The Egyptian Journal of Hospital Medicine (October 2022) Vol. 89 (2), Page 7182- 7188

Effectiveness of Low Dose Aspirin or Vaginal Sildenafil on Perifollicular Blood Flow Using Doppler ultrasound and EG-VEGF and their Impacts on ICSI Cycle Outcome

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ABSTRACT

Background: Aspirin low dose or vaginal sildenafil, the two drugs have been shown to be advantageous for women undergoing assisted conception. Using powerful Doppler imaging, it is now possible to identify perifollicular perfusion and hence the accurate selection of oocyte with good intracytoplasmic sperm injection (ICSI) outcome.
Objective: The current study aimed to investigate the effects of aspirin and vaginal sildenafil citrate on improvement of the perifollicular blood flow, EG-VEGF and conception in females following ICSI cycles.
Subjects and methods: Ninety infertile women were participated in ICSI cycles. Full investigation was made for participating women. Ovarian stimulation protocol was performed. Doppler ultrasonography was used to evaluate the perifollicular blood flow. Serum and follicular fluid EG-VEGF were measured. data analyzed using SSPS program.
Results: Total oocytes count was significantly higher in aspirin group in comparison with sildenafil and control group. No significant difference in mean serum and follicular fluid EG-VEGF. Also, no significant difference in the proportions of PFBF and endometrial vascular zones. With respect to endometrial vascular zones, there was significant variation in favor of sildenafil and aspirin groups. Positive pregnancy test was reported in 40.0% of women in aspirin group, in (33.3 %) of women in sildenafil group and in (20.0 %) of women in placebo group, the difference was insignificant.
Conclusion: Aspirin treatment resulted in higher number of total retrieved oocytes, aspirin and sildenafil did not improve endometrial thickness, but they resulted in an improvement of sub endometrial vascularity, however, biochemical pregnancy test was not affected by either pharmacological agent.
Keywords: Aspirin, Sildenafil, ICSI cycle, EG-VEGF., Doppler ultrasound.

INTRODUCTION

The developing oocyte is given access to a developmental environment such as the intrafollicular oxygen and VEGF, both of which are extremely important to the oocyte's normal development as it continues to grow. Due to the advancement of powerful Doppler imaging, it is now possible to identify with high precision the arrangement of perifollicular perfusion. When evaluated by Power Doppler ultrasonography (PDU) on the day of oocyte extraction, this ovarian perifollicular vascularity can be employed marker for oocyte competence, embryonic viability, and ultimately higher implantation potential(1).

Following IVF, it was found that oocyte from well vascularized follicles are the best to achieve higher pregnancy rates. This is evidently related to the importance of angiogenesis during early development(2).

To evaluate and characterize the vascularity of the follicles, the qualitative use of Power Doppler ultrasonography is the method that is utilized the majority of the time. Power Doppler ultrasound provides improved vision of tiny vessels compared to color Doppler imaging, as well as a higher sensitivity for identifying low velocity flow. This is because power Doppler ultrasound uses a higher frequency(3). The study by Chui *et al.* (1) is frequently used as classification regarding qualitative assessment of perifollicular blood flow(PFBF)(4). Vural *et al.* (5) studied the connection of PFBF with FF, EG-VEGF, inhibin-a, and IGF-1 concentrations, as well as endometrial vascularity, and eventually IVF results in a prospective cohort research that included 40 women with tubal infertility. Through the use of power Doppler ultrasonography, a total of 156 follicles were analyzed and placed into one of two categories: well-vascularized or poorly vascularized. Additionally, pregnant women exhibited a higher proportion of well-vascularized follicles, a well-vascularized endometrium, greater levels of FF IGF-1 and serum EG-VEGF, but equivalent levels of FF EG-VEGF and serum inhibin-a. They hypothesized that Doppler measurements of PFBF and endometrial blood flow were crucial components of the IVF and ICSI treatment processes. It is possible that PFBF and FF IGF-1, in addition to serum EG-VEGF, are both independent pregnancy outcome indicators(5).

Aspirin has been shown in certain studies to be advantageous for women undergoing assisted conception. whilst others have not. One of aspirin's alleged advantages is an improvement in uterine and ovarian blood flow; and inhibition of platelet cyclooxygenase, which prevents thrombosis in the placental blood supply by preventing thromboxane production. By increasing leukotriene synthesis, it may also be a powerful stimulator of interleukin-3 (IL-3) in modest dosages (proteins associated with pregnancy success)(6) .

On the other hand, Sildenafil citrate may attach to the catalytic site and operate as a competitive inhibitor of PDE-5, the enzyme that ordinarily catalyzes the breakdown of vasodilatory cGMP, cGMP is shielded from destruction. When PDE5 is active, cGMP is broken down, which causes the vascular smooth

Received: 14/07/2022
Accepted: 17/09/2022

muscle to contract, reducing blood flow. The buildup of cGMP causes an increase in cGMP-dependent protein kinase activity, which in turn phosphorylates several targets in the smooth muscle cell when PDE5 becomes blocked. Smooth muscle relaxes as a result of myosin light chain kinase inactivation, enhanced potassium efflux, and decreased intracellular calcium as a result of smooth muscle cell target protein phosphorylation[7, 8].

Endocrine gland-vascular endothelial growth factor (EG-VEGF) EG-VEGF has been identified in the ovary, placenta, testis, and in the adrenal glands. The name "EG-VEGF" is based on its action on the capillary endothelial cells in the endocrine glands[9].

It has been demonstrated that this factor regulates trophoblast invasion. However, as evidenced by its specific activity on human placental microvascular endothelial cells, EG-VEGF plays a significant role in placental angiogenesis. These endothelial cells are a part of the chorionic villi, which are fetal capillaries[10]. According to scientific evidence, EG-VEGF induces the same vascular arrangement, permeability, and branching of the placental microvascular endothelial cells as the well-known growth factors VEGF.

SUBJECTS, METHODS AND MATERIALS

The design of the Study design

This comparative prospective study was conducted on the 90 infertile women who participated in intracytoplasmic sperm injection (ICSI) cycles at the infertility clinic associated with the "High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al- Nahrain University / Baghdad/ Iraq." Through the period from 2020 to 2022. 90 infertile women having ICSI cycles and ranging in age from 18 to 42, with infertility durations of 2 to 15 years, were enrolled . Sub fertile females were completely examined, including gynecological and general examination and body mass index calculation (BMI). Fertility investigations were also accomplished including baseline hormonal evaluation at follicular phase.

Trans-vaginal sonography, hysterosalpingography or Hycosy were performed to evaluate cavity of uterus and assure patency of tube and / or laparoscopy

The women included in the study were categorized into three groups randomly. Patients in group I (n = 30) received low dose aspirin (81 mg) during controlled ovarian stimulation till day of HCG, while those in group II (n = 30) received sildenafil as 25 mg four times daily vaginally from day of stopping of cycle to day of HCG, and group three we

re regarded as control group (n = 30) who received no treatment.

Exclusion and Inclusion criteria:

Females underwent fresh IVF/ICSI cycles based on antagonist protocol.

Infertility causes due to female factors, male factors, combined and unexplained.

Exclusion criteria:

- Patient with chronic disease (such as asthma , cardiovascular disease (CVD), diabetes, hypertension, peptic ulcer, liver, or kidney disease).
- Patients on nitrates and alpha blockers.
- Congenital uterine malformations, uterine pathology (fibroid, polyp), and previous ovarian surgery.
- Polycystic ovarian syndrome (PCOS)

Intracytoplasmic sperm Injection (ICSI) Program

Ovarian stimulation protocol

In the current study the flexible GnRH antagonist protocol was utilized. It comprised ovarian stimulation with recombinant FSH (r-FSH) harboring 75 IU of FSH /vial by regular subcutaneous injection with doses depended on the age of women and response.

Ultrasound machine with Doppler facility (South Korea, Samsung Medison Co. Ltd., Sonoace X8) was used in order to perform the scans. Calculation of the average of 2 maximum diameters was the rule to size the dominant follicle.

At the day of oocyte retrieval after 34-36 hour from rhCG

- Assessment of Perifollicular blood flow by using color power Doppler and the highest longitudinal plane of the ovaries for all measures, PFBF was assessed. By using power Doppler analysis, the periifollicular vascularization around the prominent follicles was assessed and graded as described. The percentage of blood flow surrounding follicles measuring >17–18 mm was used to measure vascularization. thickness and morphology of the endometrium were assessed. Both layers.

Biochemical tests: serum and follicular fluid EG-VEGF

- **Sample storage and gathering**

Before centrifuging for 20 minutes at roughly 1000 g, let samples clot for two hours at room temperature or overnight at 4 ˚C in a separator tube. To test newly made serum right away, or to save samples in aliquots for later use, store them at -20°C or -80°C. Steer clear of frequent freeze-thaw cycles .Measurement of Enzyme –linked Immunosorbent assay kit for Endocrine Gland Derived Vascular Endothelial Growth factor. Then ,Oocyte preparation and maturity evaluation, intracytoplasmic sperm injection procedure, embryo transfer, support of luteal phase .

Ethical Consideration

The current work has gotten ethical approval, from "High Institute of Infertility Diagnosis and Assisted Reproductive Technologies Board, Al-Nahrain University according to the Local Medical Ethical Committee. Additionally, each woman who agreed to participate in the study gave written consent.

Statistical analysis

The statistical software for social sciences was used to analyze the data (SPSS, IBM, Chicago, USA, version 23). Quantitative data were expressed as mean, standard deviation, whilst qualitative data were expressed as number and percentage.

Chi-square test was used to compare proportions between two groups whereas independent sample t-test was used to compare means between two groups. To find the correlation between quantitative or ordinal variables, Pearson correlation was utilized.

The significance level was set at $p \leq 0.05$.

RESULTS

Table (1) showed that total oocytes characteristic of infertile women participating in the present study among study groups is shown in table 1.

Total oocytes count was significantly higher in aspirin treated group in comparison with sildenafil treated group and control group, 12.67 ±5.32 versus 9.37 ±5.57 and 9.83 ±4.79, respectively (p = 0.035). However other characteristics showed no significant difference ($p > 0.05$).

Table (1): Oocytes characteristic of infertile women participating in the present study

Characteristic	Aspirin *n* = 30	Sildenafil *n* = 30	Placebo *n* = 30	*P*
Total oocyte				
Mean ±SD	12.67 ±5.32	9.37 ±5.57	9.83 ±4.79	0.035 O*
Immature Metaphase I oocyte count				
Mean ±SD	1.50 ±1.36	2.60 ±1.24	1.60 ±1.37	0.117 O NS
Mature Metaphase II oocyte count				
Mean ±SD	6.37 ±4.21	7.77 ±4.21	6.37 ±3.59	0.301 O NS
Injected oocyte count				
Mean ±SD	7.23 ±4.22	8.77 ±4.92	6.83 ±3.69	0.191 O NS
Number of fertilized oocyte				
Mean ±SD	6.07 ±2.77	5.37 ±2.99	4.77 ±3.32	0.257 O NS

n: number of cases; **SD**: standard deviation; **O**: one way ANOVA; **C**: chi-square test; **NS**: not significant; *: significant at $p \leq 0.05$

Table (2) showed that there was no significant difference in cycle day 2 serum FSH and LH hormonal levels among study groups ($p > 0.05$). also, there was no significant difference in mean serum estradiol (E2), TSH and AMH ($p > 0.05$). Moreover, there was no significant difference in mean serum estradiol (E2) at day of trigger ($p = 0.833$).

Table (2): Serum hormonal levels of infertile women participating in the present study

Characteristic	Aspirin (*n* = 30)	Sildenafil (*n* = 30)	Placebo (*n* = 30)	*p*
FSH(CD2)				
Mean ±SD	6.40 ±1.07	5.70 ±1.41	6.49 ±1.64	0.163 O NS
LH(CD2)				
Mean ±SD	4.19 ±1.48	4.75 ±1.06	5.22 ±1.48	0.239 O NS
E_2				
Mean ±SD	46.92 ±4.80	43.86 ±4.34	42.83 ±5.16	0.540 O NS
TSH				
Mean ±SD	2.09 ±0.82	1.59 ±0.70	2.18 ±0.43	0.064 O NS
AMH				
Mean ±SD	2.96 ±0.55	2.54 ±0.11	3.26 ±0.47	0.135 O NS
E_2\Trigger				
Mean ±SD	1061.60 ±90.50	1107.30 ±16.22	1139.80 ±56.93	0.833 O NS

n: number of cases; SD: standard deviation; O: one way ANOVA; C: chi-square test; NS: not significant

Comparison of Endocrine Gland derived Vascular endothelial growth factor EG-VEGF and Doppler Ultrasound Characteristics among study groups.

Comparison of secondary outcomes among study groups is shown in table 3. There was no significant difference in mean serum and follicular fluid EG-VEGF among study groups ($p > 0.05$). With respect to sub-endometrial resistive index (RI), there was no significant difference among study groups ($p = 0.453$).

Also, There was also no significant difference in the proportions of PFBF and Endometrial Vascular Zones ($p > 0.05$). With respect to Endometrial Vascular Zones, there was significant variation in favor of sildenafil and aspirin groups ($p = 0.037$) as shown in table (3).

Table (3): Comparison of Endocrine Gland derived Vascular endothelial growth factor EG-VEGF and Doppler Ultrasound characteristics among study groups ;

Characteristic	Aspirin group *n* = 30	Sildenafil group *n* = 30	Placebo group *n* = 30	*p*
PFBF Peri follicular blood flow				
<50 % follicle vascularity, *n* (%)	9 (30.0 %)	7 (23.3 %)	14 (46.7 %)	0.142 C NS
≥50 % follicle vascularity, *n* (%)	21 (70.0 %)	23 (76.7 %)	16 (53.3 %)	
Follicular fluid EG.VEGF(pg/ml)				
Mean ±SD	31.98 ±6.43	30.18 ±4.45	31.52 ±7.90	0.218 O NS
Serum EG-VEGF (pg/ml)				
Mean ±SD	13.64 ±3.56	15.55 ±3.20	14.95 ±2.57	0.580 O NS
Endometrial Vascular Zones				
1, *n* (%)	0 0.0 %)	0 (0.0 %)	2 (6.7 %)	0.037 C *
2, *n* (%)	10 (33.3 %)	11 (36.7 %)	16 (53.3 %)	
3, *n* (%)	20 (66.7 %)	19 (63.3 %)	12 (40.0 %)	

n: number of cases; **SD**: standard deviation; **O**: one way ANOVA; **C**: chi-square test; **NS**: not significant; *: significant at $p \leq 0.05$

Comparison of pregnancy test among study groups

Comparison of primary outcome (pregnancy test) among study groups is shown in table 4. Positive pregnancy test was reported in 12 (40.0 %) of women in aspirin group, 10 (33.3 %) of women in sildenafil group and 6 (20.0 %) of women in placebo group, but the difference was insignificant from statistical perspective. (Table 4)

Table (4): Comparison of pregnancy test among study groups

Characteristic	Aspirin group *n* = 30	Sildenafil group *n* = 30	Placebo group *n* = 30	*p*
Pregnancy test				
Positive, *n* (%)	12 (40.0 %)	10 (33.3 %)	6 (20.0 %)	0.234 C NS
Negative, *n* (%)	18 (60.0 %)	20 (66.7 %)	24 (80.0 %)	

n: number of cases; **C**: chi-square test; **NS**: not significant

Comparison of characteristics based on perifollicular blood flow results

Table (5). The table demonstrated significant variation in quality of oocytes, injected oocyte count, fertilized oocyte count, endometrial vascularity, follicular fluid EG-VEGF and pregnancy test ($p < 0.05$) and the variation was in favor of high vascularized follicles.

Table (5): Comparison of characteristics based on perifollicular blood flow results

Characteristic	Poor vascularized follicles PFBF ≤50 % *n* = 30	High vascularized follicles PFBF>50 % *n* = 60	*P*
Oocyte characteristic			
Retrieved oocyte count	10.90 ±5.00	10.48 ±5.60	0.731 I NS
MI oocyte count	1.03 ±1.35	2.33 ±2.52	0.010 I ***
MII oocyte count	5.03 ±3.63	7.73 ±3.93	0.002 I **
Injected oocyte count	5.57 ±3.72	8.63 ±4.29	0.001 I ***
Fertilized oocyte count	3.43 ±1.63	6.38 ±3.12	<0.001 I ***
Fertilization rate	75.68 ±28.74	76.45 ±17.61	0.877 I NS
Embryo characteristic			
Grade I	2.00 (2.00)	2.00 (2.00)	0.635 M NS
Grade II	1.00 (2.00)	1.50 (3.00)	0.106 M NS
Endometrial vascularity Zones			
Zone I	5 (16.7 %)	0 (0.0 %)	< 0.001 C ***
Zone II	21 (70.0 %)	26 (43.3 %)	
Zone III and IV	4 (13.3 %)	34 (56.7 %)	
EG-VEGF			
Serum EG-VEGF	13.14 ±6.20	15.50 ±7.55	0.143 I NS
FF EG.VEGF	56.34 ±53.36	37.67 ±17.71	0.016 I *
Pregnancy test			
Positive	5 (16.7 %)	23 (38.3 %)	0.036 C *
Negative	25 (83.3 %)	37 (61.7 %)	

DISCUSSION

In the present study we observed that aspirin was associated with 70 % women having high vascularized ovarian follicles in comparison with 53.3 % in placebo groups, but the variation did not approach statistical level. **Rubinstein *et al*** found that low dose aspirin was associated with significant improvement of ovarian blood flow[11].

In our study, the best rate of positive pregnancy test was reported in 12 in aspirin group followed by sildenafil and lastly by placebo group, but the difference was insignificant from statistical perspective.

In one previous randomized controlled clinical trial carried out by **Davar *et al*** .[12], there was no statistically significant difference between the two groups (aspirin versus no therapy), and both the chemical and clinical pregnancy rates and the abortion rate were similar[12]. **Madani *et al***.[13] in reported that, in contrast to our findings, aspirin administration during ICSI cycles increased the rates of implantation, clinical pregnancy, and live births[12]. In 2000, **Hsieh and colleagues** [14] found that taking aspirin increased the clinical pregnancy rate, but they select women with thin endometrium[14].

In the current study, PFBF showed significant correlation to good quality oocytes and to endometrial thickness and vascularity and significantly higher biochemical pregnancy rate. Thus assessment of PFBF by Doppler ultrasound can help in predicting the outcome in women undergoing ICSI cycles and is highly recommended as en evaluation in all women subjected to ART. **Vural *et al***. [5] evaluated the correlation of PFBF with the level of EG-VEGF in the follicular fluid , vascularity of endometrial, concentrations of IGF-1 and level of inhibin-a, in addition to outcomes of IVF They used Doppler ultrasound examination to assess 156 follicles which were accordingly classified into poor vascularized (≤ 50 % perifollicular fluid vascularity) or well vascularized (> 50 % perifollicular fluid vascularity). In summary, this research showed that follicles that were well vascularized resulted in high FF EG-VEGF levels, greater count of good-quality embryos, greater clinical pregnancy proportions and an endometrium that is well vascularized[5]. On the other hand, the later study showed that pregnant ladies had higher serum and follicular fluid levels of EG-VEGF and IGF-1, well vascularized endometrium and higher proportions of well vascularized follicles, thus the authors proposed that Doppler assessment of perifollicular blood flow can be an essential procedure in women undergoing ICSI to predict outcomes and that such role may be shared by serum and follicular fluid level of EG-VEGF.

Huyghe *et al*. [15] in 2017 performed a meta-analysis in order to evaluate the predictive potential of blood flow that is perifollicular in assisted reproduction outcome and the findings were that: During the past 20 years many factors predicting pregnancy outcome in

ART were evaluated and one of the important factors was the blood flow that is perifollicular; and in the review, an acceptable conclusion can be assured in that this type of blood flow is associated with the likelihood of positive pregnancy outcome and that its predictive potential in IVF cycles is more valid than its role in predicting the role in IUI cycles[15].

In our study we found that serum follicular fluid levels of EG-VEGF were comparable among study groups, indicating that administration of aspirin or sildenafil had no effect on this marker. To our knowledge, no previous research has evaluated the effect of aspirin or sildenafil on serum or follicular fluid EG-VEGF in women undergoing ICSI cycles; However, previous clinical studies[16,17] have shown conflicting results with respect to serum levels of VEGV and not EG-VEGF.

In our study, the proportion of women with high vascularized follicles was (76.7 %) in the group receiving sildenafil which was higher than those in the placebo group (53.3 %); However, statistical wise the difference was not significant. Abdullah *et al* [18] in 2021 studied the impact of sildenafil on blood flow of ovary in ART and found that ovarian blood flow is significantly better in women receiving sildenafil in comparison with those who did not receive sildenafil[18] ,**Trakakis *et al***.[19] came to the conclusion that sildenafil therapy could enhance female ovarian response. In an experimental study, it was also discovered that sildenafil administration marginally increased ovarian angiogenesis[19].

In a 2014 experimental investigation, **Celik *et al***.[20] looked at sildenafil's impact on ovarian ischemia-reperfusion injury in rats. Their study's histological and biochemical findings supported the notion that sildenafil treatment improved the damage that was described[20]. Be aware that the implantation rate of embryos produced from oocytes from well-vascularized follicles is higher than that of embryos derived from oocytes from poorly vascularized follicles[21]. At last, the results of aspirin administration, based on our results and previous reports, are conflicting and further research work is still needed to explore the exact way by which aspirin increases pregnancy rate in some women and not in all women undergoing ICSI.

CONCLUSION

Aspirin treatment resulted in higher number of total retrieved oocytes, aspirin and sildenafil did not improve endometrial thickness, but they resulted in an improvement of sub endometrial vascularity, however, biochemical pregnancy test was not affected by either pharmacological agent.

Declaration of conflicting interest

The authors declared that there is no conflict of interest.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Author contribution:

The author Balsam Q. Mohammed performed the research, examined and reviewed results and manuscript writing with the help and supervision of Huda A. Hussaini and Wasan A. ALjubori.

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