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The influence of endometriotic serous fluid and serum on the sperm functions and its fertilizing efficient in rats

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Abstract

The objectives of this study was to investigate the influence of addition of endometriotic serous fluid (ESF) and endometriotic rat serum (ES) into culture media for *in vitro* sperm incubation, for study their influence on sperm function parameters then, exploring its potential infertility. ESF and ES were collected from rats with experimentally induced mild endometriosis. Three kinds of sperm suspension were prepared as following: Kind 1: Caudal epididymal sperm were released in 1ml of modified Earle's balanced (MEB) as control. Kind 2: Sperms were released in 0.9ml of MEB, then mixed with 0.1ml of ESF. Kind 3: Sperms were released in 0.9ml of MEF mixed with ES. All three kinds of sperm suspension were incubated at 37°C for two periods 30 min. and 1hr. Sperm parameters were examined. Adult female rats were inseminated with each kinds of sperm suspension for test their fertilizability. Results revealed that there is a significant (P<0.001) decrement in sperm parameters of kinds 2 and 3 compared to kind 1 following 30min. and 1hr. of incubation. Also sperm parameters of kind 2 and 3 are significantly decline after 30min. compared to 1hr. of incubation. Also a significant reduction (P<0.001) in results of artificial insemination were obtained. In conclusion, ESF and ES are possible contributing factors impairing fertility through impairment of both sperm function parameters and its fertilizability.

Keywords: Endometriosis, Infertility, Artificial insemination.

Introduction

The presence of endometrial tissue outside the uterine cavity is called endometrosis. It is generally divided into pelvic and extrapelvic based on location (Lin *et al.*, 2006). The pathophysiology of endometriosis remains unclear but involves a complex interaction between ectopic endometrium and host peritoneal (Hull *et al.*, 2008). Symptoms of this disease are dysmenorrhea, chronic pelvic pain and significantly reduced fertility (Elsheikh *et al.*, 2003). Endometriosis is a common finding in woman with infertility (Bulletti *et al.*, 2010). So it is one of the leading cause of disability among reproductive age and represent a major personal and public health concern (Jamieson and Steege, 1996). The incidence of infertility is 30-40% in patients with endometriosis (Mulayim and Arici, 1999). It is well recognized that, women with minimal endometriosis with no anatomical or tubal distribution/obstruction suffer in infertility for reason cannot be immediately explained (Mahutte and Arici, 2002). Several factors may responsible for infertility in female with endometriosis. These factors contributing to infertility are poor oocyte quality (Matson And Yovich, 1986; Al-Shahery, 1998), defective implantation (Simon *et al.*, 1994; Al-Shahery, 2001) impaired fertilization (Wardle *et al.*, 1985) ovulatory dysfunction (Dmowski *et al.*, 1986) and altered peritoneal fluid cytokine milieu (Gomez-Torres *et al.*, 2002), and very detailed studies were undertaken by several investigator, about the role of peritoneal fluid on fertilization of intact or with endometriosis (Mulayim and Arici, 1999). While there have been no studies to date that, have investigated whether the endometriotic serous fluid and serum of endometriotic female can play an important role in infertility of endometriosis via its effect on sperm function, where presenting in the reproductive ducts of female with endometriosis. The aim of our study was to examine the sperm function parameters and then its potential fertility when incubation with media supplemented with these fluids i.e; ESF and SE for two periods as an attempt to described mechanism by which endometriosis can cause infertility.

Materials and Methods

Study Design: Sixty five adult mature rats (20 males, 75 females) of the Sprague-Dawley, Wheihing 200-

250 g each, were maintained under conditions of controlled lighting (14L: 10D) and temperature (21-22 °C). Food and water were available *ad libitum*.

Exp.1: Preparation of endometriotic serous fluid (ESF): Endometriosis was carried out on a previous study (Venrnon and Wilson, 1985). For induction endometriosis 15 mature female rats were anaesthetized with pentobarbital sodium (30mg/ Kg) intraperitoneally. After laparotomy, the left horn was identified and distal segment 2.5cm in length was resected and then incised longitudinally, from which a 4×4mm section was removed. Each section was sutured to abdominal wall on right flank of the rat in such way the endometrium was facing the abdominal cavity. The animals remained in vivarium for 18 week to allow sufficient endometriosis to develop. Eighteen week after transplantation, female rats were sacrificed. At sacrifice endometriotic implants were examined for viability growth and the amount of serous fluid which accumulated under the explants were aspirated, and stored at -20°C until used. Blood was withdrawn by cardiac puncture under light ether anesthesia. Samples were allowed to clot and retract at room temperature for 2hrs. and the sera (ES) were separated after centrifugation for 10 minutes at 2000rmp and stored at -20°C until required for use.

Exp.2: Preparation of sperm suspensions: Sperm were obtained from the cauda epididymides of 15 mature male rats by minced gently with microsurgical scissor in 3 kinds of culture media as follow:

Kind 1: Caudal epididymal sperm were released in one ml of modified Earle's balanced (MEB), which was served as a control.

Kind 2: Caudal epididymal sperm were released in 0.9ml of MEB, and then mixed with 0.1ml of endometriotic rat serous fluid (ESF).

Kind 3: Caudal epididymal sperm were released in 0.9 ml of MEB, then mixed with 0.1ml of heat inactivated endometriotic rat serum (ES).

Sperm suspension of each kind was placed on petri dish and incubated for 30 min and 1 hr. at 37°C in a humid atmosphere of 5% CO_2 in air. After preparation of sperm suspension, one drop (10 µL) of caudal epididymal sperm was examined under 40X objective lens to study the effect of culture media on sperm parameters.

Exp.3: Artifitial insemination: Sixty adult female rats were superovulated with intraperitoneal injections of 30IU pregnant mare serum gonadotrophin (PMSG) on day two of cycle followed 72hrs., later by 30IU human chorionic gonodotrophin (hCG) (Shaher and Al-Barazanchi, 1996). The females were then caged overnight with 5 vasectomized males.

For investigation the effects of different kinds of culture media on sperm fertilizability, these females were anesthetized and the uteri exposed through the flank incisions, and 0.1ml of each kind of sperm suspensions (containing 10×10^6 sperm) was injected into the upper third of each uterine horn, at 7hrs. after hCG administration. Accordingly, the inseminated female rats were divided into 6 comparable group as follow:

G 1= 10 female rats were inseminated with sperms suspension of kind 1 which incubated for 30min.

G 2= 10 female rats were inseminated with sperms suspension of kind 2 which incubated for 30min.

G 3= 10 female rats were inseminated with sperms suspension of kind 3 which incubated for 30min.

G 4= 10 female rats were inseminated with sperms suspension of kind 1 which incubated for 1hr.

G 5= 10 female rats were inseminated with sperms suspension of kind 2 which incubated for 1hr.

G 6= 10 female rats were inseminated with sperms suspension of kind 3 which incubated for 1hr.

All inseminated female rats were allowed to survive at midgestation stages and then scarified at 14 days postinsemination. The ovaries and uteri were removed and microscopically examined.

The number of corpora lutea (CL), normal implantation and resorbing implantation sites were recorded. The percentage of lost embryo were calculated as following:

$$\%\ \text{of lost embryo} = \frac{\text{No.of C L} - \text{Sum of implantation and resorbing sites}}{\text{Total number of CL}}$$

Corpora lutea reveal to occurrence of ovulation in superovulated rats.

Statistical analysis: The data were expressed as mean±S.E. It was compared by analysis variance (ANOVA) and t-test. The different between data with P value under 0.05 was considered statistical significant (Shekarriz *et al.*, 1995).

Results and Discussion

The results of addition of endometriotic serous fluid (kind 2) and serum of endometriotic female (kind 3) into culture media, on sperm function parameters after 30min. and 1hr. of incubation are shown in (Tables 1 and 2). The data of the present study have reported that, there was decreased quality of sperm function parameters after 30min. and 1hr. of prepared samples of kind 2 and kind 3 media: There were a significant (P<0.001) decrease in the mean of sperm concentration, viability, morphology, motility, progressive motility grade A+B, while high percentage of grade C+D motility which were statistically significant (P<0.001) compared to sperm preparation of culture medium (MEB) alone (kind 1: Control). While no significant (P>0.05) differences were noted in the results of

samples obtained from kind 2 when compared with results of kind 3 which incubated for 30 minutes and 1hr. The results in this study indicated that the prolonged length period of the sperm incubation time caused a significant ($P<0.001$) reduction in the sperm function parameters in kind 3 compared with kind 2 (Figures 2 and 3). While there was no similar difference between samples of kinds 1 which incubated for 30min. and 1hr. (Figure 1). The defective impact on the percentage of sperm concentration, normal sperm, sperm motility and sperm grade activity may have resulted by the presence of many toxic factors in the ESF and SE, such as reactive oxygen species (ROS), macrophages inhibitory factor (MIF) and cytokines which are secreted by endometriotic implants into the serous fluid or produced by macrophages to the peritoneal fluid and then transfer into the serum. It is well known that the oxidative stress (OS) resulting from ROS induces lipid peroxidation and structurally and functionally alters of biochemical like protein, DNA then cell death rapidly follows (Halliwell, 1994; Choudhary *et al.*, 2010). According to the previous studies OS is product of ROS production which has been found in women with endometriosis (Agarwal and Allamanen, 2004). It has been reported that both eutopic and ectopic endometrium of patient with endometriosis have an aberrant expression of antioxidant enzymes as result of excessive free radical generation in endometriosis (Alpay *et al.*, 2006). Several previous studies refer that, oxidative stress has been implicated in male factor infertility (Mahfous *et al.*, 2010). Furthermore, many studies have been indicated that, ROS play a causative role in the etiology of defective sperm function through the peroxidation of unsaturated fatty acids in the plasma membrane (Kwanang *et al.*, 1987). As well as excessive ROS generation can overwhelm protective mechanism and initiate changes in lipid and /or protein layers of sperm plasma membrane (Sanoska and Kurpis, 2004). It was reported that, increased ROS and its stable peroxidation product i.e. melondialdehyde levels have been associated with the reduction in sperm count, motility and morphology in astheno and oligo-astheno spermic men (Colager *et al.*, 2013). Increased production of ROS has been correlated with a reduction of sperm motility (Iwasaki and Gagnon, 1992; Agarwal *et al.*, 1994). Also, Sanchez *et al.* (2006) reported that, the addition of ROS in particular H_2O_2 at the concentration of 50, 100 M to the culture media caused a significant decrease in the number of sperm with severe morphological changes such as nuclear fragmentation, chromatin dispersion and cellular agglutination. The reduction in sperm motility may have been due ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum (Guthrie and Welch, 2012). So the reduction in the sperm viability at the present study may be due to the spermidical activity of both additive kinds of fluids to culture media. Most of spermidical compounds act on plasma membrane of sperm lead to its destruction (Wilborne *et al.*, 1983). It has also been postulated that another toxic factor i.e., MIF may have a role in the damage of sperm function at the present study. Earlier studies reported that MIF has deleterious effect on sperm function at pathophysiological levels, which suggest a role in endometriosis associated infertility (Carli, 2007). The other explanation of the defective impact in the sperm parameters in our study is the role of cytokines. It is well known that, great number of cytokines especially interleukins lead to pathogenesis of endometriosis. Additionally, increment levels interleukin-β (IL-Lβ) and IL-6 in the endometrial fluid and stroma of those with endometriosis has been previously reported (Mori, 1999; Tseng, 1996). Cytokins i.e.; IL-6, IL-2 and INF–γ are produced by ectopic endometrial cells, interwork locally and systemically and the INF-γ also found in the serum of endometriotic women (Wu and Ho, 2003). According to Vassilia dis *et al.* (2005) INF-γ inhibits sperm mobility. In another study Ahmed *et al.* (2012) found that, there was a negative correlation between IL-2 and sperm motility and morphology, whilst this correlation was between IL-6 and only sperm motility.

The present research has investigated the results of intrauterine insemination with spermatozoa of all suspension kinds following 30min. and 1hr. of incubation (Tables 3, 4 and 5). Our results, refer to reduction in the number of pregnant female rats, and a significant decrement ($P<0.001$) in the mean number of normal sized implantation sites of experimental horns (groups 2, 3, 5 and 6) in comparison with those of control horns (groups 1 and 4). While a high significant increment ($P<0.005$) in the percentage of resorbing implantation sites and lost embryos were observed in G_2 and G_3 compared to G_5 and G_6. We could attributed that to the negative effect of the prolonged period of sperm incubation time on its fertilizability.

These results confirm that, the retrograde outcome of pregnant female rats which inseminated by these two kinds of sperm suspension have been attributed to many causes: The two kinds of additive fluids into culture media had negative effect on sperm function parameters as proved by the results of Exp.1 and then reflect as inhibition sperm fertilizabilty. In another study reported that, various sperm functions are crucial for successful fertilization, such as the development

of hyperactivated motility (Sikka, 2001). Also, Aitken and Baker (2004) indicated that, OS can be harmful to sperm survival and fertility and defective sperm function is the most common cause of infertility. The another cause of infertility in the present study may be the failure of implantation potential of the fertilized ovum. Finally, the decreased in the percentage of pregnancy in the present study may due to the failure of growth and development fertilized ovum and its degradation. Our results are sufficient evidence to hypothesize that, ESF and ES, particularly some constituents may have adverse effect on sperm function and its fertilizability.

Conclusions

In conclusion, our results may be useful to realize what will happen for spermatozoa when present in the reproductive tract of female with endometriosis, and then, the reduction of sperm quality which a common problem in male infertility will consider one of the mechanisms for explanation of infertility in patients with endometriosis.

Table (1): Effect of different suspension kinds on sperm parameters post 30min. of incubation.

Sperm parameters	Suspension		
	kind 1	Kind 2	Kind 3
Sperm concentration ($\times 10^6$ sperm/ ml)	30.70*/** ± 3.30	17.80* ± 2.38	19.40** ± 3.00
Sperm viability (%)	94.00*/** ± 1.94	75.20* ± 1.30	70.00** ± 3.16
Sperm motility (%)	89.00*/** ± 3.19	76.20* ± 3.11	73.20** ± 3.59
Morphologically normal sperm (%)	86.30*/** ± 5.44	73.60* ± 4.92	69.80** ± 3.11
Sperm motility Grade A (%)	22.20*/** ± 1.75	12.40* ± 1.94	14.00** ± 2.34
Sperm motility Grade B (%)	60.40*/** ± 3.36	51.40* ± 3.05	50.60** ± 2.88
Sperm motility Grade C (%)	12.70*/** ± 0.38	18.80* ± 1.04	18.20** ± 2.58
Sperm motility Grade D (%)	7.80*/** ± 1.53	19.20* ± 3.19	18.00** ± 1.82

Values are mean ± S.E; *Significant difference (P<0.001) between kind 1 and kind 2; **Significant difference (P<0.001) between kind 1 and kind 3.

Table (2): Effect of different suspension kinds on sperm parameters post 1hr. of incubation.

Sperm parameters	Suspension		
	kind 1	Kind 2	Kind 3
Sperm concentration ($\times 10^6$ sperm/ ml)	27.60*/** ± 2.18	10.20* ± 2.16	11.80** ± 1.60
Sperm viability (%)	83.40*/** ± 2.50	50.40* ± 3.04	54.00** ± 3.53
Sperm motility (%)	81.40*/** ± 3.04	45.80* ± 3.89	50.20** ± 3.70
Morphologically normal sperm (%)	79.40*/** ± 1.64	40.40* ± 2.02	44.40** ± 1.81
Sperm motility Grade A (%)	20.00*/** ± 2.34	9.00* ± 2.16	11.00** ± 1.87
Sperm motility Grade B (%)	57.80*/** ± 2.16	30.60* ± 3.44	32.80** ± 3.78
Sperm motility Grade C (%)	12.20*/** ± 1.64	34.00* ± 3.67	32.40** ± 3.28
Sperm motility Grade D (%)	11.20*/** ± 2.16	27.80* ± 2.77	25.00** ± 1.87

Values are mean ± S.E; *Significant difference (P<0.001) between kind 1 and kind 2; **Significant difference (P<0.001) between kind 2 and kind 3

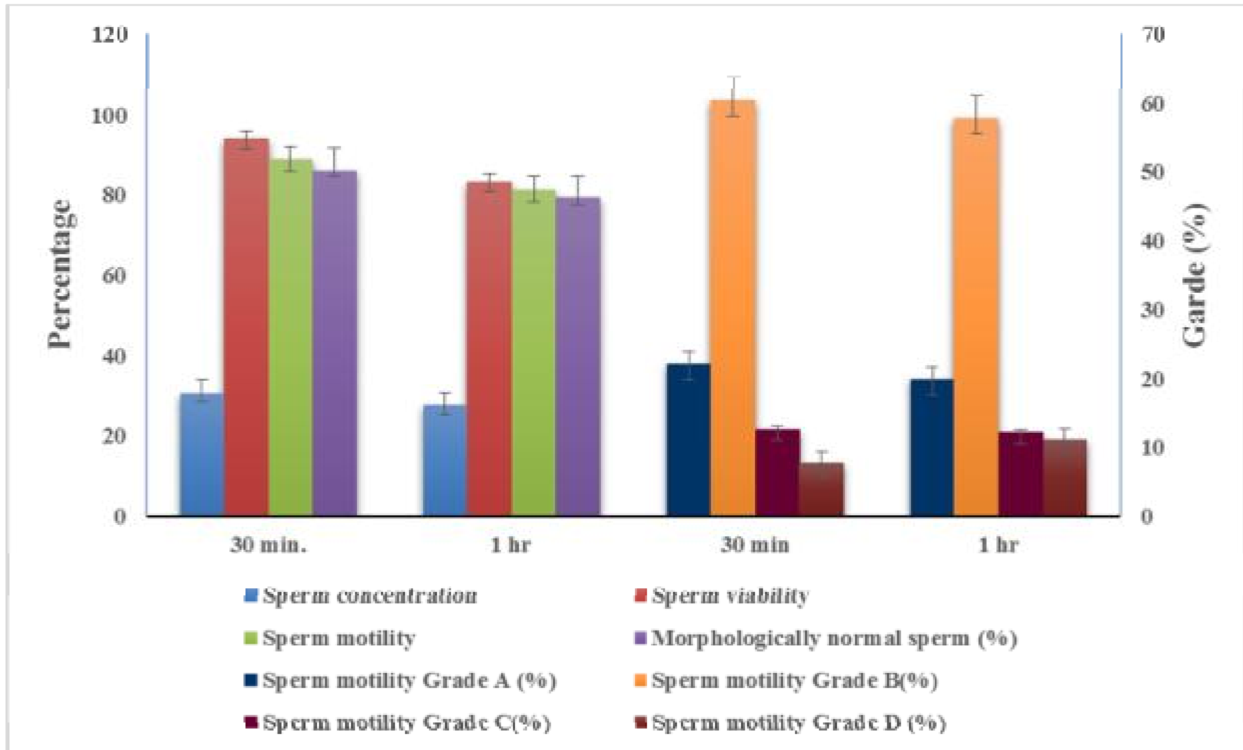

Figure (1): Comparison of sperm parameters of suspension kind 1 at different incubation times.
Values indicate a non-significant difference (P>0.05).

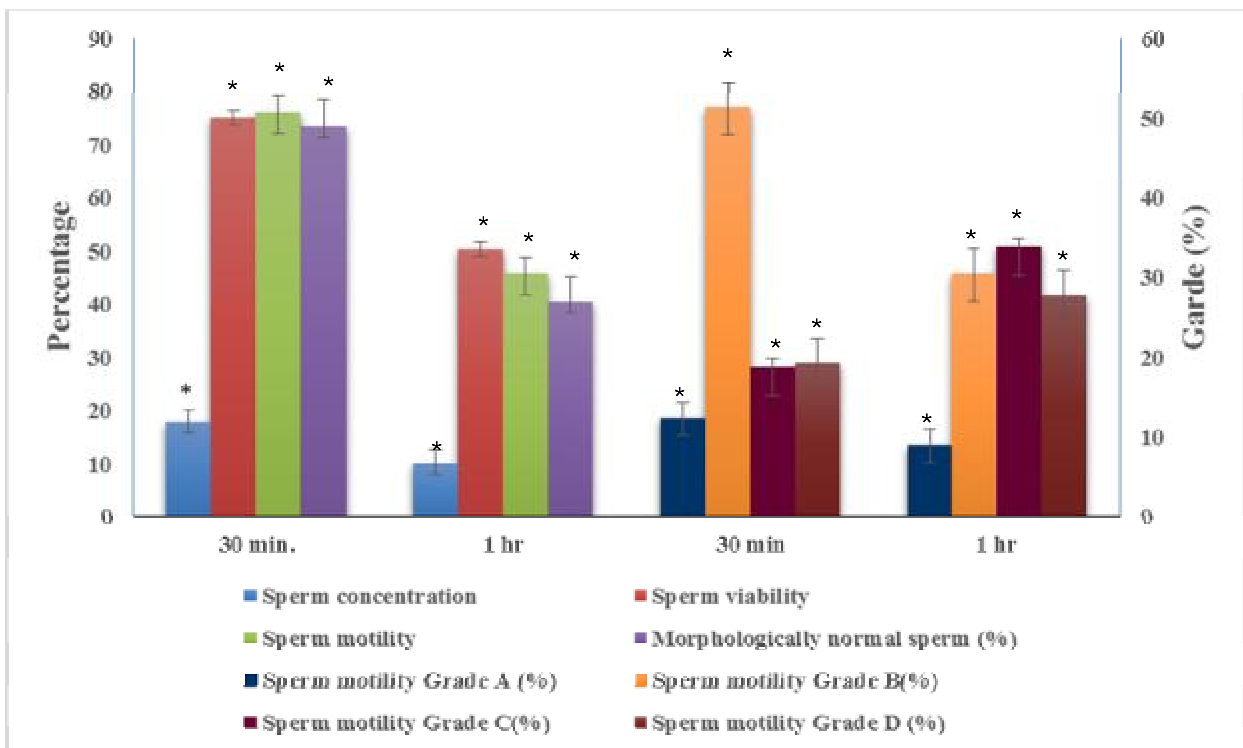

Figure (2): Comparison of sperm parameters of suspension kind 2 at different incubation times.
*Significant difference (P<0.001).

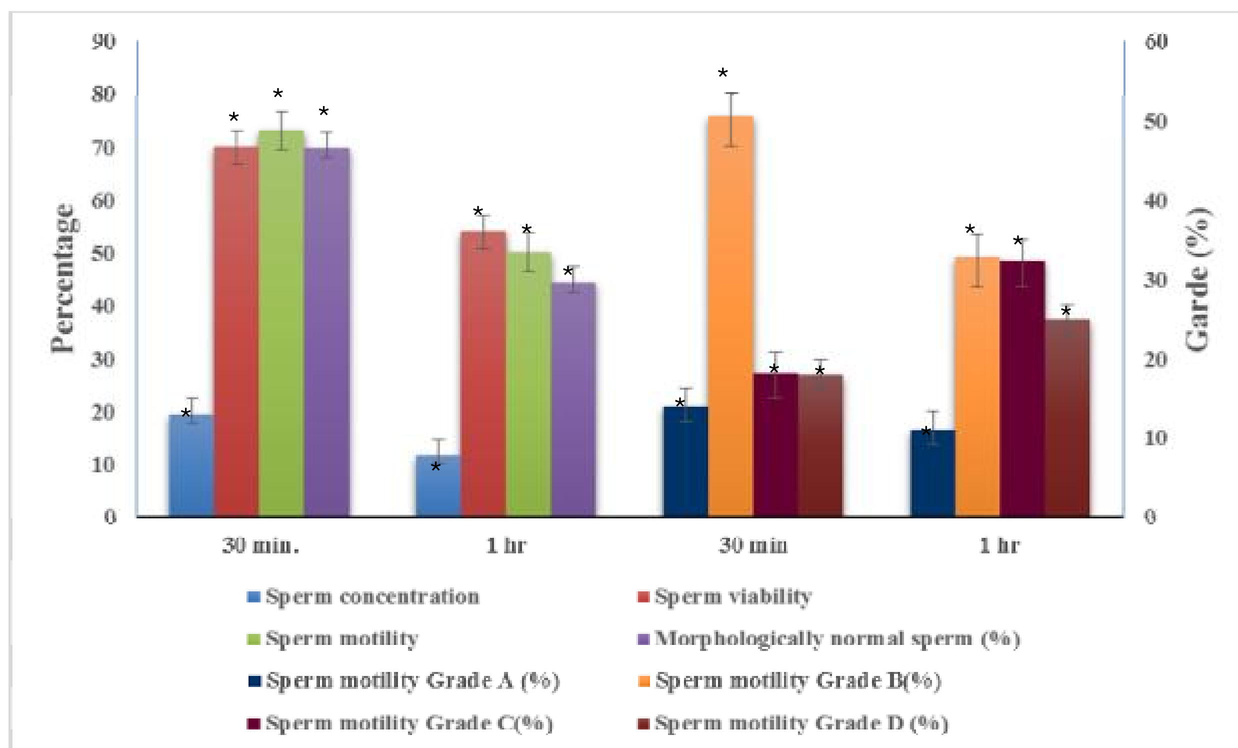

Figure (3): Comparison of sperm parameters of suspension kind 3 at different incubation times.
*Significant difference (P<0.001).

Table (3): Results of insemination of rats with sperm from different suspensions post the 30min. of incubation.

Groups	No. of inseminated rats	No. of corpora lutea mean ± S.E.	No. of pregnant rats	No. of implantation site mean ± S.E.	No. of resorbing implantation site mean ± S.E.	% of lost embryo mean ± S.E.
G_1	10	20.11±1.05	9	$14.4^{*\Delta}$ ±6.19	$6.44^{*\Delta}$ ±0.52	0
G_2	10	22.00±1.58	5	7.20^{*} ±0.44	10.8^{Δ} ±1.09	17.97±6.67
G_3	10	22.00±1.28	5	6.00^{Δ} ±1.00	10.40^{*} ±0.89	19.51±10.98

*Significant difference (P<0.001) G_1 and G_2.
ΔSignificant difference (P<0.001) between G_1 and G_3.

Table (4): Results of insemination of rats with sperm from different suspensions post the 1hr. of incubation.

Groups	No. of inseminated rats	No. of corpora lutea mean ± S.E.	No. of pregnant rats	No. of implantation site mean ± S.E.	No. of resorbing implantation site mean ± S.E.	% of lost embryo mean ± S.E.
G_4	10	19.00±0.92	8	$13.37^{\bullet\Delta}$±0.43	$6.62^{\bullet\Delta}$ ±1.09	0
G_5	10	21.50±2.24	3	$2.50^{\bullet}$ ±1.17	$11.60^{\bullet}$ ±0.58	40.40±5.29
G_6	10	23.50±0.70	2	2.00^{Δ} ±1.00	$12.50^{\bullet\Delta}$±0.70	38.22±2.18

•Significant difference (P<0.005) between G_4 and G_5.
ΔSignificant difference (P<0.005) between G_4 and G_6.

Table (5): Comparison of results of insemination of rats with sperm from different suspension at different of incubation times.

Groups / Time of incubation	No. of pregnant rats	No. of implantation site mean ± S.E.	No. of resorbing implantation site mean ± S.E.	% of lost embryo mean ± S.E.
G_1 30 min.	9	14.4 ± 6.19	6.44 ± 0.52	0
G_4 1 hr.	8	13.37 ± 0.43	6.62 ± 1.09	0
G_2 30 min.	5	7.20* ±0.44	10.80 ± 1.09	17.27• ± 6.67
G_5 1 hr.	3	2.50* ± 1.17	11.60 ± 0.58	40.40• ± 5.29
G_3 30 min	5	6.00* ± 1.00	10.40 ± 0.89	19.51• ± 10.98
G_6 1 hr.	2	2.00* ± 1.00	12.50 ± 0.70	38.22• ± 2.18

*Significant difference ($P<0.001$) between G_2 and G_5.
•Significant difference ($P<0.005$) between G_3 and G_6.

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