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Research Article

MALONDIALDEHYDE MEASUREMENTS IN SEMEN AFTER *IN VITRO* SPERM ACTIVATION BY PENTOXIFYLLINE AND *GLYCYRRHIZA GLABRA* EXTRACT

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ABSTRACT

Infertility is the inability of a sexually active, non- contracepting couple to achieve pregnancy in one year. There has been a worldwide interest in the extraction of various medicines from several plants for the treatment of different diseases, since they are natural products, easy to get and also cheap. One of these plants is the *Glycyrrhiza glabra* (*G. glabra*). The plant constituent of different compounds enhances sperm parameters *in vitro* and increases the reproductive efficiency in human and mice. Other fertility stimulant is Pentoxifylline (PF) which is a phosphodiesterase inhibitor of the methylxanthine group. It inhibits the breakdown of cyclic adenosine monophosphate (cAMP) and it is known that intracellular cAMP concentration plays a central role in sperm. Lipid peroxidation is a well-established-mechanism of cellular injury and is used as an indicator of oxidative stress. Seminal plasma malondialdehyde, which is the stable lipid peroxidation product, is one of the methods to evaluate the effect of lipid peroxidation on sperm. The objective of this study was to evaluate the lipid peroxidation in sperm when the G.glabra extract and pentoxifylline are used for sperm activation in oligoasthenozoospermic patients by detecting patients seminal plasma malondialdehyde. Fifty semen samples from infertile males with oligoasthenozoospermia, and ten healthy control semen samples was taken. Preparation of *G. glabra* concentration for sperm activation *in vitro*, preparation of pentoxifylline for sperm activation *in vitro*. Microscope examination: Sperm concentration, sperm morphology, and MDA were assessed using the thiobarbituric acid method. Significant difference between semen parameters (concentration, morphology) between fertile and infertile men, in addition to that MDA level as indicator of oxidative stress was measured which showed significant difference between fertile and infertile men. Following *in vitro* activation with *G.glabra* + pentoxifylline showed no statistical significant (NS) differences ($P > 0.05$) in the malondialdehyde compared to the activation with PBS, and compared to the malondialdehyde before activation for patients and control. Seminal plasma malondialdehyde was higher in infertile men, and activation of sperms by G.glabra and pentoxifylline will not affect the values of MDA in semen before and after activation.

Keywords: Infertility, Malondialdehyde, pentoxifylline, *Glycyrrhiza glabra*.

INTRODUCTION

Infertility is the inability of a sexually active, non- contracepting couple to achieve pregnancy in one year[1]. Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress[2]. Physiologically, the high concentrations of polyunsaturated fatty acids (PUFA) in sperm are important for maintaining membrane fluidity and flexibility during fertilization process. The mechanism by which oxidative stress induced motility loss in mammalian spermatozoa involved the induction of peroxidative damage to the sperm plasma membrane[3]. Seminal plasma malondialdehyde, which is the stable lipid peroxidation product, is a simple method to evaluate the effect of lipid peroxidation on sperm[4].

Reactive oxygen species attacks PUFA in the cell membrane, leading to a cascade of chemical reactions called lipid peroxidation.

ROS have a tendency toward chain reactions; that is, a compound carrying an unpaired electron will react with another compound to generate an unpaired electron. The reactions proceed through three main steps- initiation, propagation, and termination[6].

During initiation, the free radicals react with fatty acid chains and release lipid free radicals. This lipid free radical may further react with molecular oxygen to form the lipid peroxyl radical. Peroxyl radicals can react with fatty acids to produce lipid free radicals, thus propagating the reaction[6]. One of the byproducts of lipid peroxidation is malondialdehyde. This byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa[7].

There has been a worldwide interest in the extraction of various medicines from several plants for the treatment of different diseases, since they are natural products, easy to get and also cheap[5].

One of these plants is the *Glycyrrhiza glabra* (*G. glabra*). The plant constituent of different compounds enhances sperm parameters *in vitro* and increases the reproductive efficiency in human and mice *in vivo*[18, 19].

Other fertility stimulant is Pentoxifylline (PF) which is a phosphodiesterase inhibitor of the methylxantine group. It inhibits the breakdown of cyclic adenosine monophosphate (cAMP) and it is known that intracellular cAMP concentration plays a central role in sperm motility[20].The improvements in motility for oligozoospermics were reported to be immediate and transient while for normozoospermics various motility parameters are sustained for periods ranging between one and four hours[21]. Al-Dujaily *et al.*, 2006[18] and Abid, 2005[22] successfully used the *Glycyrrhiza glabra* and the pentoxifylline as sperm motility stimulants *in vitro*, independently.

MATERIALS AND METHODS

1) Semen samples

Sixty Semen samples were collected from fertile volunteers with normozoospermia (n=10) served as normal volunteers control and from infertile male partners (n=50 patients) with oligoasthenozoospermia of couples consulting the High Institute of Infertility Diagnosis and ART at Al-Nahrain University. The semen samples were obtained in the early morning at the Clinic after three to five days of sexual abstinence.

1. Semen sample was obtained via masturbation after an abstinence period of 3-5 days, collected directly into a clean, dry and sterile disposable plastic Petri–dish in especially allocated room for this purpose in the Institute.
2. The sample was transported to the semen examination laboratory immediately and allowed to liquefy in an incubator at 37ºC for 30 minutes. After complete liquefaction .The semen was analyzed by a macroscopic and microscopic examination using the standardization of World Health Organization[8].

2) Macroscopic examination

1. Appearance

Specimen with homogeneous, opalescent, and grayish-white in color was considered normal. Any other appearance was considered abnormal. Specimen tinged with red suggests the presence of fresh blood, while a brownish specimen may indicate the presence of old blood**.** Greenish specimens may be caused by infection. A white-yellow color may result from urine contamination or prolonged abstinence.

2. Volume

Normal ejaculate volume is between 2-6mL[8].The volume was measured by using graduated centrifuge cylinder with a conical base. The semen sample was considered hypovolumic when the volume less than 1.5 mL.

3. pH

Normal semen pH ranged from 7.2-8.0[8]. It was measured by immersing graduated Litmus paper (ranged from 6 to 14) in the semen sample while it is in the Petri-dish.

4. Liquefaction time

The semen sample was evaluated within 1 hour of collection and after the coagulum, or clot, has liquefied. Normal liquefaction time was ranged between 30 minutes at 37ºC or within 1 hour at room temperature (25ºC).

5. Viscosity

The viscosity of semen specimens was estimated by using pasture pipette. A normal sample leaves the pipette as small discrete drop. A specimen with abnormal viscosity the drop will form a thread more than 2cm long. If drops were not formed or the semen cannot be easily drawn up into a pipette.

3) Microscope examination

1- Sperm concentration

A drop of 10μl spermatozoa suspension was placed on a microscopic slide and covered

with a cover slip (22x22)mm. Concentration of spermatozoa (Sperms/million) was calculated from the mean number of spermatozoa in four high power microscopic fields under magnification of (400x). This number was multiplied by a factor of one million[9].

2- Sperm morphology

The percentage of morphologically normal sperms was performed by using the same prepared slides for sperm motility. At least 100 spermatozoa were calculated by dividing the mean number of normal Spermatozoa in four high power microscopic fields under magnification of (400x) on the number of sperm concentration[10].

3- Preparation of *Glycyrrhiza glabra* concentration for sperm activation *in vitro*

The concentration of *Glycyrrhiza glabra* of 0.1% was prepared by adding 10 mg of *G. glabra* extract to10 mL PBS in plastic test tubes with the addition of broad spectrum antibiotic (Ampicillin 0.004 gm) to prevent bacterial growth. The solution was filtered using (0.22µM) millipore filter[23].

4- Preparation of pentoxifylline for sperm activation *in vitro*

Pentoxifylline powder (Sigma, Germany) 10 mg was dissolved in 10 mL of phosphate buffered saline (PBS). These concentrations prepared daily under sterile condition using millipore filter of 0.22µM[18].

5-Thiobarbituric Acid Reactive Substances (TBARS)

Seminal MDA levels were analyzed according to Rao [11]. MDA was assessed using the thiobarbituric acid method. Briefly, semen samples were centrifuged for 7 min at 2000 g, and then 100 µl of seminal plasma (supernatants) was added in 900 µl of distilled water into glass tube. To each tube, 500 µl of thiobarbituric acid reagent (0.67 g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g NaOH and 100 ml glacial acetic acid added) was added and then heated for 1 h in a boiling water bath (all samples run as duplicates). After cooling temperature, each tube was centrifuged for 10 min at 4,000g and the supernatant absorbance of these was read on a spectrophotometer at 534 nm.

6- *In vitro* sperm activation

1. Layering technique was used for *in vitro* activation of the liquefied semen as described by Fakhrildin [24]. Each semen sample was prepared by dividing into three aliquots. The first part without any addition to the semen sample. The second part (at least 0.5mL) of semen was layered beneath a plastic conical sterile tube containing PBS with activation medium only. The third part semen was underneath the activation medium consisting of prepared pentoxifylline and licorice extract in a ratio of (80: 20) respectively. The activation medium volume was added depending on the semen volume in a proportion of (1:1). The incubation of all layering samples was 30 minutes.
2. The upper layer was taken into other tube for the three groups, and centrifugation at 1600 rpm for 7 minutes.
3. After centrifugation, the supernatant (seminal plasma) was immediately separated, and examined before storage to rule out the presence of spermatozoa in the supernatant. The seminal plasma for the three groups was aliquoted into storage ampoules and stored at -80°C until used for Thiobarbituric Acid Reactive Substances (TBARS).

RESULTS

Table -1 showed that there was statistically significant difference between the concentration of infertile men and fertile men in sperm concentration, morphology, and MDA level of the infertile patients compared to the normal volunteers.

Table 1: Semen Parameters and MDA in infertile and normal volunteers

Parameters	Patients (Mean±SEM)	Normal (Mean ±SEM)	P-value
sperm concentration $* 10^6$	11.02 ± 0.65	64.5±4.36	0.001**
morphology	51.94± 2.08	93.5 ± 0.619	0.001**
Malondialdehyde (MDA) (µM)	1.67 ±0.05	0.68 ±0.03	0.001**

Following in vitro activation with G.glabra +pentoxifylline showed no statistical significant (NS) differences (P > 0.05) in the malondialdehyde compared to the activation with PBS, and compared to the malondialdehyde before activation for patients and control as shown in table 2 .

Table 2: Comparison the results of malondialdehyde (MDA) (before activation vs. activation with PBS), (before activation vs. activation with *G.glabra* + pentoxifylline), and (activation with PBS vs. activation with *G.glabra* + pentoxifylline)

Malondialdehyde (MDA) (μM)		Before	After		*P*-Value		
			PBS	*G.glabra* + Pentoxifylline	Before vs. PBS	Before vs. *G.glabra* + Pentoxifylline)	PBS vs. *G.glabra* + Pentoxifylline
Patients	Mean± S.E.	1.67±0.05	1.62±0.05	1.59±0.05	0.474 NS	0.270 NS	0.698 NS
Control	Mean± S.E.	0.68±0.03	0.65±0.03	0.62±0.03	0.498 NS	0.180 NS	0.498 NS

DISCUSSION

Our study showed that the MDA level is higher in infertile patients with abnormal semen parameters (sperm concentration, morphologically normal spermatozoa) in comparison to normal volunteers with normal semen parameters. A number of studies have shown that lipid peroxidation affects the sperm concentration, motility, morphology and related with poor sperm quality[12, 13, 14] which coincide with our study. Kobayashi *et al.*, 1991[15], demonstrated that MDA level in spermatozoa was significantly related to the number of immotile sperm.

Some studies showed the negative significant correlation was observed between lipid peroxidation with sperm concentration, and normal morphology between fertile and infertile men[15, 16, 12, 17, 13], which coincide with current study. Increased MDA level might represent the pathologic lipid peroxidation of spermatozoa membrane and the following inhibition of sperm motility[13] this might explain our finding that the infertile patients with low active motile sperm percent had increased MDA level. In addition the results of[13] showed that seminal MDA concentrations were negatively correlated with sperm concentration and motility, and might provide a simple and useful tool in predicting sperm parameters. i.e., lipid peroxidation may play a significant role in disrupting sperm functions and semen quality especially sperm motility and morphology and may account for some cases of male infertility.

Pentoxifylline was used in this study depending on several previous studies, Al-Naimi *et al.*, 2011[25] revealed that administration of Pentoxifylline has a significant effect on the female Albino Mice genital organs especially if given in small doses for 10 weeks which might reflect itself on reproductions and the number of new generations. Al-Dujaily *et al.*, 2007[26] concluded that Pentoxifylline improves pregnancy rate when used to activate sperm function in both intrauterine and intracervical inseminations. Al-Dujaily and Alani, 2009[27] found that the use of Pentoxifylline solution to treat the uterine and cervical environment before insemination resulted in an improvement of pregnancy rates.

Using the *G.glabra* in this study was based on several studies revealed that *G.glabra* addition to the sperm activation media cause a significant increase in sperm concentration, sperm motility and grade activity of progressive forward movement of mice epididymal sperms[28]. Also Adding 20 % *G. glabra* to sEBSS medium to activate spermatozoa *in vitro* following IPI causes highly significant increase in sperm parameter sperm concentration, sperm motility, and grade activity of progressive forward movement of mice epididymal sperms[29]. Other study by Al-Dujaily *et al.*, 2006[18] revealed that the addition of *G. glabra* to the semen of asthenospermic patients may improve sperm functions. Al-Dujaily and Al-Shammary, 2008[30] concluded that using modified Tris solution with 20% *G. glabra* extract and 30% egg yolk is suitable for crystorage of human sperm and *in vitro* activation several days after ejaculation.

Tash and Means, 1982[31] revealed that the medium used with a combination of PF and *G.glabra* may attribute to the inhibition of phosphodiesterase activity and thereby increasing the cAMP which is important in sperm motility.*G.glabra* has an estrogenic activity by the presence of glibridin which is known to be phytoestrogenic and has the ability to bind to human estrogen receptors[32]. Estrogen improves sperm motility by increasing cAMP, which has been shown to be a very important factor in sperm motility percent[33]. Since MDA level was not altered after the activation by P F and *G.glabra* ,on the reverse there was a reduction in MDA level although it was not statistically different this might be explained by the small sample size, overall picture showed improvement in reduction of lipid peroxidation after using these two sperm motility stimulants.

It can be concluded that activation by PF and *G.glabra* has no harmful effects on sperms

and not accompanied by increasing lipid peroxidation.

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