*Biochem. Cell. Arch.* Vol. 19, No. 1, pp. 215-221, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

# THE IMPACT OF FOLLICULAR FLUID GROWTH DIFFERENTIATION FACTOR 8 LEVELS ON IVF /ICSI OUTCOMES

**Rusul Hashem[1], Shatha Abdul Wadood[1] and Qays A. Mahdi[2]**

[1]Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq.
[2]Kamal Al-Samarai IVF Hospital, Ministry of Health, Baghdad, Iraq.
e-mail: al_zahra.al_baydhaa@yahoo.com

**(Accepted 12 February 2019)**

**ABSTRACT : Clinical index is needed to predict the outcome of pregnancy after *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ ICSI-ET) for infertile patients. Growth differentiation factor-8 (GDF-8), also known as myostatin, is one of transforming growth factor-â superfamily localized in antral follicles in normal and PCOS ovaries but its function in female reproductive system is still unknown. Aim of the study is to assess the correlation between levels of GDF8 in follicular fluid (FF) with outcomes of *in vitro* fertilization (IVF/ICSI) in women with and without PCOS. A prospective case control study was performed enrolling (40) patients with PCOS and (40) non-PCOS women (male infertility) undergoing IVF/ICSI. The collection of follicular fluid was at the day of oocyte pick up. Sandwich enzyme-linked immunosorbent assay (ELISA) kit was used to measure the levels of FF.GDF-8. A significant higher GDF8 level was found in PCOS group compared to non-PCOS group. Also, significant higher antral follicle count (AFC) in PCOS group in comparison tonon-PCOS group. There were no significant differences between the two groups in the mean of follicle diameter, endometrium thickness, aspirated oocytes, metaphase II (M II) oocyte, fertilized oocytes, embryo at 2pro nucleus (2PN), transferred embryo, grade1(G1)embryo, maturity rate, cleavage rate, fertilization rate and pregnancy outcomes. There was a significant positive correlation between GDF8 and G1 embryo in non-PCOS group. In non-PCOS group, mean GDF8 level was significantly higher in pregnant group than non-pregnant group. In PCOS group, mean GDF8 level was significantly higher in non-pregnant group than pregnant group.Receiver operator characteristics curve (ROC) analysis revealed that GDF8 in PCOS group has a good area under the curve (AUC) and can predict pregnancy outcome with a good sensitivity, while in non-PCOS group, GDF8 had less sensitivity in predicting pregnancy outcome. Concentration of GDF8 in FF may be a promising marker for embryo quality in non-PCOS patientswhile a good predictor for pregnancy outcome and IVF success in PCOS patients undergoing IVF/ICSI-ET.**

***Key words :*** Growth differentiation factor-8, follicular fluid, polycystic ovary syndrome, *in vitro* fertilization, intra-cytoplasmic sperm injection, pregnancy outcomes.

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects women of reproductive age (Bargiota *et al*, 2012). This endocrine disorder is associated with hormonal dysfunction that maynegativelyinfluence ovarian processes and causeadverse outcomes such asestrogen reduction, irregular menstrual cycle, decreased follicle maturation, arrest of antral follicle growth,early ovarian failure and infertility (Miler *et al*, 2011). Previous evidence had suggested that a limited supply of nutrients, hormones and growth factors to the growing follicle might partially explain the arrest of follicular growth, weakness selection of dominant follicle, and defects development of corpus luteum in PCOS women. Proteins, important for follicular growth were disparity expressed in FF of women with PCOS that may in part involved in the development of an aberrant folliculogenesis in PCOS women (Ambekar *et al*, 2015). Although, PCOS patients treated by IVF/ICSI cycles, they are considerably related with high miscarriage rate (Pal *et al*, 2016; Klevedal *et al*, 2017).

Earlier study had confirmed the expression of proteins of transforming growth factor-â superfamily, their receptors and downstream effectors that play critical roles in the development of follicular growth (Kristensen *et al*, 2013). Growth differentiation factor 8 (GDF8) is a secreted proteinbelongsto this superfamily, which is expressed not only in the musculoskeletal system (McPherron *et al*, 1997), but also expressed in reproductive tissues (e.g. placenta and uterus) (Islam *et*

*al*, 2014; Peiris *et al*, 2014).

Previous studies have shown that it is possible for this protein to play multiple roles in the pathogenesis of female's reproductive disorders such as preeclampsia, PCOS and uterine fibroid (Chen *et al*, 2012; Guo *et al*, 2012; Islam *et al*, 2014).

Chang *et al* (2016a) suggests that GDF8 inhibits human granulosa cell proliferation and may play a critical role in the control of tissue remodeling during the pre-ovulatory stage.

GDF8 and its receptors are expressed in luteinized human granulosa cells and the human FF contains the mature form of the GDF-8 protein. However, GDF8 suppresses cumulus expansion, thus contributing to the ovulation dysfunction in patients with PCOS (Chang *et al*, 2015a).

Fang *et al* (2016) proved that GDF-8 plays a crucial role in maintaining successful pregnancy for infertile patients with male or tubal cause infertility treated with IVF/ICSI-ET by its effect was on progesterone levels. Serum GDF-8 levels changed during the process of controlled ovarian hyperstimulation. Before human chorionic gonadotrophin administration, higher levels of GDF8 might suppress progesterone level in serum and might ensure pregnancy; while, after human chorionic gonadotrophin administration, lower levels of GDF-8 may keep up progesterone level and thus may have a crucial role in early embryo implantation.

However, levels of GDF8 in FF of infertile patients with PCOS treated with IVF/ICSI-ET and its relation with pregnancy outcome is not yet clear. In addition, most of studies focused on *in vitro* granulosa cells model (Chang *et al*, 2015a; Chang *et al*, 2016a; Lin *et al*, 2018), while, the *in vivo* function of GDF-8 in female reproductive system is still under study and research. Accordingly, the aim of this study was to assess the correlation between levels of GDF-8 in FF with IVF outcomes in women with and without PCOS undergoing IVF/ICSI-ET. As well as, explorethe possibility of using GDF8 as a predictive indicator for pregnancy outcome.

## MATERIALS AND METHODS

### Subjects

A prospective case control study conducted at Kamal Al-Samarai IVF Hospital, Ministry of Health in Baghdad, Iraq from December 2017 to June 2018. The study included (80) infertile women undergoing IVF/ICSI were divided according to the etiology of infertility into (40) PCOS women (mean age 31.02±0.93 years) and (40) non-PCOS women (male infertility) (mean age 30.87±1.98 years) as control group. The approval for study protocol was taken from the ethical committee in College of Science, University of Baghdad. All patients signed a written informed consent. The patients has been subjected to full history taking, complete examination, gynecologic examination, hormonal profile (at cycle day2: serum E2, LH, FSH) and transvaginal ultrasound (detection of antral follicles number, size and uterus thickness).

PCOS patients were diagnosed by specialist according to the revised Rotterdam European Society of Human Reproduction and Embryology, American Society for Reproductive Medicine Criteria.

The excluded criteria was women presented with the following: any endocrine disease, type 2 daibetes mellitus, urinary system infections, endometriosis, endometrial lesions, or unilateral or bilateral hydrosalpinges without treatment.

### Controlled ovarian hyperstimulation and oocyte collection

All participants were treated according to antagonist protocol. Administration of 150-225 IU of recombinant FSH (Gonal-F®) injection was from day two of menstrual cycle. GnRH antagonist (Cetrorelix) was injected (0.25 mg) daily when the follicle reached (12-14mm) by ultrasound. Cetrorelix and Gonal-F® were continued together until either two or three follicles reached (17-18 mm). Then, the ovulation induction was done using recombinant human chorionic gonadotropin administration (rhCG 6500 IU, Ovitrelle®; Merck Serono, Italy).

Oocytes were picked up after 34-36 hours from hCG injection using needle aspiration with a transvaginal ultrasound transducer guidance.

### Follicular fluid sampling

Samples were taken during oocyte pick up and centrifuged at 3000×g for 10 min at room temperature then transferred to sterile tubes and stored at -20ºC until assayed.

### Assessment of oocyte morphology and oocyte maturation

Nuclear maturation of oocytes was dictated by the identification of the first polar body. Oocytes morphology were assessed by metaphase II (MII) oocyte (Xia *et al*, 1997; Rienzi *et al*, 2008).

### Assessment of fertilization, cleavage and embryo quality

The fertilization results were evaluated after 18 hours from ICSI depending on the appearance of two pronuclei

and two polar bodies. Cleavage was done (24 hours after fertilization). Embryo quality was graded on the second day of insemination (Van Royen *et al*, 1999).

Embryo transfer (2–3 embryos) was done on day 2 or 3 of embryonic development.

**IVF outcomes**

Oocyte Maturity rate = Total no. of mature oocytes/ Total no. of all oocytes × 100.

Cleavage rate = Total no. of informed embryos/Total no. of fertilized Oocytes × 100.

Fertilization rate = Total no. of zygotes (2 pro nucleus)/Total no. of mature oocytes MII × 100.

**Measurement of GDF8**

Human GDF-8 levels were measured in FF by human MSTN (Growth/differentiation factor 8) ELISA Kit, using sandwich ELISA method according to the manufacture's protocol from (MyBiosource incorporation), USA. The intra-assay and the inter-assay is CV<8% and CV<10%, respectively.

**Statistical analysis**

Data analysis was done by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data were appeared as mean ± standard deviation. Differences between groups were analyzed by student's t-test. Categorical variables were analyzed by Chi-square test. The association degrees between variables were analyzed by Pearson Correlation. In addition, receiver operating characteristics (ROC) was used to evaluate area under curve (AUC). The best cut off point of the studied marker, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were also calculated. A $p$ value less than 0.05 was considered statistically significant.

## RESULTS

**Outcomes of IVF/ICSI**

As shown in Table 1, mean of AFC was higher significantly in PCOS group than those with non-PCOS group (P<0.05). There were no significant differences in the mean of follicle diameter, endometrium thickness, aspirated oocytes, M II oocyte, fertilized oocytes, embryo at 2PN, transferred embryo, G1 embryo, maturity rate, cleavage rate, fertilization rate and pregnancy outcomes between the two groups (P>0.05).

**Follicular fluid GDF-8 levels in non-PCOS and PCOS patients**

Levels of GDF8 were significantly higher in PCOS group than in non-PCOS group (15.97±4.88 vs. 12.74±3.94, $P$ = 0.002, Fig. 1).

**The association between GDF8 and IVF/ICSI outcomes**

FF.GDF8 did not show any correlation with IVF outcomes in PCOS group. However, there was a significant positive correlation between FF.GDF8 and G1 embryo in non-PCOS group (r = 0.471; $p$ = 0.027, Fig. 2).

In addition, FF.GDF8 showed positive correlation (not statistically significant) with pregnancy outcome in non PCOS patients (r=0.191, $p$>0.05); while, FF.GDF8 showed negative correlation (not statistically significant) with pregnancy outcome in PCOS patients (r =-0.002, $p$>0.05).

**Follicular fluid GDF-8 levels according to chemical pregnancy outcome**

To analyze the association between FF.GDF8 levels and pregnancy outcome, we next compared levels of FF.GDF8 between pregnant and non-pregnant patients in both PCOS and non-PCOS groups.

In non-PCOS group, levels of FF.GDF8 were significantly higher in pregnant patients than in non-pregnant patients (14.74±3.01 vs. 11.40±2.70, $P$ = 0.044, Fig. 3).

In PCOS group, levels of FF.GDF8 were lower in pregnant patients compared to non-pregnant patients (16.31±3.03 vs. 19.37±3.27, $P$ = 0.048, Fig. 3).

**Receiver operating charechteristics (ROC) curve analysis**

Tha above data indicate that FF.GDF8 levels showed significant difference between non pregnant group and pregnant group in both non PCOS group and PCOS group, so we next analyze the predictive value of FF.GDF8 for pregnancy in both non-PCOS and PCOS group.

In non-PCOS group, the AUC of GDF8 concentration in FF for predicting pregnancy was 0.74 ($p$ = 0.091) (Fig. 4A), as well as in PCOS group the AUC of GDF8 concentration in FF for predicting pregnancy was 0.73 ($p$ = 0.043) (Fig. 4B). Furthermore, we analyzed the sensitivity%, specificity%, PPV%, NPV% and best cutoff value for GDF8 concentration in FF. Our data showed that, GDF8 in PCOS group with a value of 18.00 (ng/ml) predicted pregnancy with sensitivity of 61.1%, specificity of 54.5%, PPV of 45.5% and NPV of 38.9% (Fig. 4B). While, GDF8 innon PCOS group showed less sensitivity and specificity in predicting pregnancy outcome (Fig. 4A).

## DISCUSSION

The dis-regulation of hypothalamus-pituitary-ovary axis has an impact on folliculogenesis and steroidogenesis

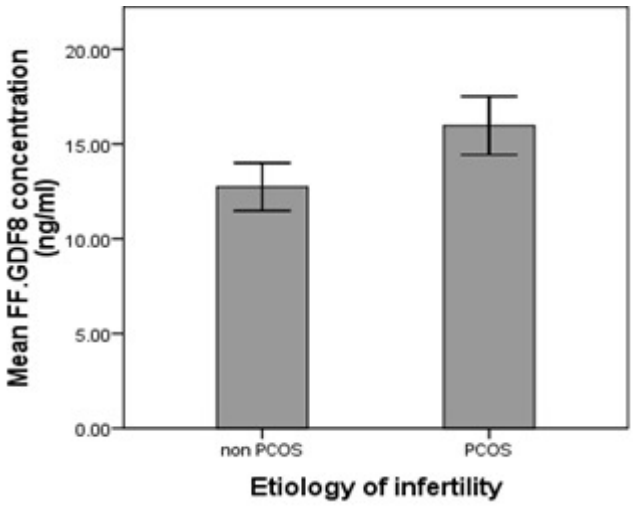


**Fig. 1 :** Follicular fluid GDF-8 levels in non-PCOS and PCOS groups.

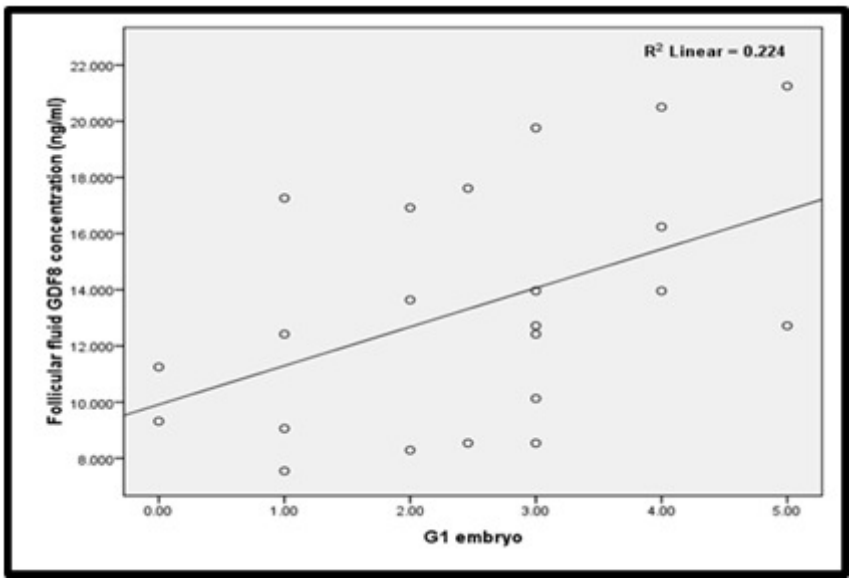


**Fig. 2 :** The association of FF. GDF8 levels with G1 embryo in non-PCOS group.

in PCOS patients. Growth factors play major role in reproductive process through their effect on ovary growth and differentiation (Baskind *et al*, 2016). Previous studies revealed the importance of GDF8 in regulation of follicular function, follicular growth and hormones level (Chang *et al*, 2015a; Fang *et al*, 2016).

**Characteristics of IVF/ICSI outcomes in non-PCOS and PCOS groups**

The result of AFC in this study, which was significantly higher in PCOS group in comparison to non-PCOS group, is in agreement with previous studies (Yang *et al*, 2015; Lai *et al*, 2017). PCOS patients are at high risk of over responding for fertility drugs, which leads to ovarian hyperstimulation complications and formation of a large number of ovarian follicles (Abbara *et al*, 2018).

Our study showed that there were no significant differences between PCOS group and non-PCOS group inM II oocyte, fertilized oocytes, embryo at 2PN, G1 embryo, maturity rate, cleavage rate, fertilization rate and chemical pregnancy outcome. A possible explanation for this is the hyperstimulation using GnRH antagonist protocol suppress pituitary gland to overcome the higher levels of LH in PCOS group. In addition, all the picked oocytes were handled *in vitro* in the same way regardless infertility cause. Consequently, IVF is an appropriate treatment strategy for women with PCOS. Similar results were reported by Saleem *et al* (2014), Yang *et al* (2015), Al-Dujaily *et al* (2017).

**Follicular fluid GDF-8 levels in non-PCOS and PCOS patients**

GDF8 concentration plays a physiological role in ovarian functions, such as, steroidogenesis (Chang *et al*, 2016b), stabilization of the extracellular matrix, follicle and oocyte maturation (Chang *et al*, 2016c). Several female reproductive diseases are results of mutations or dysregulation in GDF system (Chang *et al*, 2016d).

Growth factors secreted by oocyte and theca cell play important roles in regulation of ovarian functions (Chang *et al*, 2014, 2015b). In this study, GDF8 levels in FF increase significantly in PCOS women than in non-PCOS women.

In a previous study, Chang *et al* (2015a) proved for the first time that GDF8 is expressed in human FF and granulosa cells. Interestingly, Lin *et al* (2018) suggested that GDF8 is localized at thegranulosa cell, theca cell, oocyte, andin antral follicles of human ovary, suggesting that GDF8 mode of action isautocrine and paracrine inside the developing ovaries. Furthermore, increased expression of GDF8 and its receptors within the antral folliclecould

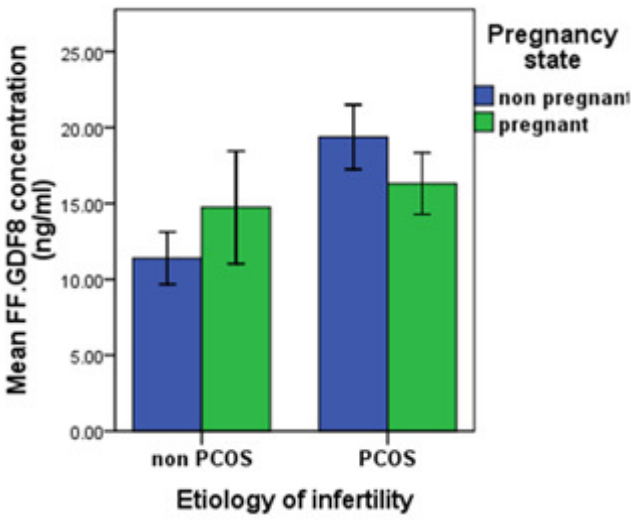


**Fig. 3 :** Follicular fluid GDF-8 levels according to chemical pregnancy outcome in non-PCOS group and PCOS group. Non-PCOS group were divided into pregnant (n=7) group and non-pregnant group (n=12) and PCOS group were divided into pregnant (n=12) group and non-pregnant group (n=18).

Together with our finding of higher GDF8 levels in FF of patients with PCOS; we suggests that disregulated GDF8 might be involve in the pathogenesis of PCOS, such as ovulatory dysfunction, arrest of follicular growth and metabolic disorder.

Future studies are recommended to demonstrate the roles of GDF8 in PCOS ovarian tissues as well as normal ovarian tissues. Furthermore, animal model studies may add to better understanding of GDF8 effect on female reproductive system disorders.

**Follicular fluid GDF-8 levels according to chemical pregnancy outcome, association with IVF/ICSI outcomes and ROC analysis in PCOS group**

Our data showed a statistically significant increase in FF.GDF8 levels in non-pregnant patients compared to pregnant patients in PCOS group. Importantly, a negative relationship (although not statistically significant) was found in this study between FF.GDF8 levels and

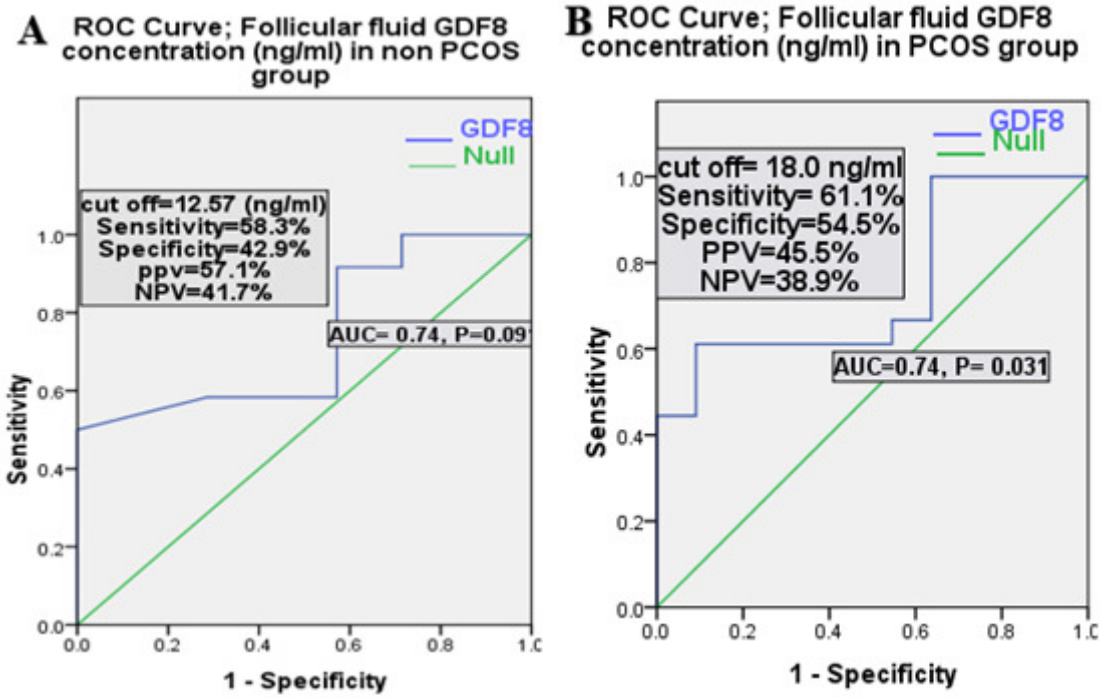


**Fig. 4 :** Receiver operating characteristics curve analysis of follicular fluid GDF8 levelsin (A) non PCOS and (B) PCOS group for predicting pregnancy. AUC= area under the curve, PPV= positive predictive value, NPV = negative predictive value.

reflect a crucial role for GDF8 in the cellular interactions between germ cells andfollicle cells during development of ovarian follicle.

An earlier study found an elevated GDF8 levels in serum women with PCOS (Chen *et al*, 2012). The presence of GDF8 in corpus luteum indicates its important role in the luteal function and the higher expression of GDF8 levels in ovarianantral follicle in patients with PCOS compared with normal ovaries (Lin *et al*, 2018).

pregnancy outcome in PCOS patients. It may be due to excessive level of GDF8 may cause pathophysiological process and maybe adversely affect ovarian function and subsequent pregnancy outcome in PCOS patients undergoing IVF/ICSI cycle. In addition, as the ROC curve was used to evaluate the diagnostic value of GDF8 levels in FF; the cutoff value with a relatively good sensitivity can be used for predicting negative pregnancy outcome. Hence, taken the previous important findings together,

**Table 1 :** Characteristics of IVF/ICSI outcomes in non-PCOS and PCOS groups.

| IVF/ICSI outcomes | Non PCOS (n=40) | PCOS (n=40) | *P* value |
|---|---|---|---|
| AFC | 8.55±2.41 | 10.67±2.79 | 0.001 |
| Follicle diameter (mm) | 17.71±1.46 | 17.70±0.87 | 0.80 |
| Endometrium thickness (mm) | 10.99±1.81 | 10.23±0.98 | 0.46 |
| Aspirated oocytes | 7.39±2.40 | 7.89±1.78 | 0.61 |
| M II oocyte | 4.84±1.79 | 4.99±2.09 | 0.82 |
| Fertilized oocytes | 5.58±2.12 | 5.53±2.09 | 0.121 |
| Embryo at (2PN) | 3.30±1.40 | 3.27±2.40 | 0.731 |
| Transferred embryo | 3.18±1.14 | 3.00±1.13 | 0.753 |
| G1 embryo | 2.08±1.35 | 2.01±1.51 | 0.81 |
| Maturity rate | 65.33±20.44 | 64.68±22.84 | 0.62 |
| Cleavage rate | 61.56±21.22 | 61.31±15.62 | 0.88 |
| Fertilization rate | 68.03±24.64 | 66.69±23.93 | 0.75 |
| Chemical pregnancy = count (%) | | | |
| Yes | 7 (17.5%) | 12(30%) | 0.189† |
| No | 33(82.5%) | 28(70%) | |
| Total | 40(100%) | 40(100%) | |

Data were shown as mean ± SD; analysis were performed by student t-test; †; statistical analysis performed by Chi-square test; significant was established at $P<0.05$; AFC = Antral follicle count; M II = oocyte at metaphase II; embryo at (2PN)= embryo at two pro nuclei; G1= grade one embryo.

we can suggest that lowering GDF8 levels in FF may be useful for better pregnancy outcome in women with PCOS undergoing IVF/ICSI-ET.

As a result, a comprehensive understanding of action, expression and levels of GDF8 in the human ovary is recommended to develop treatment for infertile women with PCOS.

**Follicular fluid GDF-8 levels according to chemical pregnancy outcome, association with IVF/ICSI outcomes and ROC analysis in non-PCOS group**

Fang *et al* (2016) found statistically significant higher levels of serum and FF.GDF8 in pregnant group than those of non-pregnant group of male or tubal cause infertile women.

Our data showed that, there was a significant difference between pregnant group and non-pregnant group according to FF.GDF8 levels in non-PCOS group. Hence, higher GDF8 levels were found in pregnant group than those of non-pregnant group. Interestingly, this study found a positive relationship (although not statistically significant) between FF.GDF8 levels and pregnancy outcome.

The relationship between oocyte quality and different biochemical markers in FF *e.g.* hormones, growth factors, interleukins, reactive oxygen species, anti-apoptotic factors, proteins, peptides, amino acids, sugars, and prostanoids have been proved (Revelli *et al*, 2009). Interestingly, in an earlier study by Wang *et al* (2007) they evaluated the relationship between oocyte morphology and embryo quality. Taken this evidence together; GDF8 might be a predictor for oocyte maturation and subsequent embryo quality.

An important point is that, GDF8 is secreted by granulosa cell and under normal physiological conditions considered as inhibitor for cumulus expansion. Furthermore, prior to ovulationin meiotic arrest, GDF8 has an important role in maintaining cumulus oophorus, acting as maturation stimulator and luteinization inhibitor (Chang *et al*, 2015a). These evidences might strengthen our finding about the relationship between GDF8 levels and G1 embryoin non-PCOS women, and support the crucial role of GDF8 in female reproductive system and in regulating ovarian functions in the human ovary.Further studies are needed to clarify the role of GDF8 on embryo quality in IVF patients.

Also, as a result of the ROC curve for non-PCOS group, FF.GDF8 levels may predict positive pregnancy outcome. However, this finding might be support the significant higher FF.GDF8 levels in pregnant patients, and the positive correlation between FF.GDF8 levels and pregnancy outcome, as well asthe significant positive correlation with G1 embryo.

## CONCLUSION

The concentration of GDF8 in FF may be an accurate marker for embryo quality in non-PCOS patients undergoing IVF/ICSI-ET. In patients with PCOS, as concentration of GDF8 in FF was significantly higher in non-pregnant patients and that GDF8 had a good sensitivity in predicting negative pregnancy outcome, the rapeutic agents targeting ovarian GDF8 to overcome its higher levels, to ensure successful IVF/ICSI-ET treatment.

**Conflict of interest :** The authors declare no conflict of interest.

**Funding :** There was no funding for current work. The study is part of Ph.D. Thesis for the first author.

## ACKNOWLEDGMENT

The authors thank the Medical Team of Kamal Al-Samarai IVF Hospital, Ministry of Health, Baghdad, Iraq. Thanks to the patients, who participate in this study by providing the follicular fluid samples.

## REFERENCES

Abbara A, Islam R, Clarke S A, Jeffers L, Christopoulos G, Comninos A N and Kelsey T W (2018) Clinical parameters of ovarian

hyperstimulation syndrome following different hormonal triggers of oocyte maturation in IVF treatment. *Clinical Endocrinology* **88**(6), 920-927.

Al-Dujaily S, Abdul-Kareem M and Selman M (2017) Role of heparin binding epidermal growth factor in the serum and follicular fluid in prediction of pregnancy outcome of infertile women with and without PCOS. *Int. J. of Adv. Res*. **5**(10), 70-77.

Ambekar A S, Kelkar D S, Pinto S M, Sharma R, Hinduja I, Zaveri K and Mukherjee S (2015) Proteomics of follicular fluid from women with polycystic ovary syndrome suggests molecular defects in follicular development. *The Journal of Clinical Endocrinology & Metabolism* **100**(2), 744-753.

Baerwald A R, Adams G P and Pierson R A (2012) Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Hum Reprod Update* **18**, 73–91.

Bargiota A and Diamanti-Kandarakis E (2012) The effects of old, new and emerging medicines on metabolic aberrations in PCOS. *Therapeutic Advances in Endocrinology and Metabolism* **3**(1), 27–47.

Baskind N E and Balen A H (2016) Hypothalamic–pituitary, ovarian and adrenal contributions to polycystic ovary syndrome. *Best Practice & Research Clinical Obstetrics & Gynaecology* **37**, 80-97.

Chang H M, Fang Y, Liu P P, Cheng J C, Yang X and Leung P C (2016c) Connective tissue growthfactor mediates growth differentiation factor 8-induced increase of lysyl oxidase activity in human granulosa-lutein cells. *Mol Cell Endocrinol*. **434**, 186–198.

Chang H M, Fang L, Cheng J C, Klausen C, Sun Y P and Leung P C (2015a) Growth differentiation factor 8 down-regulates pentraxin 3 in human granulosa cells. *Mol Cell Endocrinol*. **404**, 82-90.

Chang H M, Cheng J C, Klausen C and Leung P C (2015b) Recombinant BMP4 and BMP7 increase activin A production by up-regulating inhibin βA subunit and furin expression in human granulosa-lutein cells. *The Journal of Clinical Endocrinology & Metabolism* **100**(3), 375-386.

Chang H M, Pan H H, Cheng J C, Zhu Y M and Leung P C (2016a) Growth differentiation factor 8 suppresses cell proliferation by up-regulating CTGF expression in human granulosa cells. *Mol. Cell Endocrinol*. **422**, 9-17.

Chang H M, Qiao J and Leung P C (2016d) Oocyte–somatic cell interactions in the human ovary–novel role of bone morphogenetic proteins and growth differentiation factors. *Human Reproduction Update* **23**(1), 1-18.

Chang H M, Fang L, Cheng J C, Taylor E L, Sun Y P and Leung P C (2016b) Effects of growth differentiation factor 8 on steroidogenesis in human granulosa-lutein cells. *Fertility and Sterility* **105**(2), 520-528.

Chen M J, Han D S, Yang J H, Yang Y S, Ho H N and Yang W S (2012) Myostatin and its association with abdominal obesity, androgen and follistatin levels in women with polycystic ovary syndrome. *Hum Reprod*. **27**, 2476-2483.

Fang L, Yu Y, Zhang R, He J and Sun Y P (2016) Serum GDF-8 levels change dynamically during controlled ovarian hyperstimulation in patients undergoing IVF/ICSI-ET. *Scientific Reports* **6**, 28036.

Guo J, Tian T, Lu D, Xia G, Wang H and Dong M (2012) Alterations of maternal serum and placental follistatin-like 3 and myostatin in pre-eclampsia. *J Obstet Gynaecol Res*. **38**, 988–996.

Islam M S, Catherino W H, Protic O, Janjusevic M, Gray P C, Giannubilo S R, Ciavattini A, Lamanna P, Tranquilli A L, Petraglia F, Castellucci M and Ciarmela P (2014) Role of activin-A and myostatin and their signaling pathway in human myometrial and leiomyoma cell function. *J. Clin. Endocrinol. Metab.* **99**, 775-785.

Klevedal C and Turkmen S (2017) Fetal-maternal outcomes and complications in pregnant women with polycystic ovary syndrome. *Minerva Ginecol*. **69**(2), 141–149.

Kristensen S G, Andersen K, Clement C A, Franks S, Hardy K and Andersen C Y (2012) Expression of TGF-beta superfamily growth factors, their receptors, the associated SMADs and antagonists in five isolated size-matched populations of pre-antral follicles from normal human ovaries. *Mol. Hum. Reprod*. **20**, 293-308.

Lai Q, Xiang W, Li Q, Zhang H, Li Y, Zhu G and Jin L (2017) Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. *Frontiers of Medicine* **19**, 1-7.

Lin T T, Chang H M, Hu X L, Leung P C and Zhu Y M (2018) Follicular localization of growth differentiation factor 8 and its receptors in normal and polycystic ovary syndrome ovaries. *Biology of Reproduction* **98** (5), 683–694.

McPherron A C, Lawler A M and Lee S J (1997) Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. *Nature* **387**(6628), 83.

Miller W L and Auchus R J (2011) The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. **32**, 81–151.

Pal L, Zhang H, Williams J, Santoro N F, Diamond M P, Schlaff W D, Coutifaris C, Carson S A, Steinkampf M P, Carr B R, McGovern P G, Cataldo N A, Gosman G G, Nestler J E, Myers E and Legro R S (2016) Reproductive Medicine Network. Vitamin D status relates to reproductive outcome in women with polycystic ovary syndrome: secondary analysis of a multicenter randomized controlled trial. *J Clin Endocrinol Metab*. **101**(8), 3027–3035.

Peiris H N, Salomon C, Payton D, Ashman K, Vaswani K, Chan A, Rice G E and Mitchell M D (2014) Myostatin is localized in extra villoustrophoblast and upregulates migration. *J. Clin. Endocrinol. Metab*. **99**, 2288-2297.

Revelli A, dellePiane L, Casano S, Molinari E, Massbrio M and Rinaudo P (2009) Follicular fluid content andoocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol*. **4**, 7–40

Rienzi Laura (2008) Significance of metaphase II human oocyte morphology on ICSI outcome. *Fertility and Sterility* **90**(5), 1692-1700.

Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group (2003) Revised 2003 consensus on diagnostic criteria and longterm health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod*. **19**(1), 41-47.

Seleem A K, El Refaeey A A, Shaalan D, Sherbiny Y and Badawy A (2014) Superoxide dismutase in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection. *Journal of Assisted Reproduction and Genetics* **31**(4), 499-504.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburtg M, Van de Meerssche M, Ryckaert G, Eestermans W and Gemis J (1999) Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod*. **14**, 2345–2349.

Wang Q and Sun Q Y (2007) Evaluation of oocyte quality: morphological, cellular and molecular predictors. *Reprod Fertil Dev*. **19**, 1–12.

Xia P (1997) Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. *Hum Reprod.* **12**, 1750–1755.

Yang F, Ruan Y C and Yang Y J (2015) Follicular hyperandrogenism down regulates aromatase in luteinized granulosa cells in polycystic ovary syndrome women. *Reproduction* **150**(4), 289-296.