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Effects of Propolis Extract Medium on *In Vitro* Sperms Activation of Infertile Asthenozoospermic Men

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Abstract

Back ground: the disability to have a child is a big problem for millions of couples throughout the world. Medical herbal plants have been used in many countries to overcome such problems and many of these plants treated male infertility *in vivo* and *in vitro*. Therefore; this study has investigated the bioactive effect of propolis extract medium on certain sperm function parameters *in vitro* of infertile men.

Aim of study: This study has evaluated the beneficial pharmacological effects of propolis extract on certain sperm function characters of asthenozoospermic men.

Materials and methods: Twenty-five of semen samples from patients complaining from asthenozoospermia were analyzed and diagnosed. The layering procedure was used for *in vitro* sperm preparation and activation. Two concentrations of propolis extract (0.5 mg and 1mg / 1 ml of phosphate buffer solution) medium were used and compared with Hams-F 12 medium (control) and phosphate buffer solution (PBS). Certain sperm function parameters were recorded.

Results: The results have shown that there was a significant ($P<0.05$) effect on active sperm motility grade A when using 1mg/ml propolis medium compared to 0. 5mg/ml, Hams-F12 (control) and PBS media. A significant ($P<0.05$) improvement in sperm motility and morphologically normal sperm (MNS) percentage was observed in a media containing propolis extracts compared to before activation and after activation by using PBS and HamsF-12 media.

Conclusion: This study has found that the using of propolis extract has a significant implication on *in vitro* enhancement of sperm motility and normal morphology percentage.

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Introduction

Infertility is one of the most complicated issue of human society. World health organization (WHO) record that 10-15% of couples demonstrated some forms of infertility problem which 40% of these problems are because of male factors (1). Many causes which are effects and it responsible for the male infertility problems are decreased sperm production, decreased semen quality parameters and genetic disorders (2).

Fertility might be improved by various procedures such as chemicals, surgery, medicinal drugs and experimental experiments. Many experiments have found that botanical medicine has a major impact on testosterone levels and sperms parameters (3,4).

In combination with assisted reproductive technology (ART), some medicinal plants have been used to improve the fertility success rate and reduce the cost of infertility (5).

The widely using of medical herbal plants throughout the world is due to their important pharmacologic properties including anti-inflammatory, anti-bacterial and hypotensive agent. Herbal plants have been common among people since ancient times and a multilateral solution has emerged in recent years to use herbal drugs along with medical treatment, they get from their health care provider (6).

Interestingly, in Iraq many herbal plants are used for *in vitro* preparation of sperm using Glycyrrhiza glabra (7) Doum palm (8). One of these herbs is propolis which is a resinous mixture produced by honey bees from a special part of plants, buds

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and exudates. The waxy nature and mechanical properties promote the bees to using it seal and protect the hive against intruders and natural phenomena (9).

Propolis is a well-known medical plant since ancient times and it has been widely used by Egyptians to embalm their cadavers. The Greek and roman also used it in wound treatment and as mouth disinfectant (10). Nowadays, it was used for treatment of different diseases such as; common cold, flue-like infection, wound healing and genitals. Also, many studies found that propolis extract have a great role in protection the male reproductive system (11 ,12). Thus, the goal of this study was to evaluate the effect of using propolis extract as a medium to improve the characteristics of certain sperm function of asthenozoospermic men.

Materials and methods

-Preparation dried extract of propolis

The propolis has been obtained from the local market .25 gram of propolis powder was mixed with 250 ml water at 40 C^{O} in shaker for seven days in dark room. Once the powder was completely dissolved, the suspension was filtered by using a clean filter paper and the residue was extracted again. The filtration procedure applies every day for seven days. The yield was further dried in rotary evaporator at 30C^{o} -40C^{O} (13).

-Preparation of aqueous propolis extract for *in vitro* sperm activation

The aqueous working solution of propolis extract was prepared by dissolving different concentration of dried propolis extract, two different concentration of propolis extract were prepared by dissolving 10 mg or 5 mg in 10 ml of PBS (Sigma Aldrich) in plastic tube mixed with broad spectrum antibiotic for the purpose of *in vitro* sperm activation. The solution was filtered using Millipore (Millipore, USA) o.45 Mm.

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-Experimental design

Semen samples were obtained from 25 patients with asthenozoospermia registered in the biotechnology research center and private laboratory after ethical clearance and informed consent of all donors. Each semen sample was divided in to four aliquots groups. The semen of the first group (i.e. Control group) was treated with Hams F12 medium only. Semen portions of the second group were treated with PBS. Third group were treated with medium of 0.5mg /ml concentration of propolis extract, while the semen samples of the fourth group were treated with of 1mg/ml concentration of propolis extract.

Samples were maintained in an incubator for 1 hour at 37 ºC. Certain sperm parameters were evaluated namely: sperm concentration (million per ml), active sperm motility (percentage) and morphological normal sperm (percentage) in each group before and after *in vitro* incubation using layer technique as reported by AL-Dujaily *et al*., (14). All samples were clearly examined under the inverter microscope with 40X magnification power (Olympus, Japan) and the results were recorded.

Statistical analysis

Data analysis was done by utilizing SPSS for Windows, version 22 (SPSS Inc. Chicago, Illinois, United States). Shapiro–Wilk normality test was used to determine whether the studied parameters followed a gaussian distribution.

Data were expressed as mean ± standard deviation (SD). The Tukey's Post Hoc tests for multiple comparison was applied after ANOVA tests. A *p* value less than 0.05 was considered statistically significant (15).

Results:

The results of current study showed that the mean of sperm concentration following the activation using PBS and Extract media were significantly ($P<0.05$)

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decreased compared to before activation. The sperm concentration by using extract medium with (10mg) was tend to be higher than using Hams F12 (control) medium, PBS medium and extract medium with (5mg). However, the statistical analysis showed no significant(P>0.05) differences between them. A significant P<0.05) improvement was observed in active sperm motility grade A after *in vitro* activation by preparation and activation the semen samples with Hams F12, PBS and both extract concentration (5and 10mg) media compared to before activation result. At the same time, the sperm motility grade A using a medium of 10mg/extract (17.45±0.64) was significantly(P<0.05) higher than using Hams F -12 (8.68±0.64) PBS (6.65±0.76) and extract with (5mg) medium (7.17±0.71).

Regarding the sperm motility grade B, a significant (P<0. 05) improvement was observed when using 10mg/extract medium (72.75±0.57) compared to before activation (31.70±0.51), HamsF -12(control) medium (66.50±2.0) and PBS medium (62.00±1.71) but with no significant difference with 5mg/extract medium (71.00±1.43).

There was a significant (p<0.05) perfection in morphologically normal sperm percentage following the activation by using any medium i.e. Hams F-12, PBS and plant extract with (5mg) and(10mg) compared to before activation. However, no significant(P>0.05) differences were noticed between the media used. The number of round cells after activation using the control medium, PBS medium and extract medium using layering technique for in vitro activation was significantly (p<0.05) reduced compared to pre-activation as shown in Table -1.

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Table -1: Certain Sperm function parameters before and after *in vitro* activation the semen of asthenozoospermic infertile men by propolis extract medium (n=25).

Certain sperm function parameters		Before activation	After activation				*P* value
			HamsF-12 Control	With PBS only	With 0.5 mg extract /1ml	With 1 mg extract/1ml	
Sperm conc. (m/mL)		59.90±6.32^{a}	41.90±4.03^{b}	31.80±3.52^{b}	34.45±2.98^{b}	38.65±2.35^{b}	0.00
Sperm motility percentage	**Grade A**	3.75±0.42^{a}	8.68±0.64^{b}	6.65±0.76^{b}	7.40±0.71^{b}	17.45±0.64^{c}	0.00
	Grade B	31.70±0.51^{a}	66.50±2.0^{bc}	60.00±1.71^{b}	71.00±1.43^{cd}	72.75±0.57^{d}	0.00
	Grade C	33.35±0.98^{a}	18.00±1.65^{b}	17.10±2.10^{b}	13.65±1.37^{bc}	8.20±0.93^{c}	0.00
	Grade D	31.20±0.49^{a}	6.82±0.78^{b}	16.25±1.73^{c}	7.95±0.58^{b}	1.60±0.18^{d}	0.00
MNS		38.25±0.97^{a}	57.50±1.23^{b}	57.75±0.99^{b}	65.60±1.03^{c}	71.75±2.92^{c}	0.00
RCs		5.52±0.40^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00

Values are express as mean ± SE

Different letters mean there is a significant different at $p<0.05$.

MNS=morphologically normal sperm. RCs=Round cells

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Discussion

The results of this study found that propolis medium was activated the sperm motility of asthenozoospermic men in both concentrations. This is due to the properties of this herbal plant to bind to human estrogenic receptors that resulted from the presence of flavonoids compounds. These compounds are widely known to be a Phyto-estrogenic (16). Estrogen is known to boost the sperm characteristics including grade activity of sperm motility and also initiation of hyperactive motility (17).

At the same time, several studies demonstrate that propolis contains many active substances and it was rich in minerals such as Fe, Na, Ca, I, Mg, K, Cu, Mn and Zn (18). One of these minerals is the Ca^{2+} which trigger several important physiological events in spermatozoa such as capacitation and hyperactivation. The incubation of spermatozoa with extra cellular source of Ca^{+2} stimulate hyperactivation in mammalian spermatozoa (19).

By activating sperm adenyl cyclase, calcium can increase cAMP output from ATP and increased levels of intracellular Ca and membrane hyperpolarization have been documented during spermatozoon capacitation (20). Recent research has shown that Ca modulates sperm capacitation in a biphasic way through the modulation of sperm cAMP-dependent signaling and tyrosine phosphorylation pathways (21). It has also been shown that the intracellular Ca distribution in the male gamete of animals is remodeled during post-mating capacitation(22). Moreover, recent proteomics studies have identified such proteins, such as ryanodine receptor, troponin, and sarcoplasmic Ca-binding protein, which may lead to Ca signaling processes in the male gamete of animals at various reproductive stages, such as capacitation (23,24).

Many factors can also affect normal sperms functions and one of these factors is the oxidative stress which can affect the fertility and sperms physiology. In addition to destroying cell structures, ROS can induce lipid peroxidation and affect cell death by breaking single and double DNA, nucleocid deletion and alteration, as well as destroying DNA molecules (25).

In several trials, the strong role of propolis in relieving the adverse effects of oxidative stress on the body defense system is well established .The active compounds of propolis especially flavonoids and phenolic protect the reproductive system from toxicity and show a great effect against aluminum chloride which caused deterioration in semen quality, testicular dysfunction and testosterone levels (26) .Also the flavonoids and other active compounds in propolis have the ability to scavenging free radicals and prevent lipids and other compounds to oxidized or damaged during oxidative stress (27). These scavenging substances which decrease the levels of free radicals increase sperm motility of high-grade activity of progressive forward movement with increase the percentage of morphologically normal sperms, Preventing DNA fragmentation, enhancing semen efficiency, reducing spermatozoa cryodamage, preventing premature sperm maturation and generally enhancing sperm vitality (28).

It is concluded from the data of current work that propolis extract medium can be used for *in vitro* preparation and activation of asthenozoospermic and teratozoospermic men to be utilized for assisted reproduction.

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