Available online on www.ijddt.com
International Journal of Drug Delivery Technology 2019; 9(2); 289-294
doi: 10.25258/ijddt.9.2.27

ISSN: 0975 4415

Research Article

Study of Some Parameters of Recovered Sperm from Vitamin C-treated Endometriotic Rats Model

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Received: 4th Apr, 19; Revised: 4th May, 19, Accepted: 8th Jun, 19; Available Online: 25th Jun, 2019

ABSTRACT

The present study was designed to reveal the biochemical and parameters changes of the retrieval sperms uteri of surgically-induced endometriotic rats post 30 min and 1h of coupling. Seventy-five adult female rats were divided into 3 groups as follows: Group 1: 25 shame-operated female rats were orally administrated with 0.1 ml of d.w for 35 days. Group 2: 25 endometriotic female rats were orally administrated with 0.1 ml of d.w for 35 days. Groups 3: 25 endometriotic female rats were orally administrated with 200 mg/Kg of vitamin C for 35 days. At the end of administration, 10 female rats of each groups were mated with fertile males to study sperm parameters. Whereas, others 15 endometriotic female rats were left without mating to use their uteri for leukocytes counts, oxidative markers and $PGF_2\alpha$ assay. The retrieval sperms from uteri of endometriotic rats (G2) were showed a significant ($p\leq0.05$) lower in their functions parameters "concentration, viability, progressive motility, and the activity of LDH-C4 enzyme depending on the remaining them in the uteri compared to those values of retrieval sperms from control (G1) and unmated endometriotic rats that treated with vitamin C (G3). Also, the uterine flushing fluids of endometriotic rats (G2) exhibited that had a significant ($p\leq0.01$) increase in the levels of leukocyte counts compared to those values of control (G1) and unmated endometriotic rats with vitamin C (G3). Whereas, there was no significant ($P>0.05$) difference in the levels of $PGF_2\alpha$ in the uterine tissue of all unmated endometriotic groups (G1, G2 and G3). Surgically-induced endometriotic passive influence affected uterine milieu via an appearance of oxidative stress that increased significantly ($p\leq0.01$) as a result of leukocytes count elevation. Ditto, there passive variables in the uterine microenvironment was reflected on the sperm functions parameters leading to infertility. While, the role of vitamin C in ameliorate of all above passive variants was evident.

Keywords:

INTRODUCTION

The common benign disease that it was represented with the development of endometrial glands and stroma outside the uterus was called endometriosis. Additionally, this endometriotic lesions could be implanted at pelvic organs i.e., at liver, kidney, and bladder[1]. Many explanations for the genesis of this condition were existed such as retrograde menstruation, embryonic rest, and celomic metaplasia[2]. In addition[3], reported that the vascular endothelial growth factor C (VEGF-C) may be involved in the pathogenesis of endometriosis. The inflammation could induce damage in sperm functions such as counts, motility and acrosome reactions. Additionally, endometriosis condition was described as chronic inflammatory disease[4]. Besides the previous studies confirmed an association between infertility and endometriosis, as according to[5] who reported that, endometriosis-induced infertility was due to adverse effect on ovarian reserve, ovulation, tubal anatomy, embryo, quality and implantation. Moreover, it was reported that, the negative role of endometriotic serous fluid in the sperm parameters of rats was shown in an *in vitro* study[6]. It was also referred that, endometriosis as a chronic inflammation disease may caused infertility or miscarriage via its free radical production[7]. Ditto, it was known that, the defective sperm parameters was one important cause of infertility[8].

On the other hand, vitamin C was classified as non-enzymatic antioxidant[9]. And, it was considered as antioxidant because having two enolic hydroxyl groups that known as a strong reducing agent[10].

Although, a few information was available about on influence of vitamin C in an endometriotic implant, it was observed that, the supplemented rats with vitamin C via oral gavage, at one day post implantation of endometrial implants showed a significant dose-related reduction in the weight and volume of the cyst, than control[11].

The aim of this study was to reveal the effect of endometriosis status on the sperm parameters and some biochemical aspects pre and post treatment with vitamin C as try, to detection what occurring to the sperm during their existence in the utri of endometriotic female rats, thence to determine the involvement of male factor “that mediated by endometriosis status” in fertility reduction of female with endmetriosis.

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Table 1: The retrieval sperm parameters post 30min and 1h of coupling of all experimental groups.

Experimental groups / Recovery time		Sperm parameters Con.x10^6	Viability %	Progressive motility %
Group.1	30min	25.11±1.10[a]	89.00±3.20[a]	88.50±4.01[a]
	1h	22.00±0.91[a]	86.10±1.50[a]	80.61±3.00[a]
Group.2	30min	16.11±0.21[b]	60.21±2.00[b]	58.12±1.91[b]
	1h	8.10±0.03[c]	33.12±2.05[c]	30.10±1.50[c]
Group.3	30min	21.00±0.12[a]	82.30±3.01[a]	90.50±4.02[a]
	1h	19.12±0.20[a]	80.52±4.00[a]	71.20±2.50[a]

The values represent the mean±S.E.
The vertical different letters represent a significant ($p \leq 0.05$) difference between groups.
The similar letters represent non-significant ($p > 0.05$) difference between groups.

Table 2: The activity of LDH-C4 of the recovered sperms of experimental groups.

Experimental groups	Lactate dehydrogenase (LDH C-4) U/ml Recoverey time	
	30min	1h
Group 1	2.00±0.00[a]	1.90±0.11[a]
Group 2	1.51±0.13[b/c]	0.90±0.02[b/f]
Group 3	2.10±0.05[a]	2.00±0.00[a]

The values represent the mean±S.E.
The vertical different letters represent a significant ($p \leq 0.05$) difference between groups.
The similar letters denote non-significant ($p > 0.05$) difference between groups.
The horizontal different letters represent a significant ($p \leq 0.05$) difference between groups.
The horizontal similar letters represent non significant ($p > 0.05$) difference between groups.

MATERIALS AND METHODS

Seventy-five adult female and 10 male rats of Sprague-Dawley, weighting 190-200 g were kept under conditions of temperature (22-25°C) and lighting (14L:10D). Rats of all groups were given water and pellet *ad* libitum. The surgically-induced endometriosis was achieved as follows: The anaesthetization of 30 female rats was done by the injection of pentobarbital sodium 30 mg/Kg intraperitoneally. The abdomen of these females were shoved and the left uterine horn was stubbed.

The ablated left horn was opened longitudinally and two pieces of 4×4 mm^2 were eluted. These pieces were stitched to the abdominal muscle on the right flank of rat according to the method of[12]. While the shame operated was done by removed of the left uterine horn and the autotransplanted of the adipose tissue in female rats (control). All females were remained in vivarium for 12 weeks to develop of endometriosis. On the other hand, the second surgery was made post 4 weeks of the first operation to confirm of the endometriotic lesion. Post the and operation, the female rats were divided randomly as follows:

Group 1: 25 sham-operated female rats were orally administrated with 0.1 ml of d.w for 35 days.

Group 2: 25 endometriotic female rats were orally administrated with 0.1 ml of d.w. for 35 days.

Group 3: 25 endometriotic female rats were orally administrated with 200mg/Kg of vitamin C for 35 days.

At the end of administration, 10 female rats of each groups (at proestrus phase) were mated with fertile males. While, 15 female rats of each above group were left without mating to use their uterine for leukocyte counts, oxidative markers and $PGF_2\alpha$ assay.

Exp.1. Recovery of ejaculated semen from uterine tissue.

The technique of[13] was used with the following modulation. Briefly, the females of each group were sacrificed post 30min and 1h of copulating. The right uterine horn of each female was tied at the proximal and distal and removing from the abdominal cavity. It was transformed into the phosphate buffer saline (PBS) at 37°C with eliminated the adipose tissue and blood vessels. The uterine horn was dried and the seminal content (ejaculated semen) post two periods of copulating (30 min and 1h) expelled into microcentrifuge tube at 37°C by light pressure. The ejaculated semen was suspended in 5ml of PBS and divided into two parts.

Exp.1.1: Study of sperm parameters

The first part of recovered semen was immediately used to assessment the semen parameters sperm count, viability, and progressive motility. Also, the dilution of recovered was 1:200 when evaluating the sperm count.

Exp.1.2: Assessment of lactate dehydrogenase C4 (LDH-C4) of sperm suspension.

While, the second part of recovered semen post 2 period (30 min and 1h) of mating was used to measure the LDH-C4 activity as follows: To prepare the sperm suspension for LDH-C4 assessment, the method of[14] was followed.

In briefly, one fraction of sperm suspension was homogenized 3 times by the ultrasonic for 10 sec and centrifuged at 13000 for 30 min at 4°C. And to assay the activity of LDH-C4 the change in absorbance was evaluated by spectrophotometry at 340nm for 1min according to the method of[15].

Exp.2: Measurement of uterine leukocytes.

Five unmated endometriotic female rats used to determine the number of leukocytes in their uterine horn

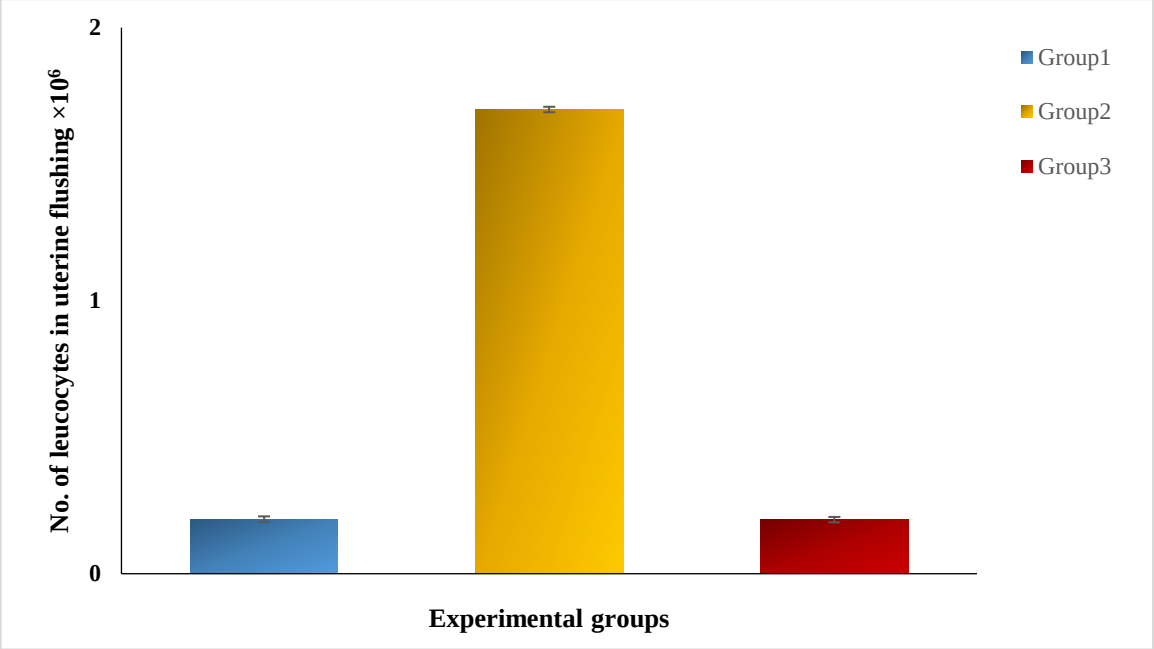

Figure 1: The number of leukocytes in the uterine flushing of unmated endometriotic female rats of all experimental groups. Bars are mean±S. E. Different letters represent significant (p≤0.01) difference between groups.

according to the method of[16]. The leukocytes were flashed from the right uterine horns with PBS and centrifuged at 8000xg for 5min.

Thereafter, it was resuspended in 0.02ml diluting solution (0.5% glacial acetic acid with 0.1% toluidine blue in d.w). All types of leukocytes i.e. neutrophils, macrophages, and lymphocyte were counted using haemocytometer.

Exp.3: Collecting of uterine flushing and assessment of its oxidant-antioxidant enzymes.

The right uterine horns of unmated female rats of all groups were eliminated and cleaned from blood vessels and adipose tissue. These horns were flushed by inserting the needle of disposable syringe filled with 1 ml of PBS at the upper end of horns. The collected uterine flushings were frozen at -20°C unity uterine for measurements. The measurements included H_2O_2 by using assay kits (Jiancheng Bioengineering, LTD, Nanjing, China) and, the serum albumin as standard was performed by using a BAK kit (Boyotime, Biotech Inc., China). While catalase (CAT) activity was evaluated according to the method of[17].

Exp.4: Measurement of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in uterine tissue.

Five endometriotic female rats without mating were used to evaluate the $PGF_2\alpha$ concentration in uterine tissue of all groups the flushed uterine horns were incubated for 1h in Krebs ringer bicarbonate solution in 5% CO_2 incubator at 37°C. Post incubation and acidification by 1M HCl at 37°C the milieu concentration of the $PGF_2\alpha$ was examined by radioimmunoassay according to[18].

Statistical analysis

T-test was achieved for comparison between experimental groups. The findings were considered as mean±S.E. the p value of ≤0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

The influence of endometriosis in retrieval sperm parameters and its LDH-C4 enzyme.

The current findings revealed that, the presence of sperms in the uterine cavity of mated endometriotic rats post two periods of copulating expiry i.e., 30 min and 1h caused a passive impact on the sperm functions parameters. Thus, there was a significant (p≤0.05) reduction in all recovered sperm parameters "concentration, viability, and progressive motility" of endometriotic rats (G2) compared to those in control: G1 (Table-1). Also, it was observed a similar significant (P<0.05) reduction in the sperm parameters when compared between two period (30 min and 1h) of sperm existence in the uterine cavity of mated endometriotic female rats (G2), and there was no significant (P>0.05) difference in both of G1 and G3 (Table-1). This reduction was compatible to the result of our previous study when the sperms were incubated in culture media that supplemented with collected fluid from endometriotic implants[6]. Moreover the recovered sperm from endometrotic rats (G2) post two periods of copulating expiry showed a significant (P≤0.05) decrement in their LDH-C4 activity compared to those of control: G1 (Table-2). The reduction in this enzyme activity was considered the underlying mechanism of the poor sperm motility. Wherein, the previous studies confirmed the role of LDH-C4 activity in creating energy for sperm motility[19,20].

Endometriosis and prostaglandin.

The previous studies revealed that, the passage of sperms from the uterus lumen to the oviduct "fertilization site" was attributed to many mechanisms such as uterine muscular contraction "which depending on the $PGF_2\alpha$" and sperms motility[21,22]. Whereas, our study showed that, there was no significant (p>0.05) difference in the uterine

Table 3: The uterine flushings oxidative markers of unmated females of all experimental groups.

Experimental Groups	Uterine flushing oxidative markers	
	H_2O_2 μ mol mg^{-1} protein	CAT Uml^{-1}
Group 1	0.53±0.04[a]	10.61±0.12[a]
Group 2	1.6±0.01[b]	3.21±0.13[b]
Group 3	0.48±0.03[a]	9.00±0.21[a]

The values represent the mean±S.E.
The vertical different letters denote a significant difference ($p<0.01$).
The similar letters denote non-significant ($p>0.05$) difference between groups.

tissue $PGF_2\alpha$ levels between all groups G1, G2 and G3 (Table-4).

This finding confirmed that, the poor of the retrieval sperm parameters from rats with endometriotic model rats (G2) was not due to the transport of an active sperms from uterus to the oviduct. Therefore the impairment in the retrieval sperm parameters could be attributed to the passive impact of uterine microenvironment that mediated by endometriosis.

Endometriosis and uterine leukocytes counts.

The results of the present study showed that, the surgically-induced endometriosis caused a significant ($p\leq0.01$) elevation in the counts of the uterine flushing's leukocytes of unmated endometriotic rats (G2) compared to those in control groups: G1 (Figure 1). This elevation could be explained according to the fact, endometriosis status produced augment permeability of the uterine blood vessels followed by transition of leukocytes to the rat uterine lumen. Additionally, the large counts of leukocytes may caused a passive effect on the uterine's microenvironment resulting to make unsuitable environment for sperms that in touch with it. Wherein many previous studies reported that, the leucocytes were considered as a source of reactive oxygen species (ROS)[23-26]. Also, it was known that, ROS was responsible for the generation of oxidative stress status. Whereby[27], demonstrated that, the oxidative stress occurred when there was overly formation of ROS.

Therefore, we can link between an elevation of uterine flushing leukocytes counts and appearance of the oxidative stress markers in the uterine flushing of endometriotic female rats (G2).

Endometriosis and some biochemical aspects of uterine flushings.

Our data revealed that, there was a significant ($p\leq0.01$) increment in the uterine flushing H_2O_2 levels of unmated endometriotic rats (G2), whereas, a significant ($p\leq0.01$) reduction in the CAT levels were observed compare to those values of control: G1 (Table).

This increment in H_2O_2 levels could be attributed to increase in leukocytes counts as it considered a main source H_2O_2. Also, the reduction in uterine flushing CAT levels expressed an elevation of its consumption that related to the oxidative stress (OS), wherein, it was known that, CAT candidates in the defence against OS. Moreover[28], reported that, catalase detoxified both intracellular and extracellular H_2O_2 through convert it to water and O_2. Ditto, it was known that, H_2O_2 was a substrate for the enzyme CAT[29].

Our findings confirmed an occurrence of OS status in the uterus of endometriotic rats. Also, these results were in agreement with other studies that, indicated the involvement of oxidative stress in endometriosis disease[30-33]. On the other hand, it was reported that, the oxidative stress was an important cause in male infertility[34,35]. According to above finding, we could assume that, an extrinsic production "by leukocytes ROS-produced oxidative stress in uterine flushing may be an important factor in etiology of sperm parameters "as we above noticed" via the peroxidative detriment to the plasma membrane and enzyme systems i.e., LDH-C4 of the exposed sperms to the uterine microenvironment. Whereby[36], mentioned that, the sperm membrane which had content of polyunsaturated fatty acid, therefore, their membrane was susceptible to oxidative stress and the double bounds of the membrane could easily be oxidized by ROS levels that present in the sperms environment.

The impact of vitamin C on the endometriosis-induced variable aspects.

On the other hand, this study revealed that, the treatment of endometriotic female rats with vitamin C (G3) contributed significantly ($p\leq0.05$) to meliorate all the retrieved sperm parameters from them.

Whereby, the concentration, viability, progressive motility and their activity of LDH-C4 enzyme were a highly significant ($p\leq0.05$) elevation compared to those values of sperms that retrieved from the endometriotic female rats (without vitamin C): G2 (Table 1 and 2). Also, these values were close to those of control: G1 (Table 1).

These results proved that, the capability of vitamin C to debilitate of endometriosis. This result was consistence with many previous studies such as the result of[37] who reported that, the administration of vitamin C (250mg/Kg) reduced the methyl parathion induced endometrial damage and apoptosis in female rats. Ditto, it was accordance with[38] who mentioned that, the vitamin C was inversely related to the pathogenesis of endometriosis.

Whereas, it was noted a significant ($p\leq0.01$) elevation in the anti-oxidant markers i.e. CAT (Table 4). It was clear that, these findings was comported with the vitamin C-induced decrement of leukocytes counts significantly. It was seemed that our results confirmed many previous studies that reported antioxidant effects of vitamin C, whereby[39] demonstrated a high antioxidant role of vitamin C in women with endometriosis by diminishing the OS markers and improving an antioxidant enzyme activity. Ditto,[36] mentioned that, the vitamin C was one of an important physiological scavengers. And,[40] observed that, the vitamin C may also prevent DNA damage by neutralizing free radicals and oxidants[10]. observed that, the vitamin C treatment (at 500mg/Kg) had suppressive and preventive effect of endometriotic induction, as well as it caused a regression of endometriotic implant volume. Thus, the treatment of endometriotic female rats

Table 4: The levels of $PGF_2\alpha$ in uterine tissue of unmated females of all experimental groups.

Experimental groups Unmated females	$PGF_2\alpha$ pg/mg of tissue
Group 1	22.13±0.012[a]
Group 2	20.50±0.03[a]
Group 3	19.00±0.00[a]

The values represent the mean±S.E.
The similar letters refer non-significant (P>0.05) difference between groups.

with vitamin C (G3) exhibited a significant ($p \leq 0.01$) reduction in each of uterine flushing leukocytes counts (Figure 1).

This result was comported to the study of[41] who revealed that, the treatment of endometriotic patients with vitamin C (1000mg) led to reduce the inflammatory markers. Additionally the vitamin C treatment (G3) caused a significant ($p \leq 0.01$) reduction in the pro-oxidant prevented DNA damage via neutralizing free radical and antioxidant. Besides, it was reported that, the treatment of endometriotic women with vitamin C (500mg) for 4 month increased antioxidant enzymes activity, as well as, decreased in the oxidative stress marker compared to control[42].

Finally, our results elucidated that, the treatment of endometriotic rats with vitamin C led to abrogate of uterine flushings inversely change that produced by endometriosis status.

In conclusion, endometriosis-induced unsuitable of uterine microenvironment that redounded on the quality of sperm functions and parameters could explain one of causes of poor fertility that related to endometriosis.

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