semen parameters (4). Moreover, cryopreservation has been shown to be associated with an increase in the proportion of sperm with DNA fragmentation (5).

In the last years, many studies were concerned with the herbal plants and sperm motility stimulants substances added for culture media *in vitro*. Furthermore, different researches were accomplished using a media containing PX and/or *G.glabra* that resulted in positive effect on active sperm motility. The PX was added to activate sperms and used for Human IUI (6, 7) and *G.glabra* was added to the medium used for mice IVF program (8). However the best *in vitro* sperm preparation and activation results has been observed following mixing the PX and *G.glabra* together.Therefore the aim of this work was to study whether ,there was any harmful effect on the sperm DNA integrity following the preparation and activation of mice epididymal sperm *in vitro* by medium containing PX and/or *G.glabra* before and after cryopreservation technique.

Materials and Methods:

Animal management: Mature fertile mice(Mus musculus) 812- weeks old and 25- 35 gm of weight were obtained from the Animal House at the National Center for Drug Control and Research-Ministry of Health. Room temperature was between 22 -24 °C, the air of the room was changed continuously using ventilating fan. Photoperiod was automatically controlled of (13± 2 hours light from 6 A.M. to 7 P.M. daily regimen).

-Study Design: Male mice were sacrificed by cervical dislocation and the epididymis was dissected out. The sperms were obtained from caudal region by flushing method with 1 ml culture medium (Ham's F-12, Sigma- Aldrich Group, Germany) then each sample divided into four media:

1- Medium (1): control medium (free-treated with PX and *G.glabra)*.The epididymal sperm activation was done by adding Ham's F-12(0.5 ml).

2- Medium (2): The epididymal sperm activation was done by adding (0.5 ml) of *G.glabra* (0.1 mg/ml ,ratio20%).

3- Medium (3): The epididymal sperm activation was done by adding (0.5 ml) of PX (0.1 mg/ml).

4- Medium (4): The epididymal sperm activation was done by adding (0.5 ml) of PX(0,1mg/ml) and *G.glabra* (0.1 mg/ml, ratio 20%).

All the suspensions were kept in the incubator for 30 minutes at 37°C. After incubation, 0.5 ml of the prepared sperms were taken and placed in cryovial containing cryoprotectant.

-Epididymal sperm cryopreservation: The cryopreservation method used was according to Frank (9). Each prepared epididymal sperm was mixed with cryoprotectant (prepared by adding glycerol to the Ham's F-12 and then filtered by using filter Millipore 0.22 µ and exposed to the UV light for sterilization) and placed in cryovial (Nunc Tm, Denmark, capacity 1.8 ml). Each cryovial was hold by cryovial holder (Nunc Tm, Denmark) and left at room temperature for 10 minutes. The samples were hanged in liquid nitrogen vapor for 30 minutes, then embedded in the liquid nitrogen.

-Thawing: After one month of cryopreservation, the thawing of sample was done through rapidly transferring cryovial from liquid nitrogen to the water bath (Kotterman,) at 37°C until melting ice, for at least 5 minutes. The sample was mixed with 1:1 ratio culture medium (Ham's F-12) and then incubated for 10 minutes. Then certain sperm functions parameters and DNA integrity test were examined. The thawing process was done according to Frank (9) .

-Acridine orange protocol: This test was done according to Tejada (10).

-Comet Assay Protocol: A detailed elaboration of the alkaline comet assay procedure used to quantify DNA damage was accomplished as described by Singh (11).

Ethics: This study was approved by the ethics committee. All animals received humane care in accordance with the Institution>s guideline and criteria for humane care as outlined by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Statistical analysis: The data analysis was performed by using SPSS (Statistical Package of Social Science; version 17.0 LED Technology, USA) and Microsoft Excel Work Sheet 2007). Paired sample t- test was applied by SPSS statistical program to compare between the results of cryopreserved and control groups. The differences between the values were considered statistically significant at P value 0.05(12).

Results:

-Effect of *in vitro* sperm activation on certain sperm function parameters before and after activation and cryopreservation:

There was a highly significant (P<0.001) improvement in active sperm motility grades A, B and A+B of sperms treated with PX, Gg and PX+ *G.glabra* media compared with before activation and control Ham's F-12 medium. A highly significant (P<0.001) decrease in active sperm

motility grades A, B and A+B after cryopreservation was observed with PX, *G.glabra* and PX+ *G.glabra* media compared with the control Ham's F-12 medium before and after cryopreservation. There was a highly significant (P<0.001) decrease in morphologically abnormal sperms (MAS) after activation with (PX, *G.glabra* and PX+ *G.glabra* media) than that of before activation and control medium as shown in figure (1).

- Results of DNA integrity tests:

1. Results of Acridine Orange:

The results of acridine orange were shown in image -1 and figure (2), A highly significant (P<0.001) decline was observed in DNA denaturation after activation and after cryopreservation in all treated media that used for epididymal sperm activation (PX, *G.glabra* and PX with *G.glabra*) compare to before activation. However, by using Ham's F-12 medium, the DNA denaturation of epididymal sperm activation *in vitro* demonstrates a significant (P<0.05) reduction after activation and cryopreservation of treated media. After cryopreservation DNA denaturation showed a significant (P<0.05) increase in control Ham's F-12 medium as compared to that after activation (Figure 2).

-Results of comet assay:

In general, intact cells under fluorescent microscope appear round (all DNA incorporated in head of comet, image 2 A), while cells with DNA damage have a tail (comet) that illustrated in (image 2 B), the length of tail of comet was directly related to the amount of DNA damage in the cells.

The results of comet assay after activation and cryopreservation with (PX, *G.glabra* and PX + *G.glabra* media) revealed a highly significant (P<0.001) decrease in comet length, tail length, DNA in tail and tail moment in treated media compared with the before activation and control Ham's F-12 medium before and after cryopreservation. Also the results show a highly significant (P<0.001) increase in DNA of sperm head in treated media compared with the before activation and control Ham's F-12 medium before

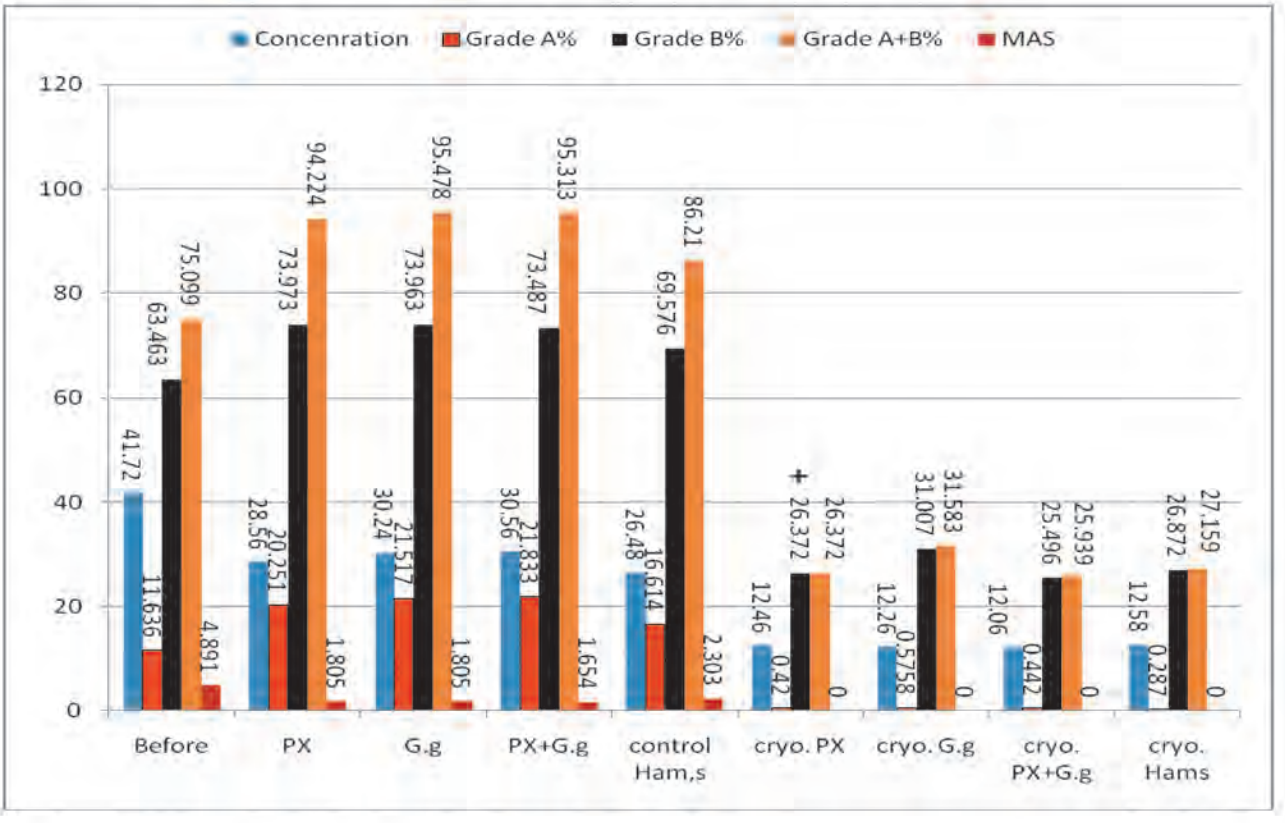


Figure (1): Histograms showing certain sperm function parameters after *in vitro* sperm activation and cryopreservation with PX, *G.glabra* , PX + *G.glabra* and Hams F-12 media.

* Highly significant (P<0.001) decrease in sperm concentration in before and after activation and cryopreservation.

** Highly significant (P<0.001) increase in active grade motility between before and after activation.

+ Highly significant (P<0.001) decrease in active grade motility between before and after cryopreservation.

MAS: Morphologically Abnormal Sperm. PX: Pentoxifylline *G.glabra : Glycyrrhiza glabra*

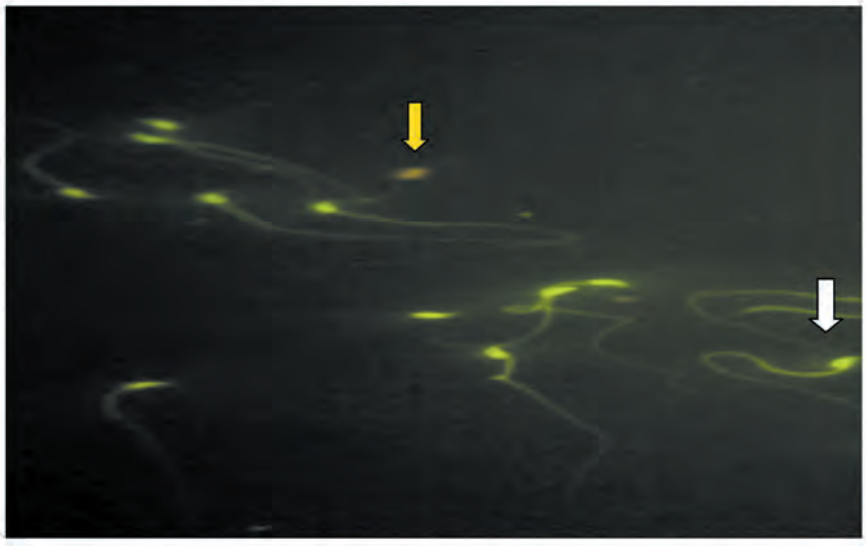


Image (1): Acridine orange test showing sperm normal DNA (white arrow) and sperm DNA denaturation (yellow arrow)

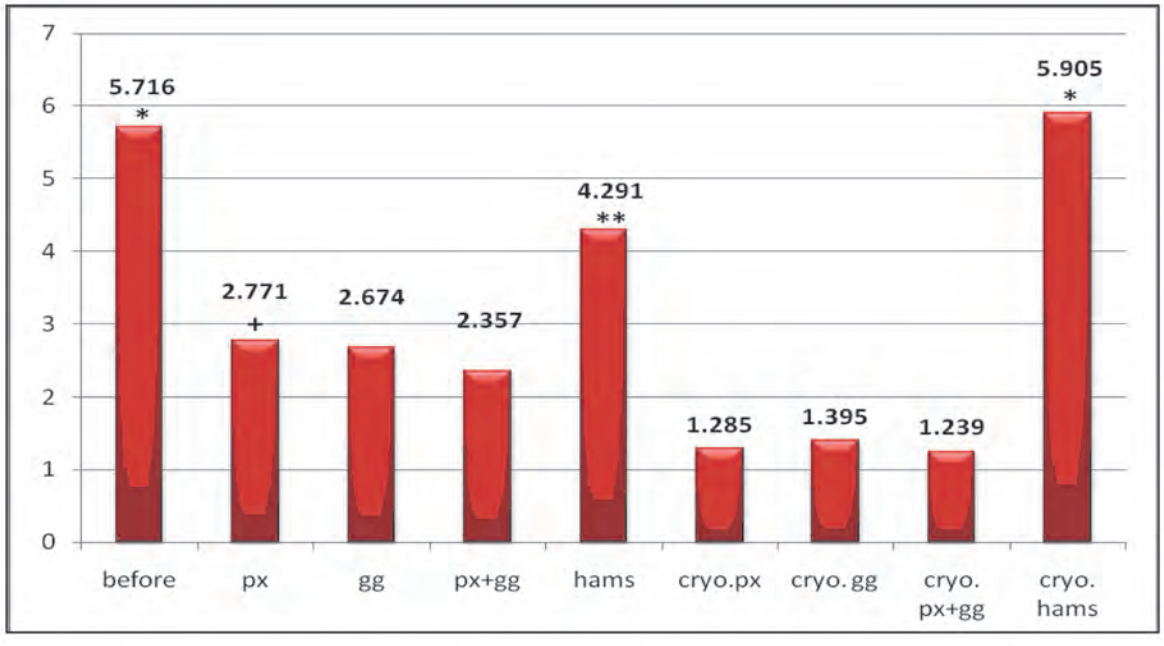


Figure (2): Histograms showing the percentage of DNA denaturation after activation and cryopreservation on epididymal sperms.

*P<0.001 a highly significant difference in before and after activation and cryopreservation and then to the control Ham's F-12 medium cryopreservation.

**P<0.05 a significant difference in treated PX medium and control Ham's F-12 medium.+P>0.05 no significant difference in treated PX, PX + *G.glabra* and *G.glabra*..

PX: Pentoxifylline *G.glabra: Glycyrrhiza glabra*



Image (2): SCGE stained with EtBr cell that have intact DNA (white arrow) normal and cell that have DNA damage form a comet (yellow arrow) abnormal.

Table (1): Comet assay results after and before *in vitro* activation and cryopreservation with PX and Ham's F-12 media.

Parameters	Before activation	After activation		After cryopreservation	
		Treated PX	control Ham's	Treated PX	control Ham's
Comet length	1.909±0.199	1.541±0.082*	1.600±0.933 *	1.040±0.782 *	1.938±0.125
Head diameter	1.342±0.500	1.287±0.450	1.248±0.318	6.464±0.336	1.385±.080
DNA in head	90.912±0.599	95.848±0.419*	94.478±0.428*	96.904±0.267*	90.887±0.597
Tail length	56.689±4.539	25.317±3.499*	35.209±3.233*	9.376±0.882*	55.293±4.143
DNA in tail	9.088±0.599	4.152±0.418*	5.521±0.428*	3.096±0.266*	9.113±0.596
Tail moment	1.501±0.467	6.779±1.836	6.899±1.636	1.159±0.238	1.179±0.211

Values are expressed as mean ±SEM. ANOVA
*Highly significant (P<0.001) differences between before and after activation and cryopreservation.

Table (2): Comet assay results before and after *in vitro* activation and cryopreservation with *G.glabra* and Ham's F-12 media.

Parameters	Before activation	After activation		After cryopreservation	
		Treated *G.glabra*	Control Ham's	Treated *G.glabra*	control Ham's
Comet length	1.909±0.199	1.527±0.635*	1.600±0.933*	1.300±0.937*	1.938±0.125
Head diameter	1.342±0.500	1.401±0.537+	1.248±0.318*	1.122±0.494	1.385±.080
DNA in head	90.912±0.599	97.613±0.179*	94.478±0.428*	94.538±0.367	90.887±0.597
Tail length	56.689±4.539	12.589±0.714*	35.209±3.233*	17.80±1.067*	55.293±4.143
DNA in tail	9.088±0.599	2.387±0.179*	5.521±0.428*	5.462±0.367	9.113±0.596
Tail moment	1.501±0.467	0.677±0.074*	6.899±1.636*	1.997±0.227*	1.179±0.211

Values are expressed as mean ±SEM, ANOVA
*Highly significant (P<0.001) differences between before and after activation and cryopreservation.
*Significant (P<0.05) differences between before and after activation.
G.glabra: *Glycyrrhiza glabra*

Table (3): Comet assay results after and before *in vitro* activation and cryopreservation with PX + *G.glabra* and Ham's F-12 media.

Parameters	Before activation	After activation		After cryopreservation	
		Treated PX+ *G.glabra*	control Ham's	Treated PX + *G.glabra*	control Ham's
Comet length	1.909±0.199	1.499±0.591*	1.600±0.933*	1.234±0.509*	1.938±0.125
Head diameter	1.342±0.500	1.363±0.377*	1.248±0.318*	1.112±0.248*	1.385±.080
DNA in head	90.912±0.599	97.640±0.160*	94.478±0.428*	97.253±0.182*	90.887±0.597
Tail length	56.689±4.539	13.596±0.749*	35.209±3.233*	12.119±0.742*	55.293±4.143
DNA in tail	9.088±0.599	2.359±0.161*	5.521±0.428*	2.746±0.181*	9.113±0.596
Tail moment	1.501±0.467	0.686±0.079*	6.899±1.636*	0.773±0.108*	1.179±0.211

Values are expressed as mean ±SEM ,ANOVA

Discussion

Epididymal sperm activation *in vitro* with the PX, *G.glabra* and PX+ *G.glabra* media noticed positive effect on certain sperm function parameters and DNA normality after activation and cryopreservation by using comet assay and acridine orange stain tests. The action of PX is mediated by inhibition of phosphodiesterase enzyme leading to an increase in cAMP and the *G.glabra* contains Ca++ with different compounds will stimulate the motility of sperm (13) (14). The decrease in the percentage of MAS may resulted from direct activation technique, in addition to the positive effect of both PX and *G.glabra* in stimulating the swim up only of sperm with normal form to move upward. These results are in agreement with those obtained by Al-Dujaily and Malik (15). Moreover, the decrease in DNA abnormality following cryopreservation can be explained by the interaction of PX with the lipid content of the sperm cell membrane, which contains a high proportion of polyunsaturated fatty acids. The positive interaction of PX with polyunsaturated fatty acid may decrease the production of lipid peroxidation which is the source of reactive oxygen species (ROS) (3).

On the other hand, *G.glabra* contains different antioxidants with different cryoprotectants such as glucose, sucrose, fructose, and maltose. All will exert a positive effect on DNA following cryopreservation of epididymal sperm. However, there was a decline in sperm motility by using the four media following cryopreservation. This finding can be observed because of the nature of epididymal sperm that cannot be equivalent to ejaculate sperm. The caudal region is responsible for protection, maturation and transportation of new product of sperms (17). The mature sperm may resist the external environment whereas the epididymal sperm may contain cytoplasmic droplet which is the source of ROS (18).

The cryopreservation process of epididymal sperms is different from ejaculate sperm because of the increased sensitivity of the latter to DNA damage resulted from enhanced nuclease activity present in the ejaculate sperm of mouse (19). Also Membrane properties between epididymal and ejaculated sperms are different, which may affect sperm survival after cooling and freezing (20). The other explanation that may affect positively in decreasing DNA abnormality is that several dilutions occurs during activation and cryopreservation lead to decrease in abnormal cells and absence of leukocytes may generate ROS. This finding is compatible with other studies that found spermatozoa (21) (22) and leukocytes (23) (24)are the potential sources of ROS .

In addition to PX activation in the mixture medium the *G.glabra* constituents contributed as antioxidants and hepatoprotective properties. Glycyrrhizin and glabridin inhibit the generation of ROS by neutrophils at the site of inflammation (25) (26). *In vitro* studies have demonstrated that licorice contains isoflavones, hispaglabridin A and B, inhibit Fe3+ to induce lipid peroxidation in rat liver cells (27). Other researches indicated that glycyrrhizin lowers lipid peroxide values in animal models of liver injury caused by ischemia reperfusion (28).

Finally, according to the properties of PX, *G.glabra* and glycerol after activation and cryopreservation-thawing process, there was a best result in active sperm motility and DNA integrity which may indicate that PX and *G.glabra* can be used for activation as well as cryoprotectant with the glycerol. All media used for *in vitro* sperm activation (PX, *G.glabra* and PX + *G.glabra* media) kept sperms from freezing damage and did not have any toxic effect on the DNA .

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The Role of E-cadherin in the embryo implantation and its site in the endometrium

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Abstract:

Background:
Endometrial receptivity is defined as a temporary unique sequence of factors that make the endometrium receptive to the embryonic implantation. Histological evaluation, now considered to add little clinically significant information, should be replaced by functional assessment of endometrial receptivity. A large number of functional molecular mediators have been identified to date, including adhesion molecules. Thus, endometrial biopsy samples can be used to identify molecules associated with uterine receptivity to obtain a better insight into human embryo implantation.

Objectives:
to investigate E-cadherin expression in the endometrium throughout the menstrual cycle of fertile females

Patients, Materials and Methods:
Under simple analgesia, fractional endometrial biopsies from anterior, posterior, fundal walls, and the cervix of (32) fertile females(age= 29.91± 5.9 year, parity= 3.62.1±) were taken at different days of the menstrual cycle. The immunohistochemical (IHC) stained tissues were analyzed using computerized image analysis for measurement of PI of E-cadherin which is ratio of the number of stained cells to the number of total cells. PI were measured in three regions: stromal, membranous, and glandular epithelium.

Results:
E-cadherin expression in the endometrium is up-regulated in the proliferative phase and down-regulated in the secretory phase of the menstrual cycle. The PI of E-cadherin in the glandular epithelium of the anterior wall of the endometrium was significantly lower than that of posterior and fundal walls (P=0.027). The PI of E-cadherin in the endocervical epithelium was significantly higher than that in the anterior, posterior, and fundal walls of the endometrial cavity (P=0.004).

Conclusion:
Conclusion: E-cadherin might have a role in embryo implantation

Key Words: Endometrial receptivity; adhesion molecules;E-cadherin.

Introduction
Endometrial receptivity is defined as a temporary unique sequence of factors that make the endometrium receptive to the embryonic implantation (1). It is the window of time when the uterine environment is conductive to blastocyst acceptance and subsequent implantation (2).

Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst developmental stage and a synchronized dialogue between maternal and embryonic tissues (3). The process of implantation may be classified into three stages: apposition, adhesion and invasion (4). Even though the blastocyst can implant in different human tissues, surprisingly in the endometrium, this phenomenon can only occur during a self-limited period spanning between days 20 and 24 of a regular menstrual cycle. Throughout this period, namely the window of implantation (5), the human endometrium is primed for blastocyst attachment, given that it has acquired an accurate morphological and functional state initiated by ovarian steroid hormones(6)(7)(8).

Implantation involves a complex sequence of signaling events, consisting in the acquisition of adhesion ligands together with the loss of inhibitory components, which are crucial to the establishment of pregnancy(9). Histological evaluation, now considered to add little clinically significant information, should be replaced by functional assessment of endometrial receptivity. A large number of molecular mediators have been identified to date, including adhesion molecules, cytokines, growth factors, lipids and others. Thus, endometrial biopsy samples can be used to identify molecules associated with uterine receptivity to obtain a better insight into human implantation (9).

Cadherins constitute a group of glycoproteins responsible for the calcium-dependent cell-to-cell adhesion mechanism. They are divided into subclasses E- (epithelial), P- (placental), and N- (neuronal) cadherins that are distinct in immunological specificity and tissue distribution. They promote cell adhesion via a homophilic mechanism. In regard to implantation, E-cadherin represents the most studied subclass.E-cadherin is a cell surface transmembrane glycoprotein, which belongs to the family of calcium-dependant CAMs, that mediates cell–cell adhesion through homeotypic binding. E-cadherin is located in the adherents junctions that are specialized regions on the lateral side of the epithelial plasma membrane and is believed to be critical for the establishment and maintenance of these junctions in epithelial cells (10,11). E-cadherin is expressed by a variety of tissues and plays an important role in embryogenesis formation during gastrulation, neurulation and organogenesis (12). Studies on mouse embryo implantation have shown that targeted mutations in the E-cadherin gene result in defective preimplantation development (13). The role of E-cadherin in human embryo implantation is not fully known, but based on its expression pattern; it has been suspected that it is of importance for this process. E-cadherin mRNA levels were shown to be significantly higher during the luteal phase (14). Nevertheless, these menstrual cycle variations were not detected at the protein level by immunohistochemical studies (15).

The regulation of E-cadherin availability at the epithelial cell surface enables cellular adhesion control. Down-regulation of E-cadherin expression correlates with the acquisition of metastatic potential by carcinomatous cells. Subsequently, the tissue architecture is lost resulting in cell dissociation and dispersion (16). Intracellular calcium is essential in the E-cadherin regulation. Indeed, a rise in its concentration activates key signaling pathways that mediate cytoskeletal reorganization and disassembly of E-cadherin at the adherents junctions. Alterations in intracellular calcium concentrations affect epithelial cell adhesiveness and polarity by triggering CAMs redistribution (17). This phenomenon could be of importance in EECs expressing E-cadherin. Interestingly, calcitonin expression is induced by progesterone in the human endometrial epithelium specifically during the mid-secretory phase of the menstrual cycle (18). Indeed, calcitonin is known to be a potential regulator of implantation (19). Progesterone, probably via endometrial calcitonin induction leading to increased intracellular calcium, could regulate E-cadherin expression. Thus, it is possible that E-cadherin possesses a dual function. In the preliminary phases, its expression at the cell surface is required to ensure adhesiveness. In contrast, E-cadherin may be subsequently down-regulated to enable epithelial cells dissociation and blastocyst invasion(9).

Objectives

to investigate E-cadherin expression in the endometrium throughout the menstrual cycle of fertile females

Materials and Methods

Thirty-two fertile females, between 20- 40 years of age, participate in the current study as a control (volunteers). They are parous with no history of abortion, gestational trophoblastic disease, preterm labour or ectopic pregnancy. Control group are collected from patients attend gynaecological department in Al- Yarmouk Teaching Hospital who agreed to participate in the study as a volunteers with informed consent form.

Under simple analgesia, fractional endometrial biopsies from anterior wall, posterior wall, fundus, and endocervix were taken for the control group at different days of the menstrual cycle to investigate the changes in the expression of the studied marker (E- cadherin) throughout the cycle and to estimate the differences in the level of expression between different walls. The formaldehyde fixed paraffin embedded tissues are stained with immunohistochemical stain. To quantify immunostained cells objectively, a computerized image analysis program (Aperio Image scope) was used. Several parameters per sample were computed: the percentage of immunostained surface (compared with the counterstained surface), the mean staining intensity, and an immunostained score (percentage of immunostained surface _ mean staining intensity).

Results

In table (1) shows that the E-cadherin was measured in the endometrium at three sites: anterior, fundal, and posterior walls in addition to the endocervical epithelium from early proliferative phase to the midsecretory phase. Three epithelial regions were evaluated for E-cadherin expression: membranous, stromal, and glandular epithelium .

The expression is increasing with time during the proliferative phase (eg. at anterior stromal epithelium it increases from (4 ±1) and peak at late proliferative phase (40± 6), then decline gradually towards midsecretory phase(32 ±3 at day 20 to 8± 2 at day 25) (implantation window). Note that the expression of fundal wall and ;to a lesser extent; the posterior wall continue to rise to the midsecretory phase,while the expression of the anterior wall and the cervix decline at the same period figuns (1,2,3).

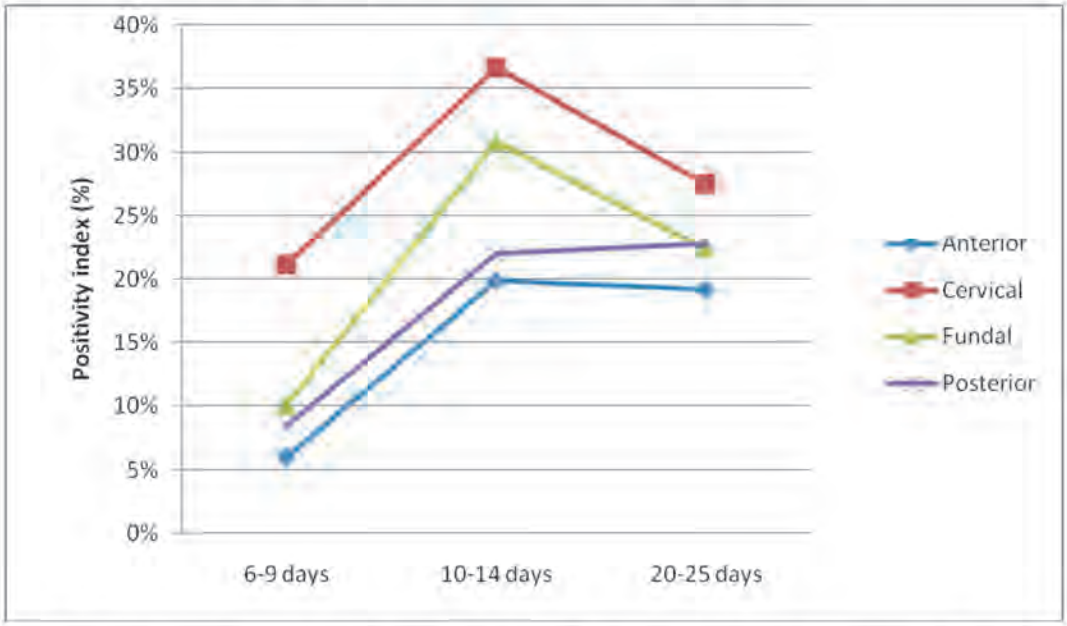
Table(1): Mean E- cadherin expression from early proliferative phase towards midsecretory phase in the anterior, posterior and fundal walls.

Site of endometrium		E- cadherin expression							
		Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Site	Region	6th day	7th day	8th day	9th day	10th day	14th day	20th day	25th day
Anterior	Stromal	4±1	7±2	10±1	12±1	20±4	40±6	32±3	8±2
	Membranous	5±1	5±1	6±1	9±1	15±2	29±5	24±6	16±2
Posterior	Glandular	4±1	4±1	4±1	5±1	8±1	26±11	26±4	6±3
	Stromal	8±2	10±1	10±1	18±1	22±1	32±2	27±4	19±8
	Membranous	8±1	6±1	8±1	10±1	16±4	33±3	33±5	20±8
Fundal	Glandular	5±1	4±1	7±1	10±1	15±2	27±7	25±5	13±7
	Stromal	12±1	14±1	14±1	16±1	24±2	44±12	43±8	32±4
	Membranous	8±1	10±1	10±1	11±1	13±0	31±14	40±11	23±11
	Glandular	5±1	6±1	8±1	10±1	14±2	25±6	29±6	18±7

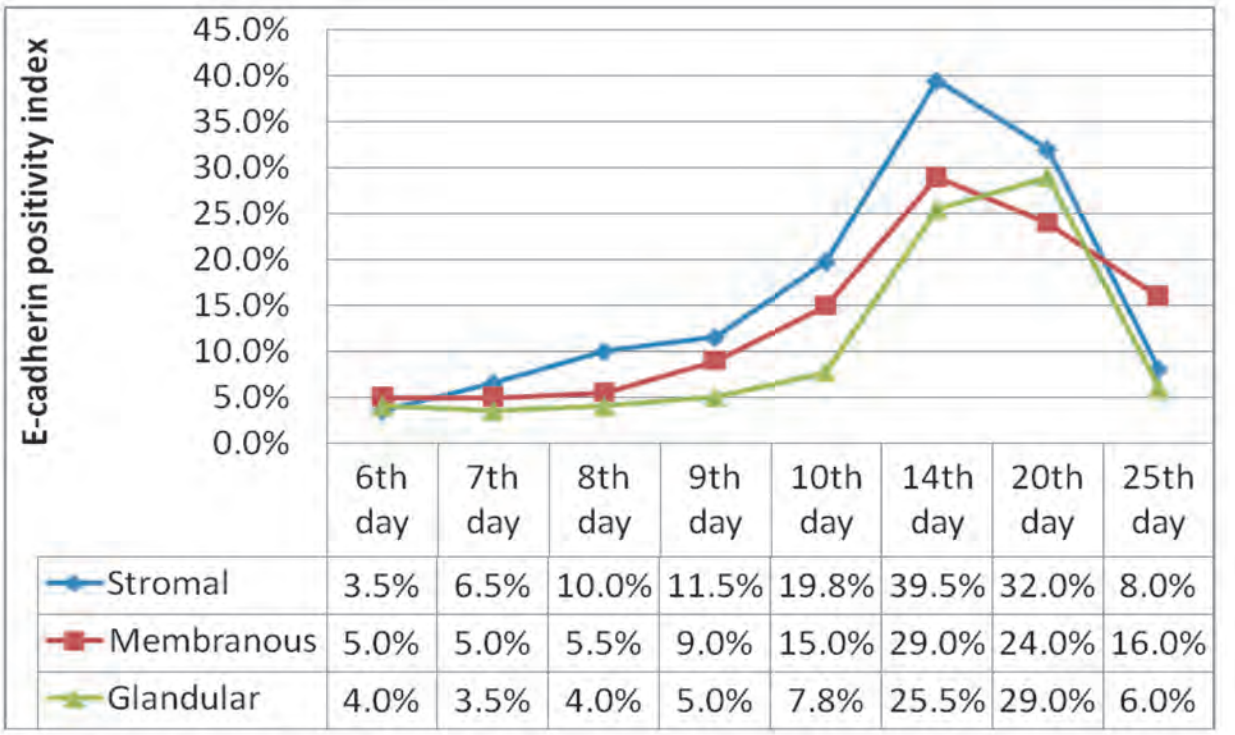
Figure (1)shows PI of E-cadherin in the three walls of endometrial cavity. Note that the cervix has the highest level at peaks (37%) while the anterior wall has the lowest level at peaks(20%).

Figure (2) shows that the PI of epithelial E-cadherin is increasing steadily at early proliferative phase (day 6 to day 9) then peak sharply at late proliferative phase(day 10 to day 14) then decrease in the secretory phase. The glandular epithelium shows the lowest level of PI at peaks (up to 29%) compared with 39.5% in the stromal epithelium while the decrement is sharp in the glandular epithelium (down to 6%) and more steadily in the membranous (down to 16%) and to lesser extent stromal epithelium(down to 8%).As in the anterior wall, the increase starts steadily at early proliferative phase and peaks at late proliferative phase then decrease at early and midsecretory phase. The membranous epithelium shows the highest peak of PI followed by stromal epithelium. The glandular epithelium has the lowest PI level at peaks and the most sharp decrement in the secretory phase. Similar to the anterior wall, steady early followed by late sharp in increase is noticed. Again, the glandular epithelium has the lowest level at peaks.

Figure (2) shows that the PI of E-cadherin is increasing during the proliferative phase, peaks at late proliferative phase and ovulation, and decreasing in the secretory phase .The same results are shown in figure (3) steady elevation in stromal, membranous, and glandular regions in the three walls during day 6,7,8, and 9, respectively.

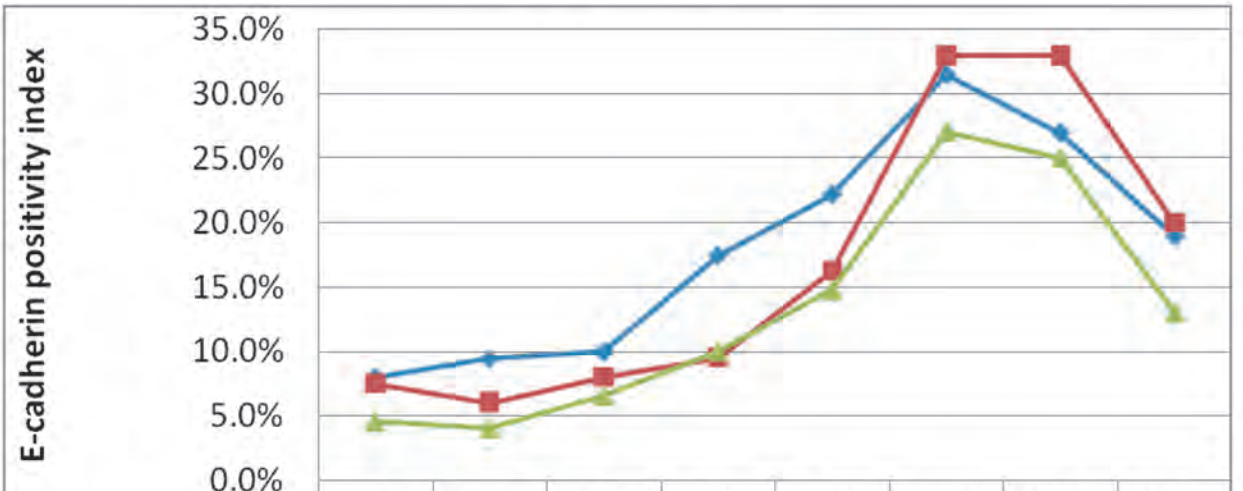


Figure(1): E-cadherin expression in different endometrial walls and the cervix at different days of the menstrual cycles.



	6th day	7th day	8th day	9th day	10th day	14th day	20th day	25th day
Stromal	3.5%	6.5%	10.0%	11.5%	19.8%	39.5%	32.0%	8.0%
Membranous	5.0%	5.0%	5.5%	9.0%	15.0%	29.0%	24.0%	16.0%
Glandular	4.0%	3.5%	4.0%	5.0%	7.8%	25.5%	29.0%	6.0%

Figure (2): E-cadherin positivity index in the anterior wall of the endometrium.



	6th day	7th day	8th day	9th day	10th day	14th day	20th day	25th day
Stromal	8.0%	9.5%	10.0%	17.5%	22.3%	31.5%	27.0%	19.0%
Membranous	7.5%	6.0%	8.0%	9.5%	16.3%	33.0%	33.0%	20.0%
Glandular	4.5%	4.0%	6.5%	10.0%	14.8%	27.0%	25.0%	13.0%

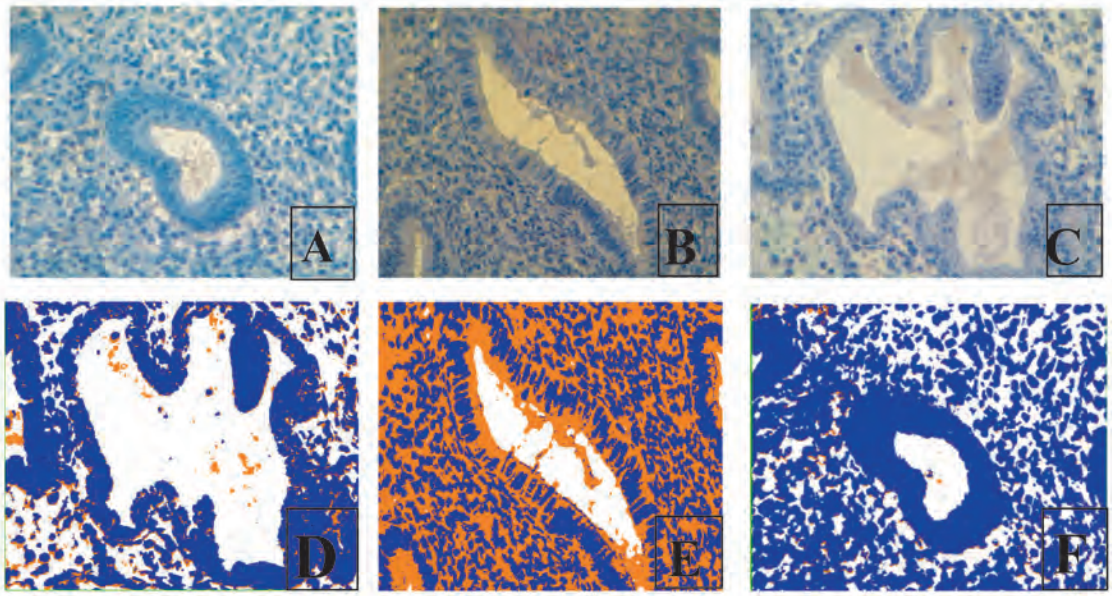
Figure (3): PI of E- cadherin expression in the posterior wall of the endometrium.

In table (2) PI is measured in three regions: stromal, membranous, and glandular epithelium during day 10 of the cycle .In the anterior wall, there was a significant difference in PI of E-cadherin expression between the stromal (20%), membranous(15%), and glandular(8%) (P=0.027). A significant difference was found between the same regions in the fundal wall(24%, 13%,and 14% for stromal, membranous, and glandular regions respectively)(P=0.001). No such significant difference were found in the posterior wall (P=0.171). In table (2), a significant difference was found between the three walls in the glandular epithelium (P=0.037) while no significant differences were found between the three walls in stromal and membranous epithelium..

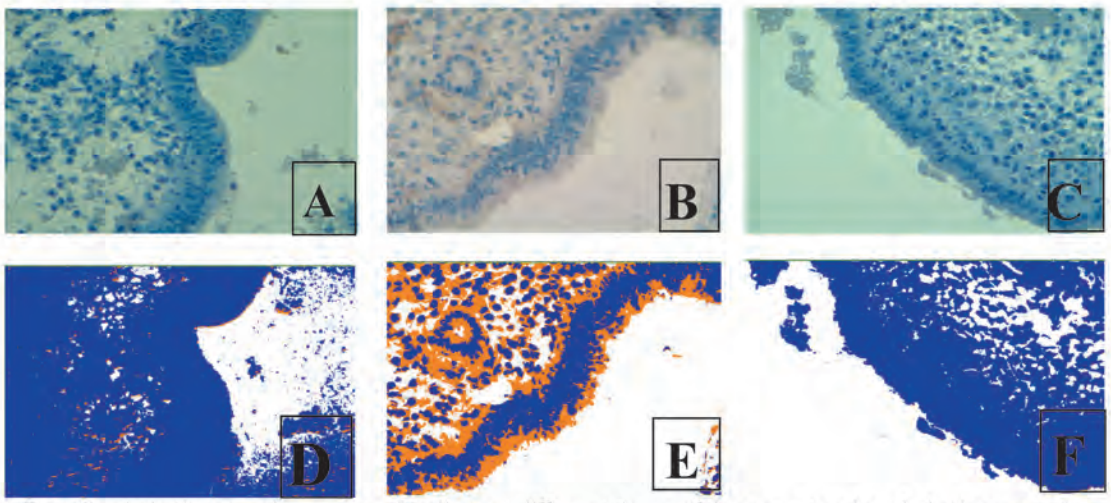
Table (2):The mean PI of E-cadherin expression in the anterior, posterior, and fundal walls of the control group

Control		Anterior		Fundal		Posterior		p value
		Mean	SD	Mean	SD	Mean	SD	
Histological Layer	Stromal	20%	4	24	2	22%	1	0.461
	Membranous	15%	2	13%	0	16%	4	0.689
	Glandular	8%	1	14%	2	15%	2	0.037
P Value		0.027		0.001		0.171		

In figures 4 and 5 ,A more detailed analysis of the PI in the three histological regions, showed that there is a significant difference between stromal, membranous, and glandular epithelium (P=0.027) which may be attributable to the low PI of the glandular epithelium. A more significant difference were noticed in the fundal wall(P=0.001). This may be attributable to the higher PI of the stromal epithelium. No such significant difference were found in the posterior wall (P=0.171). There is no significant(P>0.05) difference between the three walls in mean PI.



Figure(4): E-cadherin in the glandular epithelium at different times of the menstrual cycle in the control group. IHC staining of endometrial glands at early proliferative phase(A), late proliferative phase(B), and mid secretory phase(C). D, E, and F are computerized image analysis for PI of E-cadherin in A, B, and C



Figure(5): E-cadherin in the membranous epithelium at different times of the menstrual cycle in the control group. IHC staining of endometrial surface epithelium at early proliferative phase(A), late proliferative phase(B), and mid secretory phase(C). D, E, and F are computerized image analysis for PI of E-cadherin in A, B, and C.

Discussion

The PI of E-cadherin day 3, 4, and 5 were excluded from the statistical analysis first because the value of PI is very low (near zero) at the time of shedding that might affect the statistical results. Secondly because the value of PI is very high indicating that the sample is from the basalis layer and not from the functionalis(21)

The PI of E-cadherin is increasing during the proliferative phase, peaks at late proliferative phase and ovulation, and decreasing in the secretory phase This may reflect the relatively steady slow elevation of estradiol during early follicular phase (18). The follicular phase is of variable length in part because of the uncertainty about when its functional onset , the intercycle FSH elevation, truly takes place(11). It may be indicated by the sharp increment that starts at day 10 of the cycle and reaches peaks afterward. Yet, this variability appears not to affect the endometrial priming and has no practical consequences on the endometrial responsiveness in the ensuing luteal phase.(11)

The PI of E-cadherin is higher in the stromal epithelium than membranous and glandular epithelium.

The elevation in stromal E-cadherin more than other region may be related to the higher level of mitotic activity in this region(19.)

It can be observed that the peak of E-cadherin is reached at day 14 in most cases ,however the peak of glandular and membranous epithelium was more or less equal in the anterior wall while there is discrepancy between the two peaks in other walls. Delay of the glandular epithelium in reaching peaks until the secretory phase is due to continuation of gland mitosis in that phase as observed by Noyes, et al. (22).Since the adhesiveness is positively correlated with E-cadherin level (17) and that glandular secretion requires lowering of this adhesiveness, this may explain the continuously observed low level for E-cadherin in the glandular epithelium in all walls. Low level of peaks for membranous and glandular epithelium in the anterior wall might point to a suitable site of implantation.

Concerning the style of decrease in PI of E-cadherin expression after peak, it is obvious that the decrease in the PI of the anterior wall is sharp in glandular epithelium and more steady in the stromal and membranous epithelium. This may reflect the rapid changes during the implantation window in the favorable sites since loose adhesiveness in the glandular epithelium enables the secretion from the endometrial glands which is the main source of uterine endometrial secretion (uterine milk) that is the nutrient to the developing embryo(23).

In the posterior wall, the decrease in PI is steady in all regions with obvious delay in the membranous epithelium while in the fundal wall, the decrease in PI is steady in stromal epithelium, delayed and sharp in the glandular and membranous epithelium. These two walls may show high level compared with anterior wall to maintain integrity of the endometrium while the embryo is invading the anterior wall.

The lowest level of PI of the membranous epithelium remains higher than that of other regions except for the fundal wall where the lower level of E-cadherin PI in the stromal epithelium(32%) is larger than other region(23%,18% for membranous and glandular epithelium respectively). This may be necessary to maintain integrity of the endometrial epithelium.

In some aspects these result are consistent with Shih et al.(24) who found that the expression of E-cadherin in glandular cells was observed mainly in the proliferative phase. The E-cadherin expression decreased significantly in the secretory phases both in the functionalis and in the basalis compared to levels in the proliferative phase. Stromal cells showed positive staining for E-cadherin in the proliferative and early secretory phases, but the positive disappeared in the mid- and late secretory phases. Surface epithelial cells showed positive staining for Ecadherin throughout the menstrual cycle with a slight predominance in the secretory phase(10) .No predominance of E-cadherin in surface epithelium were noticed in this study, the same observation was found by other study that very low or no protein expression of E-cadherin, in luminal and glandular epithelial cell in the mid-secretory endometrium of healthy fertile controls(14). These findings suggest that temporal down-regulation or loss of E-cadherin expression during the window of implantation might be necessary to enable epithelial cell dissociation and blastocyst invasion, in agreement with the results of recent animal study (14).

In the three histological regions, considering that the anterior wall is more suitable for implantation, showed low PI of the glandular epithelium and higher PI of the stromal epithelium with no difference were found in the posterior wall. Since there is no significant difference between the three walls in mean PI, then it is the relative differences between these histological sites and not the level of PI which is important. Thus ,this study concluded that E-cadherin play an important role in endometrium receptivity.

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Studying the risk factors for ectopic pregnancy among spontaneous and assisted conception

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Abstract:

ackground:
An ectopic pregnancy, or eccyesis, is a complication of pregnancy in which the pregnancy implants outside the uterine cavity. Theoretically, differences between conception via ART and natural conception may affect the risk of ectopic pregnancy. Data on the risk factors for developing ectopic pregnancy after IVF are still inconsistent. There is controversy whether ART increase or decrease the ectopic pregnancy.

Objectives:
To describe the occurrence or risk factors of ectopic pregnancy following IUI, IVF and spontaneous pregnancy

Patients and Methods:
The recorded cases of ectopic pregnancy in the period between January 2010 to January 2011 in four teaching hospitals and two private hospitals were surveyed. New cases in the period between July 2011 to July 2012 in two teaching hospitals were also included in this study. The data was collected from medical records and interview.

Results:
A total of 86676 pregnancies were recorded during the period of the study with, the number of ectopic pregnancy out of total pregnancies in Baghdad was 408 during the period of this study. It was found that the type of conception was significantly associated with infertility ($P < 0.001$), ovulation induction ($P<0.001$), dilatation and curettage ($P = 0.016$), and history of contraception ($P =0.002$).

Conclusion:
Although several risk factors for ectopic pregnancy are known, the causes of increasing incidence of ectopic pregnancy remain unknown. The risk factors of ectopic pregnancy following IUI, IVF and spontaneous pregnancy included history of infertility, medication used for ovulation induction, history of contraception, and dilatation curettage as these factors are tightly linked with the types of conception. Increasing knowledge of risk factors for ectopic pregnancy may improve understanding of the causes of infertility.

Key Words: Ectopic pregnancy, IUI, IVF, Risk factors

Introduction

An ectopic pregnancy, or eccyesis, is a complication of pregnancy in which the pregnancy implants outside the uterine cavity (1). The incidence of ectopic pregnancy has increased all over the world from 0.5% Thirty years ago, to a present day 12%-. Most ectopic pregnancies occur in the Fallopian tube (so-called tubal pregnancies), but implantation can also occur in the cervix, ovaries, and abdomen. An ectopic pregnancy is a potential medical emergency, and, if not treated properly, can lead to death. There are a number of risk factors for ectopic pregnancies. However, as many as one third (2) to one half of ectopic pregnancies, no risk factors can be identified. Risk factors include: pelvic inflammatory disease, infertility, use of an intrauterine device (IUD), previous exposure to DES, tubal surgery, intrauterine surgery (e.g. D&C), smoking, previous ectopic pregnancy, and tubal ligation(2).

Assisted reproductive technology (ART) is a general term referring to methods used to achieve pregnancy by artificial or partially artificial means. Examples of ART include *in vitro* fertilization, intracytoplasmic sperm injection (ICSI), cryopreservation, and intrauterine insemination (IUI) (3).

Data on the risk factors for developing ectopic pregnancy after IVF are still inconsistent. Between fresh non donor IVF and embryo transfer cycles, the significant risk factor for ectopic pregnancy was tubal factor infertility, and endometriosis, rather than male factor infertility. Higher ectopic pregnancy rate could be associated with zygote intrafallopian transfer, assisted hatching, large embryo transfer volume, deep fundal transfer, and frozen embryo transfer. . Higher implantation potential per embryo at the blastocyst stage may increase the risk of ectopic pregnancy than cleavage stage. Especially, according to numbers of embryos transferred, different risk of ectopic pregnancy after IVF was noted (4).

The aim of this study is to evaluate risk factors of ectopic pregnancy following IUI, IVF and spontaneous pregnancy.

Patients and Methods

The recorded cases of ectopic pregnancy in the period between January 2010 to January 2011 in four teaching hospitals and two private hospitals were surveyed. Two teaching hospitals in Al-Karkh side: Al-Yarmouk and Al-Kadhymia Teaching Hospitals are included in this study as well as two teaching hospitals in Al-Rusafa side Al- Ulwyia and Baghdad Teaching Hospitals and the two private hospitals: AL-Salama Hospital in AL-Karkh side and AL-Jarah hospital in Al-Rusafa side.

New cases in the period between July 2011 to July 2012 in two hospitals: Al-Kadhymia and Al-Yarmouk teaching hospitals were also included. In addition to the cases of IUI from the High Institute of Infertility Diagnosis and Assisted Reproductive Technology and IVF cases from private center for IVF in Baghdad.

This study is conducted to compare the risk factors of ectopic pregnancy among women who conceived after IVF and IUI and among women with spontaneous pregnancy. The data were collected from medical records and interview regarding patients age, parity, gestational age, ectopic pregnancy risk factors like history of previous abortion ,infertility treatment ,current use of intrauterine contraceptive device (IUD), history of previous tubal surgery (i.e. tubal ligation, sterilization reversal) history of appendicectomy, caesarean section, appendicitis complicated by peritonitis, endometriosis, ovulation induction, IUI or IVF procedures (done in or outside the Iraq) , in addition to smoking habits, sexual and infectious history, characteristics of ectopic pregnancy , and the treatment procedure used.

Statistical analysis: The data were analyzed by using SPSS version 16 and Microsoft office 2007; numerical data were presented using Mean ± SD. Statistical tests used include: (Student t test used to compare the mean, chi square test used to compare the frequency, and Fish Exact test).P value <0.05 was considered significant.

Results

A total of 86,676 pregnancies were recorded during the period of the study with 408 pregnancies were outside the uterus proper as shown in table (1).

Table 1: Number and percentage of ectopic pregnancy from the total number of pregnancies included in the study.

	Ectopic	In topic	Total pregnancy
Number	408	86268	86676
Percent	0.47	99.53	100

The proportion of different types of conception: IVF, IUI and spontaneous conception among ectopic pregnancy is shown in table (2).

Table 2: Proportion of different types of conception among ectopic pregnancy.

Types of conception	Ectopic No. (%)	*P* Value
IVF	23 (5.64%)	<0.001
IUI	9 (2.20%)	
IVF &IUI	32 (7.84%)	
Spontaneous	376 (92.16%)	
Total	408 (100%)	

A significant association was found between different types of conception (IVF, IUI, and spontaneous conception) among ectopic pregnancy.

For the cases included, the mean age of the studied group and the mean age at marriage are shown in the table (3). There is a significant association between increasing age and types of conception (P =0.004), while there is a non significant association between age at marriage and the types of conception (P=0.129).

Table3: Mean age of studied group and mean age at marriage.

	Group1 (spontaneous)	Group2(IVF,IUI)	*P* value
Number	376	32	---
Mean age	30.04±6.42	32.72±4.66	0.004*
Mean age at marriage	25.38±5.20	23.89±4.95	0.129

*significant association between increasing age and types of conception.
The statistical test is (t- test).

Table4: Frequency of curettage in the studied group of pregnant women.

D&C	Group1 (spontaneous) No. (%)	Group2 (IVF or IUI) No. (%)	Total	*P* Value
No	282 (75%)	30 (94%)	312	0.016
Yes	94 (25%)	2 (6%)	96	
Total	376 (100%)	32 (100%)	408	

Statistical test is Chi-square test, DF =1
Significant association was found between D&C and the types of conception.

There was a significant association between history of infertility and types of conception ($P < 0.001$) as shown in table (5).

Table4.5: Type of infertility in the studied group of ectopic pregnancy.

Infertility	Group1 (spontaneous) No. (%)	Group 2 (IVF,IUI) No. (%)	Total	P Value
No	258 (68%)	0 (0%)	258	<0.001
Yes	118 (32%)	32 (100%)	150	
Total	376 (100 %)	32 (100%)	408	

Statistical test is Chi-square test, DF =1

The mean duration of infertility for the studied cases is shown in table 6

(Table 6): Duration of infertility in the studied group.

	Group1(spontaneous)	Group 2(IVF,IUI)	*P* value
Number	118	32	---
Mean duration	3.68±2.55	5.89±3.54	0.004*

Significant association between duration of infertility & types of conception.*
Statistical test is(t- test).

The duration of infertility was significantly associated with types of conception as P=0.004

Table 7: Association between past history and types of conception.

Past history	Group1(spontaneous) No. (%)	Group2 (IVF or IUI) No. (%)	*P* value
Ovulation induction	147 (39%)	30 (94%)	<0.001*
PID	39 (10%)	2 (6%)	0.630 †
Surgery	58(15%)	7 (21%)	0.439 *
Previous EP	17 (5%)	4 (12.5%)	0.072 †
Contraception	73 (19%)	3(9%)	0.002*
Vaginal douching	49 (13%)	4 (13%)	1.000 †
Previous disease	5 (1%)	0(0%)	0.934 †
Smoking	22 (6%)	1(3%)	1.000 †

† Statistical test is Fisher Exact test *Statistical test is Chi-square test

In this study a significant associations were found between types of conception and ovulation induction (P <0.001) and contraception (P =0.002). No significant association was found between types of conception and PID (P = 0.630), surgery(P =0.439), previous EP (P=0.072), vaginal douching(P =1.00), previous disease(P =0.934) , and smoking (P =1.00) .

Discussion

In this study, there were significant increases in ectopic pregnancy following IVF and IUI as we found that the percentage of EP following IUI and IVF is (7.84%) from total EP, this is in agreement with other studies which were conducted by Farquhar (2). Similar finding were found by Laurie Barclay (3) who estimated a rate of EP after IVF between 2 and 11%. Clayton also found that the rate of EPs per 100 IVF births was 5% (4).

Nowadays, the increase in rate of EP following IVF and IUI is quiet happened. Increasing number of couples attending IVF center in addition to increasing acceptance of IVF\PGD as a method of sex selection are some of the attributable factors.

Different ART have different risk on the incidence of EP, as the rate of ectopic pregnancy in women undergoing IVF with transcervical transfer of fresh embryos is 2.2 %, and the rate is significantly increased (up to 3.6%) with zygote intrafallopian transfer (4),while with assisted hatching it reach 5.5 % as it was found by Jun et al.(5) . These high rates were significantly decrease with donor oocytes as the risk is lower (1.6%) and when the embryos are transferred to a surrogate mother, the risk is 0.9%(6). One of the important determents factors for the outcomes in IVF is the time of embryo transfer. Lensy(7)found that difficult ET on day 2 rather than day 3 might increases the rate of EP, while Milki found that embryos transferred on day 3 and those on day 5 had similar rates of ectopic pregnancy (8). In addition to technique, the volume of embryo transfer media, the number and quality of embryos transfer could be determent factors that affect the rate of ectopic pregnancy as found by Weigert et al. (9). The medication used for ovulation induction might increase the risk as found by Shaista (10) and Cohen(11) Both researchers found that clomiphen citrate had two fold increase in the incidence of EP. With the use of gonadotropins the rate of ectopic pregnancies was 2.7% (12) and (13).

A question may rise here: Can this be prevented? The answer is no, since the exact mechanism and risk factors are not identified yet. The mechanism of implantation is just to be elucidated. What can be done is the early detection by serial β- hCG and early vaginal ultrasound scan then ectopic pregnancy can be treated medically before need for surgical intervention (14).

In the present study, many risk factors for EP were evaluated. Woman age was a significant risk factor for EP (P=0.004), similar result was found by Buoyer (15). The association of ectopic pregnancy with increasing women age is likely due in part to an increased prevalence of fallopian tube scarring (16).

In this study significant association were found between types of conception and curettage (P=0.016) because of increased prevalence of surgical evacuation of the retained product of conception due to the patient preference. There is also significant association between types of conception and duration of infertility (P=0.001) and ovulation induction (P=0.001). It was found that there is misuse of induction especially with clomid which may be used without medical consultation and in some cases and under social pressure, clomid is taken even when male factor is the cause of infertility (17). Other significant association was found between types of conception and contraception (P=0.002) .The injectable progesterone which affect the tubal motility , is more preferable by a considerable number of women due to their poor compliance with combined oral contraceptive pills. The same finding was found by Bouyer (15) who found also an association between EP and PID, previous ectopic, and surgery.

In this study, there were difficulties in determining the effect of female genital Chlamydial infection on reproductive outcome which arise from lack of uniform Chlamydia screening, a lack of sexual transmitted disease clinic and tracking, moreover lack of agreement on the diagnostic methods for diagnosis of Chlamydia infection(18).

Waylen (19) had found that cigarette smoking significantly increases the risk of tubal ectopic pregnancy as the nicotine in cigarettes stimulates contraction in the fallopian tubes and this can cause spasm resulting in temporary blockage of the tubes so the embryo cannot pass through, in addition to its damaging effect on the cilia of the fallopian tubes. The current study did not found association with smoking (P=1.0).This may be explained by the fact that smoking in females in our community is not that prevalent as in the western communities, in addition some pregnant denied smoking due to social considerations.

In conclusion the increase impact of ectopic pregnancy in term of women risk and financial issues call for increase research about the causes of high prevalence of EP in order to act upon to reduce the risk and squally of ectopic pregnancy.

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Relationship between potency of hypo osmotic swelling test and antisperm antibody assay for fertile and infertile men in Thi-Qar governorate

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Abstract:

Background:
The sperm plasma membrane activity and absence of antisperm antibodies in infertile patients ejaculate significantly associated with fertilization potential of human spermatozoa. The sperm viability detected by hypoosmotic swelling test (HOST) and autoimmune infertility detected by antisperm antibody assay presented diagnostic and prognostic tools for male infertility factors.

Objective:
This study was devised to compare the results of hypoosmotic swelling test and antisperm antibody assay for normozoospermic men and infertile patients affected by autoimmune infertility.

Methods:
Thirty semen samples were collected by masturbation and prepared by direct layering technique. Hypo-osmotic swelling test was performed by mixing 0.1 ml of semen with 0.9 ml of a 150 mOsm/ L NaCl as a hypo-osmotic solution and direct immunobead assay were used to determine the presence of Antisperm Antibodies (ASA) bound on sperm surface. However, sperm concentration, sperm motility, and normal sperm morphology were evaluated according to World Health Organization (WHO) criteria and subjected to HOS test and ASA assay. The sperm prepared and incubated for 30 minute in 5% CO_2 at 37ºC after *in vitro* sperm preparation.

Results:
The results of the present study reported that the percentage of HOS test score in antisperm antibodies positive sperm samples (52.30 ± 5.2) was significantly lower than that noticed in antisperm antibodies negative (70.30 ± 3.4) sperm samples.

Conclusion:
It was concluded that the use of HOS test as a simple and diagnostic and prognostic tools for identify male infertility. Further studies are suggested to assess the effect of ASA on sperm plasma functional integrity after ICSI and *in vitro* fertilization and embryo transfers (IVF-ET).

Key words: Male infertility, hypo osmotic swelling test, antisperm antibody assay.

Introduction

The hypo-osmotic swelling (HOS) test originated as a laboratory index of the functional integrity of sperm plasma membrane (1). The HOS-test measures the ability of sperm plasma membrane to transport water when exposed to hypo-osmotic solutions, thus inducing cell swelling and plasma membrane stretching. If water transport does not occur, it can be assumed that the sperm membrane is functionally inactive and that it cannot be functional during the fertilization process (2). Since sperm plasma membranes may be physically intact but functionally inactive, the HOS test gives additional information on the sperm functional status (3).

In this regard the World Health Organization (WHO, 1999) has advised its use as an additional test in the routine semen analysis (4). However, the clinical usefulness of semen samples with low HOS test scores are associated with normal fertilization but low pregnancy rates during IVF techniques (5). Recently, Check's group reported that it is not clear if sperm from subjects affected by antisperm autoimmunity have some plasma membrane alteration leading to low HOS test scores or if the antisperm antibodies directly affect the sperm plasma membrane functionality (6). For that reason, the low HOS-test scores were found to be associated with lower pregnancy rates and fertilization potential of human spermatozoa (7). The men with low HOS test (<50%) rarely achieved a pregnancy with intercourse or conventional intra-uterine insemination (IUI) or even IVF-ET. (8) The defects give the impression to be related to a toxic factor attached to the sperm membrane. Some of these sperm may attach to the zona pellucida and transfer the toxic factor to the oocyte and eventually the embryo, and the defective embryo membrane may prevent proper implantation. Thus, it is not the single sperm fertilizes the egg that is problem; instead, it the supernumerary sperm attached to the zona pellucida (9).

The presence of antisperm antibodies (ASA) has been associated with decreased fertility ability *in vivo* as well as *in vitro* (10). Since approximately 15% of the male population have ASA, it would be beneficial to have a procedure capable of eluting of antibodies from the sperm and altering the sperm membrane configuration (11). One adverse effect of ASA on human spermatozoa may be inhibition of sperm progression through cervical mucus as demonstrated by a poor post-coital test by reduce percentage of sperm positive for both IgA and IgG (12). The possible mechanism was that the culture medium supplemented with human serum albumin (HSA) absorbs the antibody or antigen complex from the sperm membrane (13). The mechanism seems to be related not so much to impaired fertilization but to inhibition of implantation rates. The defect may be present in male with normal or subnormal semen specimens (14). Therefore, the compartment of males with antisperm antibodies (ASAs) has a higher frequency of low HOS-test scores. The possibility exists that ASAs may impair the functional integrity of sperm plasma membrane (15).

The Intra-uterine insemination (IUI) may be an effective therapy for sperm that have an impaired motility (16). Sometimes antibody on the sperm surface cause poor recovery of motile spermatozoa after sperm preparation technique, since agglutinated and poorly progressive spermatozoa are not recovered (17). Conversely, the reduced sperm motility may be related with an intrinsic sperm defect with the simultaneous presence of ASA (18). As a result, the presence of ASA may be etiologic; although the male ejaculate is normally devoid of complement, injury to the male ejaculatory system may have cause complement to leak into it outside (19). This study was designed to see if human sperm hypo-osmotic swelling test and plasma membrane functional integrity are adversely affected by antisperm antibodies attached to sperm surface.

2. Subjects, Materials and Methods

2.1. Subjects

Thirty infertile men 15 for normozoospermic men and 15 for infertile patients affected by autoimmune infertility were obtained from Al-Hussein Teaching Hospital/ Thi-Qar Health directorate/laboratory section. The selection of infertile patients was based on the physical examination and each infertile patient was to have semen samples including the HOS-test and immunobead assay for antisperm antibodies (ASA).

2.2. Semen preparation technique

The semen was prepared using a direct layering technique. However, 1ml of prepared IVF culture medium (Medi-Cult Company, Denmark) was added to the test tube, and then 1ml of the liquefied semen was layered beneath a culture medium. After incubation for 30 minute in 5% CO_2 at 37°C, 10µl of the mixture was aspirated by Pasture pipette and examined under light microscope at 40X magnification for assessment parameters of sperm parameters according to WHO criteria (1999).

2.3. Hypo-osmotic swelling test

The HOS test was performed after examination of standard semen parameters by mixing 0.1ml of semen with 1.9 ml of a 150 mOsm/ kg NaCl as a hypo-osmotic solution. The mixture was incubated for 30 minute at 37°C in 5% CO_2. Then, 10 µl of the mixture was placed on a slide and mounted with a cover and examined immediately at a magnification of 40X objective under a light microscope. A total of 100 spermatozoa were counted in at least ten different fields, and sperm tails were classified into seven distinct subtype of coiling in various regions. The percentage of HOS reacted spermatozoa (with coiled and swollen tail) and non-reacted spermatozoa (with straight or non swollen tails) were calculated according to WHO criteria (1999).

2.4. Antisperm antibodies assay (ASAs)

A direct immunobead assay (IBT) was performed for each semen samples. The percentage of sperm with ASA was noted. Therefore, the washed sperm were mixed with IgG or IgA beads and read microscopically for the percentage and attachment sites of sperm binding to the head. At least three beads had to be attached to be considered positive. A level of ≥ 50% was considered positive and ≥ 20% to 49% weakly positive according to WHO criteria (1999).

2.5. Statistical analysis

Statistical analysis was performed with the SPSS version 12.00 by the Statistical Package for Social Sciences software to compare difference between pairs of groups. P-value < 0.05 was used as a level of statistically significance.

3. Results

Thirty infertile subjects affected by autoimmune infertility (15 patients) with mean age 31.35 ± 0.66 years and duration of infertility 4.65 ± 0.22 years and normozoospermic men (15 patients) with mean age 34.12 ± 0.33 and duration of infertility 3.51 ± 0.11 were involved in this study. The result of the present study demonstrated that the percentage of sperm HOS test score for infertile subjects affected by autoimmune infertility (52.30 ± 5.2) significantly lower than those without antisperm antibodies (70.30 ± 3.4) after *in vitro* sperm activation and sperm processing. However, it was noticed a highly significant (P<0.001) differences in sperm parameters were assessed post *in vitro* sperm activation for both groups as compared to pre-activation. In the meantime, significant (P<0.001) and markedly reduction in sperm concentration were observed for *in vitro* post-activation.

Table (1): Standard semen parameters and hypo-osmotic swelling test for infertile patients affected with antisperm antibodies (ASAs positive).

Semen parameters	Pre-activation	Post-activation
Sperm concentration ($\times 10^6$ sperm/ml)	51.6 ± 2.1	37.2 ± 1.3 *
Sperm motility (%)	37.2 ± 7.0	45.5 ± 1.1 *
Normal sperm morphology (%)	41.5 ± 2.1	48.7 ± 0.2*
HOS-test (%)	43.1 ± 1. 2	52.30 ± 5.2 *

Values are Mean ±S.E.M
Total No. of patients=15

*: means significantly (P<0.001) difference between pre-activation and post-activation

Table (2): Standard semen parameters and hypo-osmotic swelling test for normozoospermic men (ASAs negative).

Semen parameters	Pre-activation	Post-activation
Sperm concentration ($\times 10^6$ sperm/ml)	63.1 ± 4.2	42.4 ± 8.0 *
Sperm motility (%)	54.6 ± 7.4	61.2 ± 6.3*
Normal sperm morphology (%)	47.2 ± 6.1	58.4 ± 3.6 *
HOS-test (%)	61.5 ± 1.4	70.30 ± 3.4 *

Values are Mean ±S.E.M
Total No. of patients=15
*: means significantly (P<0.001) difference between pre-activation and post-activatio

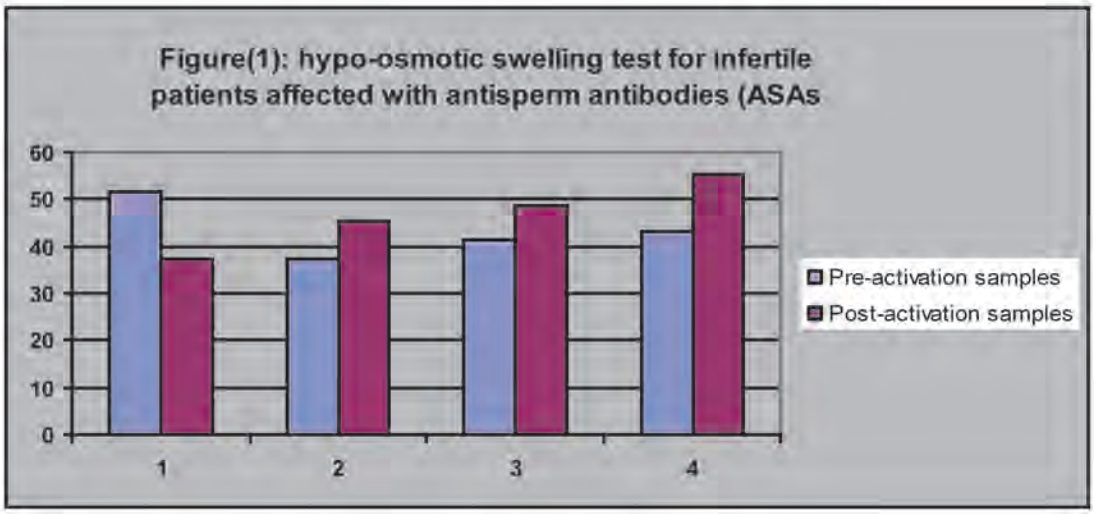


Figure (1): Standard semen parameters and hypo-osmotic swelling test for infertile patients affected with antisperm antibodies (ASAs positive).

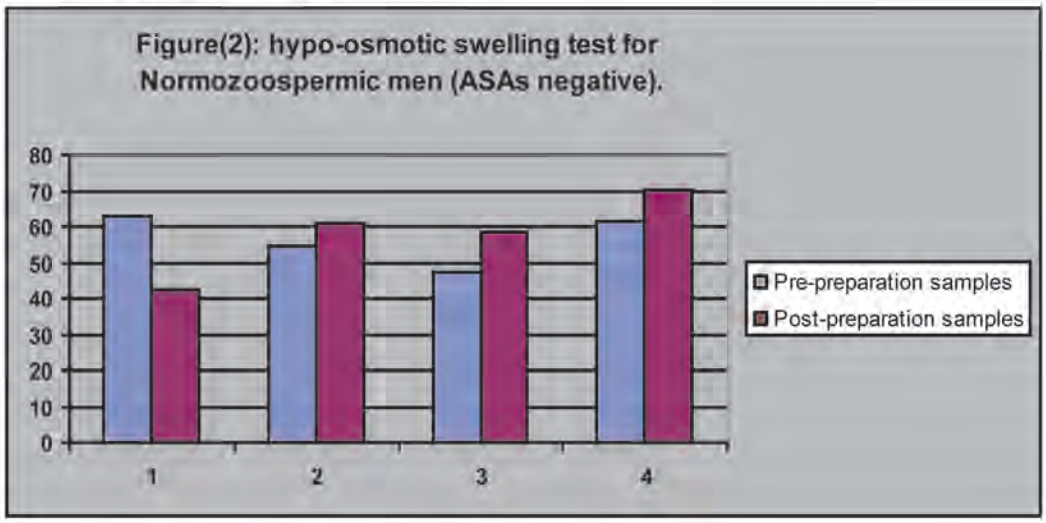


Figure (2): Standard semen parameters and hypo-osmotic swelling test for normozoospermic men (ASAs negative).

Discussion

The HOS-test is an important laboratory process during semen analysis for male infertility assessment and measures the functional integrity of sperm plasma membrane (20). The functional integrity of sperm plasma membrane is an important factor in sperm metabolism, capacitation, acrosome reaction, and the binding of spermatozoa to the egg surface. However, the sperm swelling induce when exposed to the hypo-osmotic solutions due to enter of water within sperm cytoplasm (21).

The sperm plasma membrane can be considered functionally active, thus suggesting the normal functionality of the plasma membrane of these swollen sperm. On this basis, it can be assumed that a dead sperm has a functionally inactive plasma membrane so that it does not swell when exposed to hypo- osmotic solution. In contrast, a live sperm has a physically intact plasma membrane but one that could be functionally inactive, thus not swelling when exposed to hypo-osmotic solutions (22). It is accepted that sperm samples from fertile subjects have normal HOS test scores and that those from infertile subjects with low HOS test scores show low pregnancy rates during assisted reproductive techniques (23).

The antisperm antibodies may fairly modify sperm plasma membrane integrity leading to low HOS test score (24). The present data demonstrated that sperm with ASA bound to their plasma membranes showed low HOS test scores, and this non-specific alteration of the plasma membrane permeability or fluidity may participate in determination of infertility leading to low fertilizing potential of spermatozoa. The potential clarification for low HOS test score in ASA positive sperm samples is that ASA modify water permeability. In this regard it has been demonstrated that water transport across cell plasma membranes utilizes specific water channels named aquaporins (25). Therefore, it is possible that ASA bound to sperm surface may block these water channels, thus altering

sperm water permeability. The antibody cross-linking could prevent plasma membrane distensibility, thus reducing sperm swelling when exposed to hypo-osmotic medium (26).

The role of sperm plasma membrane permeability to water in regulating important sperm functions (27). Though, when human sperm exposure to hypo- osmotic medium activates an influx of water within sperm cytoplasm. This water influx induces a sperm volume increase and plasma membrane stretching, leading to the opening of osmosensitive calcium channels, calcium influx within sperm cytoplasm and activation of acrosome reaction (28). Conversely, the osmosensitivity of sperm acrosome reaction in man and role of external osmolarity in regulation of mammalian sperm functions are well known (29).

The effects of ASA for autoimmune infertility in reducing plasma membrane water permeability and reduced HOS test score could induce also a reduction of the sperm responsiveness to the putative hypo-osmotic stimuli fundamental for sperm activation during the fertilization process, as suggested by the low osmolarity of female genital tract secretions with respect to that of semen (30). Indeed this hypothesis was demonstrated to be true since sperm with ASA bound to their surface show a marked reduction of [Ca+2] rise and acrosome reaction percentage increase induced by sperm exposure to hypo-osmotic medium, as evidenced in sperm from normozoospermic subjects without ASA (31).

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In vitro effects of terpinoid compounds of *Euphorbia helioscopia* L. extracts on rats epididymal sperm activity rats

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Abstract:

Background:
Many plants have medical importance due to the presence an active compounds in plants extraction acting as a drugs for treatment of several diseases in human and animals. *Euphrobia helioscopia* extract was used to treat diabetes, removal the warts of the skin, stimulate the nervous system and also used against constipation.

Objective:
Study the effect of terpinoid extract of *E.helioscopia* on rat epididymal sperm activity and fertility efficacy of male rats.

Methods:
This study was conducted at Biol. Dept., College of Science/ University of Babylon and Medical College, Mahidol University- Thailand. The study was designed to purify the active compounds of the Terpinoid of *Euphorbia helioscopia* extract by Nuclear Magnetic Resonance (NMR), and studying their effects on sperm motility and grade activity of the epididymal sperms of the rats. Six main active compounds were isolated from the terpinoid extract of E. *helioscopia* due to the differences in color , weight and nature by using NMR. The main terpinoid compounds were named A,B,C,D,E and F.
Two concentration (0.5 mg/ml and 1 mg/ml) of each active compound of *E. helioscopia* extract were added to sperm suspension *in vitro* and incubated for 30 minutes , then sperm motility and grade activity were assessed and the results were compared to those values before adding the active compounds (control).

Results:
The results showed that 0.5 mg/ml of C and D active terpinoid compounds caused significant ($p<0.05$) decrease in sperm motility compared to before adding the extract, while the same concentration of B,C,D,E and F active terpinoids compound caused a significant ($p<0.05$) decrease in grade activity. The addition of 1 mg/ml of terpinoid active compounds (C,D and F) caused a significant ($p<0.05$) decrease in sperm motility, while the grade activity significantly ($p<0.05$) decreased by adding B,C,D,E and F active terpinoid compounds compared to control. The sub – compounds (C1,C2,D1,D2,D3 and D4) had a powerful decreasing effect on the sperm motility and grade activity compared to control and other active compounds.

Conclusion:
It was concluded that terpinoid compounds of *E. helioscopia* extract have adverse effects on rat epididymal sperm activity.

Key words; *Euphorbia helioscopia*, **sperm motility, rats**

Introduction

Medicinal plants were used in traditional medicine to treat various diseases in humans and the animals, although the large number of medicinal herbs remains in the world and require verification of their validity and to treat many conditions(1,2). *E. helioscopia* were belongs to the family *Euphorbia*ceae (Family Alsossbep Spurge family), which includes about 283 genera and 7300 species. The members of this family distributed in most parts of the world, particularly in the tropics and subtropics and also extend to temperate regions in both hemispheres. *Euphorbia* genus is one of the biggest races of this family in terms of more than 2000 species spread across the world and approximately 44 species in Iraq (3).Five types spread widely in Iraq. The genus *Euphorbia* contains several types, some of them have not contain active compounds free of active substances used or nonmetal plants and others have broad medical uses. Species deployed in Iraq are E. pilulifera L. ; E.pulcherrima W; E. heterophylla L. ;E. marginata; and E. *helioscopia* L. (4).

All parts of the plant are toxic, however, the impact of these parts on the animal varies between one part and another, the material secreted by the stem of the plant has toxic effect more than the other parts of the plant. Despite that the plant is considered poisonous it has medical uses, including the use of leaf of E. *helioscopia* in various parts of the world such as Asia, Africa and South America as a popular treatment against diabetes, also used the juice of milk was widely used in removal of warts of the skin, and the plant was used as a stimulator for the nervous system and against constipation (5).

This plant was known as greatly traditional medicine in poor populations in Asia, it was medicinal plants. The dry leaves were the main part of this plant used as a medicine in many disorders of physical chronic conditions such as arthritis and influenza bronchial and skin diseases (6). The studies on the role of this plant *E.helioscopia* on male fertility is almost non-existent , so that this study was performed to determine the effect of terpinoid extract of *E.helioscopia* on sperm activity and fertility efficacy of male rats.

Materials and Methods:

Extraction and Isolation:

Dried and powdered aerial parts of the plant material *Euphorbia helioscopia L.*(2 kg) were placed in Soxhlet apparatus with chloroform solvent and concentrated by rotary evaporator overnight pump. The crude extract (115.06 g) was mixture by using different solvent according polarity (MeOH : CH2CL2 : hexane) . The column chromatography was used with diameter 15 cm and length 16 cm the number of silica gel was 7734 used(2000 g) and used thin layer of chromatography .

The six fractions of active compound (A-F). Dissolve 0.1 from each fraction in chloroform and take 4 ml in special tube and testing by NMR. These fractions were further submitted to silica gel column chromatography to obtain (D1. D2 . D3 .D5 .C1.C2) all these compounds were different in weight and nature . (7) .

Semen Analysis:

Epididymal semen samples were obtained from Albino rats. Sperm motility was performed as followings:

A drop of well mixed undiluted semen was placed on the surface of a warm dry clean slide. A cover slip was placed on the drop of the semen. The slide is allowed to rest on the microscope until the streaming of the sperm has stopped. Then the drop was viewed at 400X (8). A minimum of around 200 sperm was counted; both motile and immotile sperms were counted in at least 5 separate fields (9).

The motility of spermatozoa in each sample was graded 0, 1, 2, 3, or 4 according to (10,11 and12).

0 = no movement
1 = movement but not forward progression
2 = movement with slow forward progression
3 = movement in an almost straight line with good speed
4 = movement in a straight line with high speed.

The percentage of sperm motility was calculated by:

Motility % = number of motile spermatozoa / total number of spermatozoa (motile and immotile) × 100 .

The above mentioned estimation was considered as control (Before treatment).

Test the effectiveness of separated compounds:

Two concentrations of terpinoid extract were prepared (0.5 mg/ml and1 mg/ml) and used to study their effects on sperm activity. Test tubes containing phosphate buffered saline (PBS). The tubes containing a mixture of sperms and the compound were incubated in an incubator at a 37C ° for half an hour after which the sperm motility and grade activity were assessed (9).

Six main active compounds were isolated from *E. helioscopia* extract due to the differences in color, weight and nature by using NMR. The main terpinoid compounds were named A,B,C,D,E and F. The weight of six active compound were: A=1.487gm; B=6.77gm; C=17.25;D=29.4gm;E=42.31gm and F=12.99gm. 0.1 gm of each sample was dissolved in chloroform.

Two concentrations (0.5 mg/ml and 1mg/ml) of each active compound of terpinoid extract were added to sperm suspension *in vitro* and incubated for 30 minutes , then sperm motility percent and grade activity were estimated and results were compared to those values before adding the active compounds (control).

Fifteen gm of C and D active compounds were dissolved in different solvent like acetone, hexane, methanol, and dichloromethane, and kept in 4 C° for two days after noticing the presence of crystals. Then these samples were dissolved in methanol and filtered with filter paper. Four sub – compound isolated from D compound (D1,D2,D3 andD4), and two sub –compounds from C (C1 and C2).

Two concentrations (0.5 mg/ml and 1 mg/ml) of each sub - active compound of C&D terpinoid extraction were added to sperm suspension *in vitro* and incubated for 30 minutes , then sperm motility and grade activity were estimated and

the results compared to those values before adding the active compounds (control).

Statistical analysis

Analysis of data was performed by using SPSS (Version 10) in the home computer. Results are expressed as mean ± S.E. Statistical differences were determined by Least Significance Differences (LSD) test for multiple comparisons between different groups after performing ANOVA (13).

Results:

The results showed that 0.5 mg/ml concentration of C and D active terpinoid compounds caused a significant ($p<0.05$) decrease in sperm motility percent compared to control (Figure-1-), while the same concentrations of B,C,D,E and F active terpinoid compounds caused a significant ($p<0.05$) decrease of grade activity (Figure-2-).

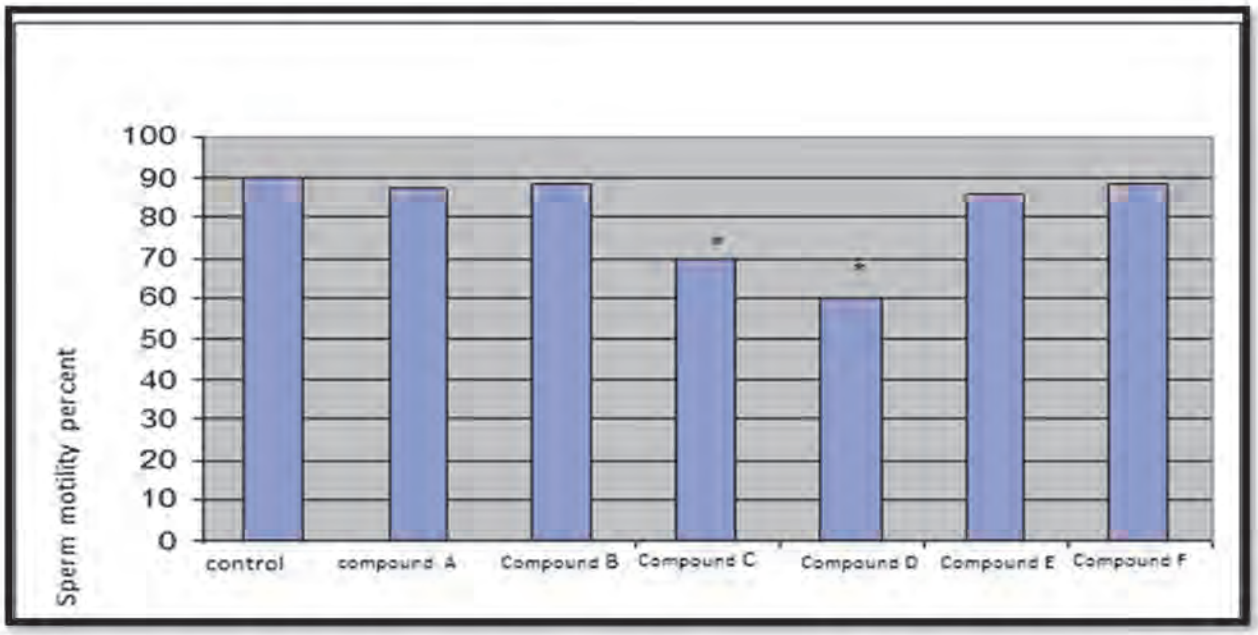


Figure-1- *in vitro* effect of active terpinoid compounds (0.5mg/ml) of *E. helioscopia* on sperm motility rat epididymal sperms after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens. L.S.D=25.56

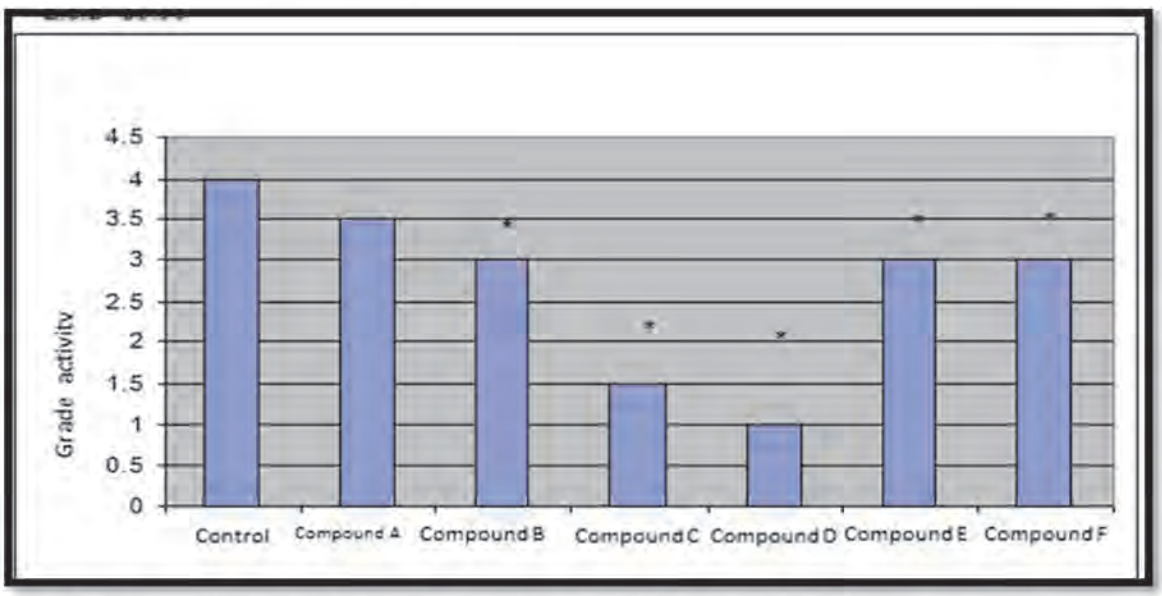


Figure-2- *in vitro* effect of active terpinoid compounds (0.5mg/ml) of *E. helioscopia* on grade activity Rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. = 0.95

The addition of 1 mg/ml of terpinoid active compounds (C,D and F) caused a significant ($p<0.05$) decrease in sperm motility percent (Figure-3-), while the grade activity was significantly ($p<0.05$) decreased by adding B,C,D,E and F active terpinoid compounds compared to control (Figure-4-)

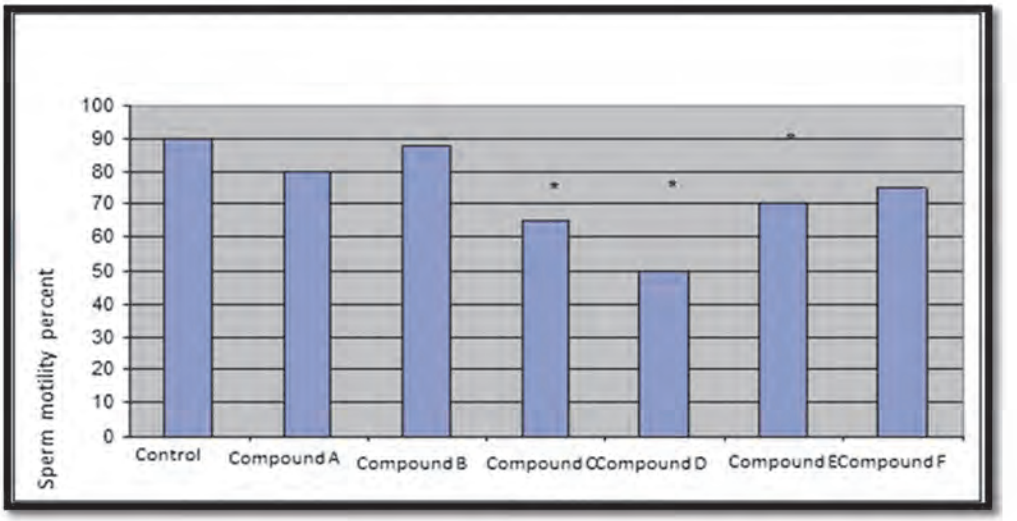


Figure-3- *In vitro* effect of active terpinoid compounds (1mg/ml) of *E. helioscopia* on sperm motility of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens,L.S.D. = 19.98

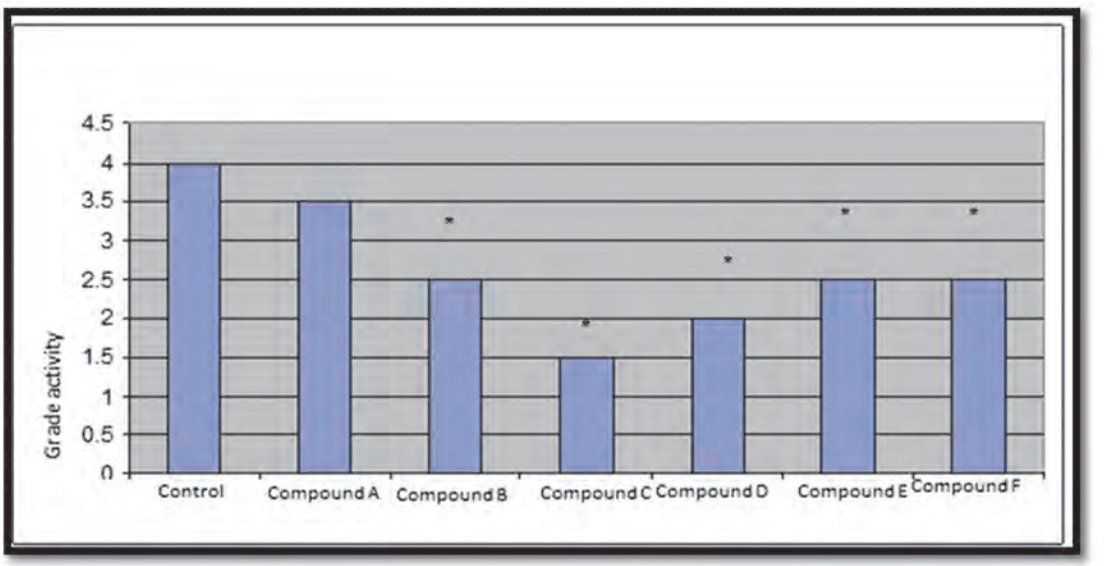


Figure-4- *In vitro* effect of active terpinoid compounds (1mg/ml) of *E. helioscopia* on grade activity of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. = 1.15

The incubation of rat epididymal sperm suspension with can D sub-compound each kind of above mentioned sub - compound for both concen trations (0.5mg/ml and 1mg/ml) caused significant ($p<0.05$) decrease in sperm motility percent and grade activity compared to control (Figures 5 to 8).

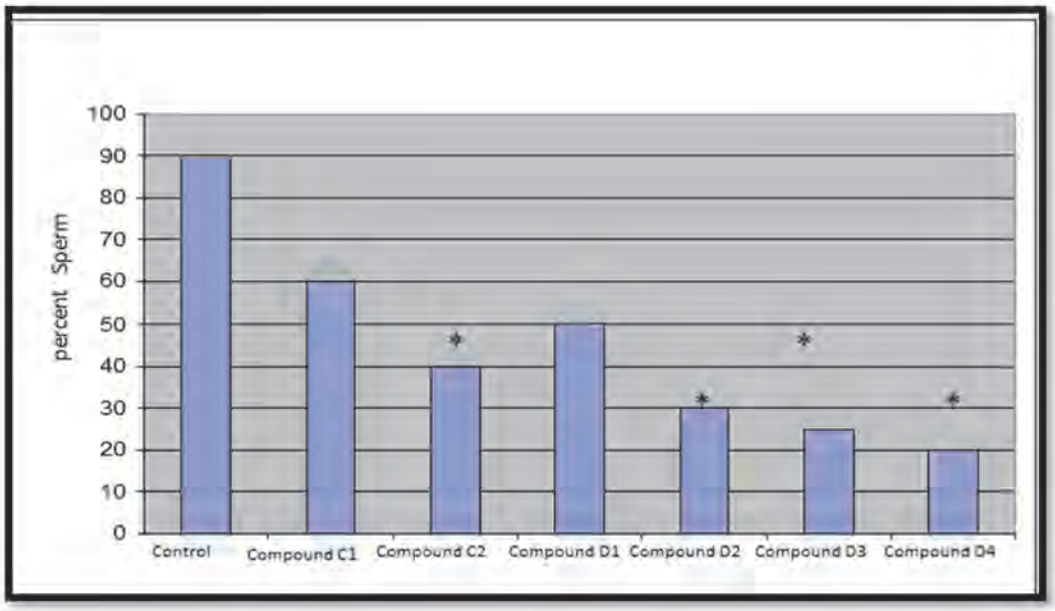


Figure-5- *In vitro* effect of active terpinoid (C&D)compounds (0.5mg/ml) of *E. helioscopia* on sperm motility of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. = 22.45

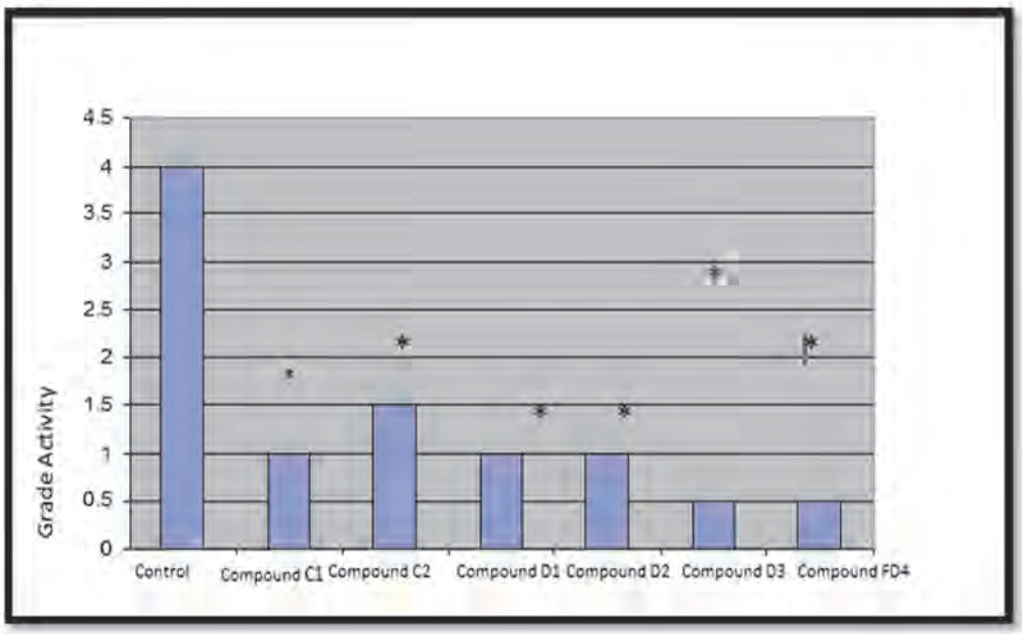


Figure-6- *In vitro* effect of active terpinoid (C&D)compounds (0.5mg/ml) of *E. helioscopia* on grade activity of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. 2.55

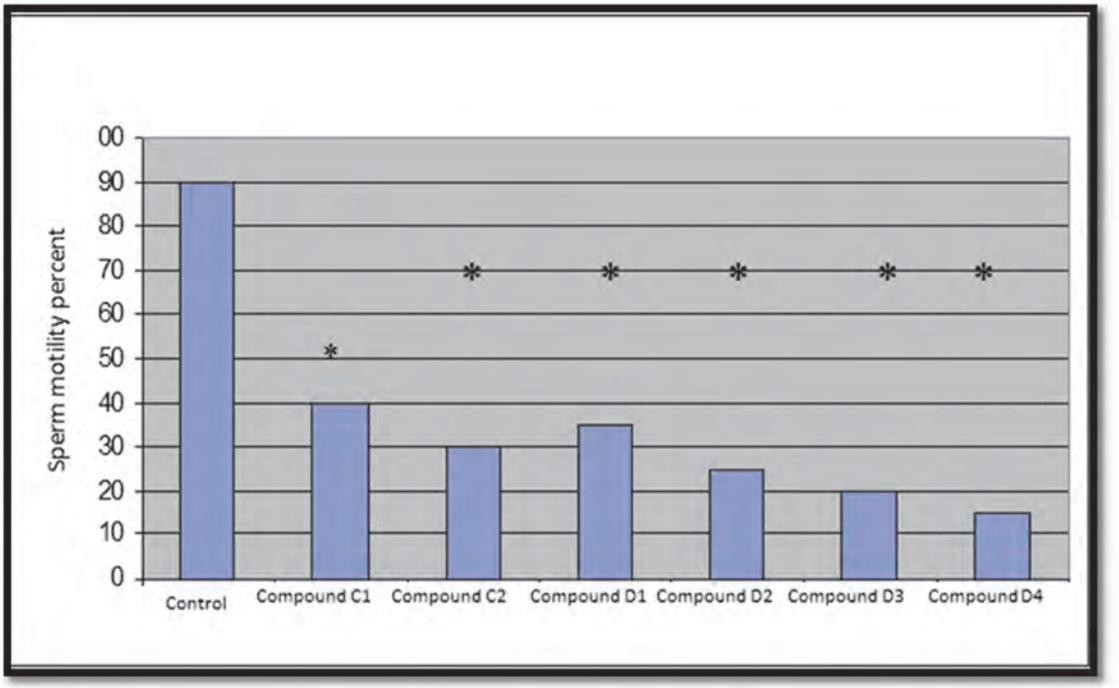


Figure-7- *In vitro* effect of active terpinoid(C&D) compounds (1mg/ml) of *E. helioscopia* on sperm motility of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. = 29.56

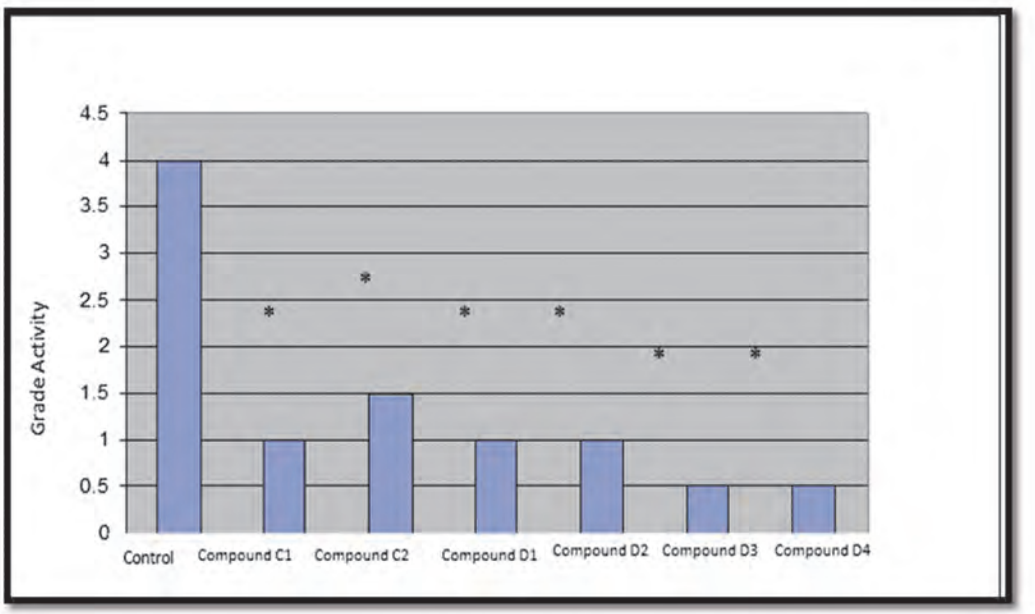


Figure-8- *In vitro* effect of active terpinoid (C&D) compounds (1mg/ml) of *E. helioscopia* on grade activity of rat epididymal sperm after 30 minutes of incubation
*($p<0.05$) significant decrease
N=4 Rat semen specimens
L.S.D. = 2.34

Discussion:

The sperm motility is the most important sperm parameter because The capacity of human sperm fertilization principally depends on sperm motility and membrane integrity. In this study sperm motility and grade activity has been affected significantly when incubated for 30 minutes with 0.5mg/ml and 1mg/ml of active terpinoid compounds of E. *helioscopia*. This results agree with other study (14), which revealed that concentration of 0.08 g/mL of the extract affected the motility, and at a concentration of 0.16 g/mL, the sperm motility was reduced to 20% immediately (within 20s) when the sperm incubated with extract of Achyranthes aspera and Stephania hernandifolia plants.

The significant decrease of sperm motility and grade activity as a result incubation with terpinoid compounds of *E. helioscopia* extract may be as a result to increase of oxygen reactive species (ROS). Reactive oxygen species, such as superoxide anion and hydrogen peroxide, are known to impair sperm motility and membrane integrity by inducing membrane lipid peroxidation (LPO),(15). The sperm membrane allows transport of ions and molecules selectively, and this is essential for normal sperm motility.

Most of the herbal spermicidal agents induce spermicidal effects by disrupting the plasma membrane as they act on sperm surface(16). Plant derivatives also cause drastic inhibition in sperm membrane-specific enzymes like acrosin and hyaluronidase, the most important enzymes in the process of fertilization(17).

The treatment of human spermatozoa *in vitro* with 100 mg/ml of Ruta graveolens extraction caused significant decline in sperm motility and viability(18), this result agree with our study.

One study(19) demonstrated that the oral administration of phenolics, alkoaloids and terpinoids compounds of *E. helioscopia* extract caused a significant decrease in sperm parameters, sex organs weight, diameter of seminiferous tubules, spermatogenesis cells, Leydig`s cells and a decline in several hormones like LH,FSH and Testosterone. Also the fertilization test revealed that the non-treated female mated with male orally treated cannot become pregnant until 6th week after last dose of *E. helioscopia* extract.

According to our results and other studies on E. *helioscopia*, it was concluded that E. *helioscopia* extraction caused a decrease in rat epididymal sperm activity, and it was recommended to study the effect of terpinoid compounds on human sperm activity to use them as a contraceptive.

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تأثير المركبات التربينية لمستخلص نبات *Euphorbia helioscopia* L. في نشاط نطف الجرذان في الزجاج

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الخلفية العلمية:
هناك العديد من النباتات ذات الأهمية الطبية طبقا لوجود المركبات الفعالة في مستخلصاتها والتي تعمل كأدوية لعلاج العديد من الامراض في الانسان والحيوان. مستخلصات نبات اللبينة تستعمل لعلاج السكري وازالة بثور الجلد ومحفز للجهاز العصبي كما تستعمل ضد الامساك.

الهدف:
تحديد تأثير المستخلص التربيني لنبات اللبينة في فعالية نطف البربخ والكفاءة للجرذان البيض.

طرائق العمل:
أجريت الدراسة في قسم علوم الحياة/ كلية العلوم/ جامعة بابل وكلية الطب/ جمعة ماهيدول/ تايلند. صممت الدراسة لتنقية المركبات الفعالة للمستخلص التربيني لنبات اللبينة باستعمال جهاز NMR , ودراسة تأثيرها في النسبة المئوية للنطف المتحركة ودرجة نشاط النطف للنطف المسحوبة من برابخ ذكور الجرذان. عزلت ستة مركبات فعالة من المستخلص التربيني للنبات طبقا للتغير في اللون والوزن والطبيعة باستخدام جهاز NMR , وسميت المركبات التربينية الرئيسية A,B,C,D,E,F . تم استعمال تركيزين وهما ٠,٥ ملغم/ مل و ١ ملغم/ مل لكل مركب من كل مركب فعال للمستخلص التربيني وأضيف الى معلق النطف في الزجاج وحضن لمدة ٣٠ دقيقة, بعد ذلك تم تقدير النسبة المئوية للنطف المتحركة ودرجة نشاطها وقورنت النتائج مع تلك قبل اضافة المستخلص (مجموعة السيطرة).

النتائج:
أظهرت النتائج ان التركيز ٠,٥ ملغم/ مل لكل من المركبات التربينية الفعالة نوع C و D سببت نقص معنوي ($p\leq0.05$) في النسبة المئوية للنطف المتحركة مقارنة مع مجموعة السيطرة, في حين لوحظ ان نفس التركيز من المركبات التربينية الفعالة F,E,D,C,B سببت نقصان معنوي ($p\leq0.050$) في درجة نشاط النطف. أما أضافة ١ملغم/ مل من المركبات التربينية الفعالة C,D,F سببت نقص معنوي ($p\leq0.05$) في النسبة المئوية للنطف المتحركة, بينما سببت المركبات التربينية الفعالة E,D,C,B وF للمستخلص نقص معنوي ($p\leq0.05$) في درجة نشاط النطف مقارنة بمجموعة السيطرة.
أشارت النتائج أن للمركبات الفعالة الثانوية C1 ,C2,D1,D2,D3,D4 التأثير الاقوى في تقليل النسبة المئوية للنطف المتحركة ودرجة نشاط النطف مقارنة بمجموعة السيطرة والمركبات الفعالة الاخرى.

الاستنتاجات
يستنتج من هذه الدراسة أن المركبات التربينية الفعالة لنبات اللبينة التأثير الضار على نطف البربخ للجرذان البيض.

الكلمات الدالة: نبات اللبينة *Euphorbia helioscopia* L.,حركة النطف,الجرذان

العلاقة بين فعالية فحص كفاءة النطفة البشرية تحت الضغط الازموزي الواطىء مع فحص الاضداد المناعية المضادة للنطف للرجال الاسوياء والعقيمين في محافظة ذي قار

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خلفية الدراسة:
أن فعالية الغشاء البلازمي للنطفة البشرية ووجود الأجسام المضادة للنطف في عينات السائل المنوي لمرضى العقم مرتبط بشكل مباشر بقابلية وقدرة النطفة على الإخصاب. أن قياس حيوية النطف من خلال فحص انتفاخ الغشاء البلازمي تحت الضغط الازموزي الواطئ والعقم المناعي للرجال المشخص من خلال فحص الأجسام المناعية المضادة للنطف يعتبر أداة تشخيصية ذات دلالة عالية يمكن من خلالها تحديد العوامل المسببة للعقم الذكري.

هدف الدراسة:
هدفت هذه الدراسة الى مقارنة نتائج فحص كفاءة انتفاخ الغشاء البلازمي للنطفة البشرية تحت الضغط الازموزي الواطىء مع فحص الاجسام المناعية المضادة للنطف للمرضى المصابين وغير المصابين بعقم المناعة الذاتية للرجال.

طرق العمل:
تم اختيار المرضى وعددهم ثلاثون مريضاً وفق فحوصات السائل المنوي وموزعون على مجموعتين بطريقة التقنية الطباقية المباشرة. أن فحص انتفاخ الغشاء البلازمي للنطفة تم تحضيره بمزج (٠,١ ملليتر) من السائل المنوي مع من (٠,٩ ملليتر) من المحلول الازموزي الواطى (محلول التناضح الواطى) كذلك تم اعتماد اختبار ارتباط الاجسام المناعية المباشر بسطح الحيمن لتحديد مدى تواجد هذه الاجسام من خلال تقنية الكريات الوامضة على راس النطفة. تم اعتماد اختبارات تركيز النطف, حركة النطف, الحركة التقدمية للنطف, والنسبة المئوية للنطف السوية حسب معايير منظمة الصحة العالمية مع اجراء اختبار انتفاخ التناضح الواطى و فحص الاجسام المناعية المضادة. تم تحضير السائل المنوي في (٣٧درجة مئوية و٥٪ثاني اوكسيد الكاربون) بعد اجراء عملية تحضير النطف.

النتائج:
أظهرت نتائج هذه الدراسة ان نسبة اختبار انتفاخ التناضح الواطى للنطفة اظهر انخفاظاً مع وجود فرقاً معنوباً عالياً للمرضى ذوي اضداد النطف مقارنة بالمرضى غير الحاوين على الاضداد.

الاستنتاجات :
نستنتج من خلال هذه الدراسة ان فحص كفاءة انتفاخ الغشاء البلازمي للنطفة البشرية هو فحص بسيط ومعتمد واداة تشخيصية وتعريفية مهمة في تحديد عوامل العقم الذكري.هنالك توصية باجراء دراسة لمعرفة مدى ثاثير الاجسام المناعية على الغشاء البلازمي للنطفة بعد اجراء عملية الاخصاب الخارجي وحقن الحيمن عن طريق سايتوبلازم خلية البيضة.

الكلمات الدالة: العقم الذكري, فحص انتفاخ النطفة, فحص الأجسام المضادة للنطف, العقم المناعي

دراسة عن عوامل الخطورة للحمل خارج الرحم الناتج عن الحمل الطبيعي والحمل المساعد

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الخلفية العلمية:
الحمل خارج الرحم هو احد مضاعفات الحمل وفيها ينبت الحمل خارج التجويف الرحمي وبشكل نظري فان الاختلافات بين الحمل بواسطة تقنيات الاخصاب المساعد والحمل الطبيعي يمكن ان تؤثر على احتمالية خطر الحمل خارج الرحم. المعلومات حول عوامل الخطورة لحدوث الحمل خارج الرحم بعد الاخصاب الخارجي لازالت غير مؤكدة وهناك اختلاف حول ما اذا كانت تقنيات الاخصاب المساعد تزيد او تقلل من الحمل خارج الرحم.

الاهداف:
دراسة حالات اوعوامل تكرار الحمل خارج الرحم بعد الحمل الاعتيادي والحمل المساعد .

المرضى وطرق العمل:
تم مسح الحالات المسجلة للحمل خارج الرحم للفتره من كانون الثاني ٢٠١٠ الى كانون الثاني ٢٠١١ في اربع مستشفيات تعليميه واثنان من المستشفيات الاهلية . كما شملت الدراسة الحالات الجديدة للفترة من تموز ٢٠١١ الى تموز ٢٠١٢ في اثنين من المستشفيات التعليمية. جمعت المعلومات من السجلات الطبية والمقابلات وقد سجلت ٨٦٦٧٦ حالة حمل خلال فترة الدراسة بضمنها ٤٠٨ حالة حمل خارج الرحم.

النتائج:
عدد حالات الحمل خارج الرحم هي ٤٠٨ من عموم حالات الحمل في بغداد خلال فترة الدراسة. حيث وجد ان هناك ارتباط كبير بين نوع الاخصاب وحالات العقم ($P<0.001$) و تحريض التبويض($P<0.001$) وجرف الرحم الالي ($P=0.016$) واستعمال موانع الحمل ($P=0.0012$).

الاستنتاجات:
على الرغم من معرفة الكثير من عوامل الخطوره للحمل خارج الرحم إلا ان سبب زيادة نسبةحدوث الحمل خارج الرحم غير معروفة. في هذه الدراسة وجد ان عوامل اختطار الحمل خارج الرحم بعد الحمل الاعتيادي والحمل المساعد هي حالات العقم والادوية المستعملة لتحريض التبويض, وجرف الرحم الالي واستعمال موانع الحمل حيث وجد انها ترتبط ارتباطا كبيرا مع نوع الاخصاب. ان زيادة المعرفة بعوامل الخطورة لحدوث الحمل خارج الرحم يمكن ان تزيد من ادراك مسببات العقم.

الكلمات الدالة: الحمل خارج الرحم, التمنية داخل الرحم, الاخصاب الخارجي, عوامل الخطورة

دور مادة الايكادهرين في عملية انبات الجنين ومكانه في بطانة الرحم

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الخلفية العلمية:
استقبالية بطانة الرحم هي سلسلة موحدة من العوامل التي تجعل بطانة الرحم م لانبات الجنين. يجب استبدال التقييم النسيجي , والذي يعد حاليا عاملا يضيف القليل من المعلومات المهمة سريريا, بالتقييم الوظيفي لبطانة الرحم. تم تعريف العديد من الوسيطات الوظيفية الجزيئية وبضمنها الجزيئات الرابطة.يمكن استخدام عينة بطانة الرحم لتحديد الجزيئات المرتبطة باستقبالية الرحم للحصول على رؤية افضل لانبات الاجنة في الانسان.

الاهداف:
تهدف الدراسة الى التحقق من وجود الايكادهرين في بطانة الرحم خلال الدورة الحيضية للنساء الخصبات

المرضى وطرق العمل:
تم اخذ خزعة بطانة الرحم المجزاة من الجدار الامامي،الخلفي،قعر الرحم وعنق الرحم لـ(٣٢) امراه خصبة تم تحليل الانسجة المصبوغة بالصبغة الكيميائية المناعية بواسطة تحليل الصور بالحاسوب لقياس مؤشر الايجابيه للايكادهرين في ايام مختلفة من الدورة الحيضية.

النتائج:
يتزايد مؤشر الايجابية للايكادهرين في الطبقة الطلائية الوظيفية في بطانة الرحم خلال الطور التكاثري بينما يتناقص خلال الطور الافرازي من الدورة الحيضية.ان مؤشر الايجابية في النسيج الطلائي الغددي للجدار الامامي يكون اقل من الجدار الخلفي، غور الرحم، وعنق الرحم في مجموعة السيطرة($P=0.037$) . كما ان مؤشر الايجابية للايكادهرين في بطانة قناة عنق الرحم هو اعلى من مؤشر الايجابية في الجدران الثلاث للتجويف الرحمي($P=0.016$).

الاستنتاجات:
يمكن ان يكون لمادة الايكادهرين دور في عملية انبات الجنين .

الكلمات الدالة: استقبالية بطانة الرحم; الجزيئات الرابطة; الايكادهرين.

تاثير الحفظ القري على طبيعة الحامض النووي للنطف البربخية للفئران بعد التحضير في الزجاج باستخدام البنتوكسفيلين وعرق السوس

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الخلفية العلمية:
بينت الدراسات التاثير الايجابي للمواد المحفزة لحركة النطف كالبتوكسفيلين ونبات عرق السوس عند استخدامها في التحضير في الزجاج مما يؤدي الى زيادة واضحة في الحركة التقدمية لها. مع ذلك لا توجد معلومات عن تاثير هذه المواد على المادة الوراثية للنطفة وخاصة بعد الحفظ بالقري

الهدف :
صممت التجربة الحالية لمعرفة اي تاثير سلبي للوسط الذي يحوي على خليط من البنتوكسيفلين والمستخلص المائي لعرق السوس على الحامض النووي قبل وبعد الحفظ بالقري للنطف .

المواد وطرائق العمل:
استخدم خمسون ذكر فار خصب وناضج بعمر ٨-١٢ اسبوع. تم الحصول على النطف البربخية من منطقة ذيل البربخ وتم تحضير ها في الزجاج باستخدام تقنية التنشيط المباشر باستخدام اربع انواع من الاوساط الزرعية : وسط البنتوكسفيلين ووسط عرق السوس ووسط الخليط بابنتوكسفيلين وعرق السوس ووسط خالي من اي اضافة(السيطرة). تم فحص معايير النطف الوظيفية الرئيسية وسلامة الحامض النووي باستخدام فحص Acridine orange وفحص comet assay قبل وبعد الحفظ بالقري

النتائج:
بينت الدراسة الحالية وجود تحسن معنوي في الحركة التقدمية مع انخفاض معنوي عالي في الحامض النووي غير الطبيعي عند فحص Acridine orange وفحص comet assay بعد التنشيط في الزجاج باستخدام الاوساط الزرعية الاربعة عند المقارنة بالنتائج قبل التنشيط. اظهرت نتائج النطف البربخية بعد التنشيط والحفظ بالقري انخفاض معنوي باستخدام الوسط الزرعي الحاوي على البنتوكسفيلين وعرق السوس بشكل منفرد او مختلط بالمقارنة مع الوسط الخالي من محفزات الحركة بعد الحفظ بالقري وجميع الاوساط قبل الحفظ بالقري.

الاستنتاجات:
طبقا لنتائج الدراسة الحالية فقد ظهر عدم وجود اي تاثير سمي للاوساط اتي استخدمت فيها محفزات الحركة بشكل منفرد او مختلط على الحامض النووي بعد التنشيط في الزجاج والحفظ بالقري عند اجراء فحص Acridine orange وفحص comet assay.. ومن الملاحظ بان افضل نتيجة تم الحصول عليها عند استخدام خليط البنتوكسفيلين مع مستخلص عرق السوس. هذه المعلومات يمكن الاستفادة منها في تنشيط النطف البربخية والخصوية التي يتم الحصول عليها من الرجال الذين يعانون من اللانطفية الانسدادية.

الكلمات الدالة: البنتوكسفيلين. عرق السوس, سلامة الحامض النووي,فحصوصات وراثية خلوية ,الحفظ بالقري

تقيـم الأنظمة المختلفة لحفظ الخصية بالقري بأستخدام -٨هيدروكسي -٢ دي أوكسي كوانوسين كمؤشر لضرر الدنا

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الخلفية العلمية:
العلاج الكيميائي والعلاج الأشعاعي يمكن أن يدمر أو يقلل بشدة من عملية تكوين النطف وبالتالي يهدد الخصوبة على المدى الطويل. ولا يوجد حتى الآن علاج طبي يضمن الحفاظ على الخصوبة بعد العلاج الكيماوي والعلاج الإشعاعي. في السنوات الأخيرة ونتيجة التطور الحاصل في التقنيات المساعدة على الانجاب وامكانية استخدام النطف الخصوية او النطف البربخية في الاخصاب الخارجي بواسطة استخدام طريقة الحقن المجهري للحيامن داخل الهيولي ، فأن الحفظ القري لنسيج الخصية هو خيار في الحفاظ على خصوبة الذكور الذين يفقدون الخلايا المنوية نتيجة العلاج الكيماوي والعلاج الإشعاعي، والذكور مع فقد النطف.

الهدف :
بناءا على ماتقدم، صُممت هذه الدراسة لتقيم الضرر التأكسدي للحامض النووي في نسيج الخصية بعد دورة الحفظ القرّي/ الأذابة عند استخدام انواع مختلفة من المواد الحافظة من القرّي وكذلك لمشاهدة التغيرات النسجية التي تحدث بعد هذه الدورة في نسيج الخصية لذكور الفئران البالغة كنموذج للأنسان.

طرق العمل:
أستخدم في هذه الدراسة خمسون فأراً من ذكور الفئران البالغة الخصبة التي تتراوح اعمارها مابين ٨ الى ١٢ اسبوع. في كل فأر، قيمت احدى الخصى نسيجيا وبالفحص الكيميائي المناعي النسيجي (بأستخدام -٨هيدروكسي -٢ دي أوكسي كوانوسين كمؤشر لضرر الحمض النووي التأكسدي) بدون الحفظ القري (مجموعة السيطرة). بينما الخصية الأخرى قيمت نسيجيا وبالفحص الكيميائي المناعي النسيجي (بأستخدام -٨هيدروكسي -٢ دي أوكسي كوانوسين كمؤشر لضرر الحمض النووي التأكسدي) بعد الحفظ قرّيا لمدة ستة اسابيع باستخدام أنواع مختلفة من المواد الحافضة من القرّي (مجموعة الحفظ القري).

النتائج:
وجد خلال الفحص المجهري للشرائح المأخوذة من العينات المحفوظة قرًّيا (الخصية) ان التغيرات تختلف باختلاف نوع المادة الحافظة من القرّي المستخدمة (الكليسيرول ، البروباندايول وثنائي ميثيل سلفوكساید)، اذ اظهر الفحص المجهري للعينات المحفوظة قرّيا بأستخدام مادة ثنائي مثيل سلفوكساید بأنه لا توجد اختلافات كبيرة مقابل مجموعة السيطرة، ولكن المقاطع النسيجية التي حصل عليها من الأنسجة المحفوظة قرّيا بأستخدام مادة الكليسيرول أظهرت تغيرات شكلية وهيكلية معتدلة مقارنة مع مجموعة السيطرة. بينما أظهرت العينات النسيجية المعرضة للبروباندايول كمادة حافظة من القري تغيرات شكلية وهيكلية شديدة مقارنة مع مجموعة السيطرة.
أما نتائج الفحص المناعي النسيجي لهذه الدراسة اظهرت زيادة معنوية ($P<0.05$) في الضرر التأكسدي للحمض النووي مابين العينات المحفوظة قرّيا (٤٦,٧٨٪ و٦٣,٤٥ ٪ و٣٢,٥٩ ٪ تمثل الكليسيرول، البروباندايول وثنائي مثيل سلفوكساید على التوالي) مقارنة مع العينات من مجموعة السيطرة (١٩,٢٧٪) بعد ستة اسابيع من الحفظ القرّي.
الأستنتاجات: نستنتج في ضوء ماتقدم من النتائج حصول تغيرات نسجية في نسيج الخصية المحفوظة قرًّيا تختلف بأختلاف المواد الحافظة من القرّي، ووجود ضرر تأكسدي في الحمض النووي بعد الحفظ القرّي تختلف نسبته باختلاف نوع المادة الحافظة المستخدمة في القرّي، كذلك نستنتج ان مادة ثنائي مثيل سلفوكساید هي المادة الأفضل لحفظ نسيج الخصية قرّيا.

الكلمات الدالة : الحفظ القري، ضرر الحمض النووي التأكسدي،فرط الاوكسجين النوعي، -٨هيدروكسي -٢ دي أوكسي كوانوسين

تأثيرالحفظ بالقري على بعض معاييرالنطف للمرضى العقيمين

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الخلفية العلمية :
كل الرجال لديهم الرغبة الطبيعية لان يكون لديهم أطفال بشكل سوي. ان الحفظ بالتجميد للنطف هو جانب هام من جوانب الحفاظ على خصوبة الإنسان. مع التقدم في التقنيات المساعدة على الإنجاب، الدواعي على الحفظ بالتجميد للنطف آخذ في التوسع.

الأهداف:
لتقييم تأثير الحفظ بالتجميد باستخدام عدة Sperm Freeze TM على بعض المعايير الوظيفية للنطف.

المواد وطرائق العمل:
تم اجراء تحليل السائل المنوي لخمس وتسعين عينة من المرضى الرجال وفقا للمعايير القياسية لمنظمة الصحة العالمية (١٩٩٩). وقد قسموا المرضى إلى أربع مجموعات تبعا لتركيز الحيوانات المنوية والحركة وقد تم الحفظ بالتجميد في النيتروجين السائل باستخدام عدة Sperm Freeze TM لتجميد العينات وأجريت عملية اذابة العينات بعد ثلاثة أشهر.

النتائج:
اظهرت النتائج بصورة عامة انخفاضا كبيرا وملحوظا في قيم المعايير الوظيفية للنطف بعد الاذابة في كل من معدلات تركيز وحركة النطف خاصة الحركة التقدمية وشكلياء النطف الطبيعية بالمقارنة مع قبل الحفظ بالتجميد. بينما كانت هنالك زيادة هامة وملحوظة في نسبة النطف غير المتحركة لكل المجاميع باستثناء المرضى المصابين بقلة النطف اذ ازدادت زيادة ملحوظة بعد التجميد.

الاستنتاجات:
بعد تجميد السائل المنوي انخفضت المعايير الوظيفية للنطف وبشكل ملحوظ

الكلمات الدالة:الحفظ بالقري، المعايير الوظيفية للنطف، منظمة الصحة الدولية.

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