ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Effect of Activin A, Fibrillin-3, and Follistatin on Oocytes Quality and Embryonic Development Following ICSI Cycle

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Abstract

Objectives: The aim of this study is to elucidate the effect of Activin A, Fibrillin-3, and Follistatin hormones on the oocytes number and quality ,embryonic development, and pregnancy rate (PR) following intra cytoplasmic sperm injection (ICSI) cycle. Subject, Materials and Methods: Two hundred women undergoing ICSI treatment were chosen randomly. Controlled ovarian stimulation was done with two different protocols. Measurements of Activin A (pg/ml), Fibrillin-3 (ng/ml) and Follistatin (ng/ml) were assessed in serum and follicular fluid on the day of ovum pick up. Intra cytoplasmic sperm injection was performed and the oocyte and embryonic development were evaluated and determined in relation to the levels of these hormones. Results: There was a significant difference between number of MII oocytes and high quality embryos (G1) in pregnant women than that of non-pregnant women. The follistatin follicular fluid (FF) is the only hormone level which has a significant relation with total oocytes and good MII oocytes. Both the mid-cycle level in serum and follicular fluid of activin-A show a negative correlation of around -0.2 with the total number of embryos and the number of G1 embryos. For both Activin A and Fibrillin-3, there was a high or relatively high correlation within the pregnant subject and low till very low correlation in the non-pregnant subjects, independent of the stimulation protocol. Conclusions: The Follistatin FF has a significant correlation with good MII oocytes that could be used as a predictor for successful ICSI outcome.

Keywords: *Activin A, Follistatin, Fibrillin-3, ICSI, Controlled ovarian stimulation, Metaphase II oocytes, Embryos.*

Introduction

Recently, the reproductive hormones, activin A, and Follistatin have been found to play an important role in folliculogenesis, oocyte maturation and corpus luteum function by changing the pattern of granulose cell expression which in turn affects the success of fertilization potential [1]. Assisted reproduction is a complicated process involving multiple stages of ovarian stimulation, ovum pick up, then fertilization, embryo cleavage and implantation. The ultimate goal of all assisted reproductive procedures is to get a viable intrauterine pregnancy as a step to get a healthy baby. Good quality of all reproductive components and primarily embryos have a positive impact on success rate .Thus, better selection of developed embryos is one of the greatest challenges in in vitro fertilization (IVF) programs [2]. Consequently, the morphological approach of choosing good oocytes and high quality embryos at 2-8 cell stages based on number, equality of size and the fragmentation percent [3]. Oocyte evaluation for maturity is more accurate following the removal of the cumulus-corona cells in preparation for intracytoplasmic injection (ICSI) [4].

Oocyte maturation based both on nuclear and cytoplasmic maturation that should be completed in a coordinated manner to ensure optimal condition for subsequent fertilization [5]. Thus in spite of the presence of the first polar body which considered to be a marker of oocyte nuclear maturity ,recent studies showed that only those displaying a meiotic spindle can in fact be considered as true mature metaphase II (MII) stage oocyte [6].

On the other hand, the follicular fluid (FF) act as a medium by which signaling mediators are transported in and out of the follicle, also within the follicle between various cell types [7] .It is a reasonable thinking that some biochemical characteristics of the FF may play an important role for determining oocyte quality and the subsequent capability to achieve fertilization and embryo development [8]. Different hormones have been biosynthesized in the ovaries such as the activin A, follistatin [9], and fibrillin-3 [10].

Activin increase FSH secretion while follistatin which is an activin-binding protein, neutralizes activin bioactivity, thus it inhibit FSH secretion by blocking activin bioactivity. They exert their effect through local autocrine and paracrine effects on granulosa cells through action on specific receptors [11] .Fibrilline-3 is a glycoprotein located mainly in the brain, but also in the gonads [10]. The significant role of fibrillins has recently emerged in the control of growth factor signaling, fibrillins regulate transforming growth factor ß family (TGF-ß) bioactivity in tissues by binding latent TGF-ß binding proteins [12].

To the best of our knowledge there are no studies evaluating the possible effect of these three hormones collectively on the number and quality of oocytes and embryos in IVF program. Recently, the reproductive hormones, activin A, and Follistatin have been found to play an important role in folliculogenesis, oocyte maturation and corpus luteum function by changing the pattern of granulose cell expression which in turn affects the success of fertilization potential [1].

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Materials and Methods

A prospective study which involves two hundred infertile females selected from the Consultant Clinic in Kamal Al-Samaria IVF Center in Baghdad-Iraq and Janpalfijn IVF Center in Ghent -Belgium through the period from August 2014 to July 2015. The females' age ranged between 20-45 years. Exclusion criteria were patients with elevated FSH levels, polycystic ovary syndrome (PCOS), and those with endocrinological pathology such as elevated prolactin, thyroid dysfunction and diabetes. Two protocols were used for controlled ovarian hyper stimulation: The long protocol GnRH-a treatment and the short GnRH antagonist protocol.

The hCG (Ovitrelle®, Merck Serono -USP) 6500-13000 IU or (Pregnyl®, Merck Serono Company USP) 5,000–10,000 IU were given ~30 hours after the last FSH or HMG injection. Pregnyl would be administered if the following criteria were present: at least 3 follicles ≥17mm in diameter with 17ß estradiol levels of at least 3500 pmol/L (920 picogram/ml) (Morley et al., 2012).Then oocyte retrieval was carried out 34 - 36 hours after the hCG injection under local or general anesthesia or analgesia, oocytes were harvested by needle aspiration through the posterior fornix with TVU guidance.

Oocytes were assessed for their morphology and maturation after removal of the surrounding cumulus cells by 10% hyaluronidase enzyme 80 IU/ml in HEPES-buffered medium (Scandinavian IVF science, Gothenburg, Sweden) and repeatedly pipetted in hyaluronidase for a max of 15 sec. The normal MII oocyte showed a smooth surface round or ovoid first polar body within the perivitelline space, a clear cytoplasm with homogeneous fine granularity, and a colorless zona pellucid with regular shape [13].

Any abnormal MII oocyte weather cytoplasmic and/or extracytoplasmic abnormalities were recorded collectively. Fresh sperm were collected at time of oocytes pick up by masturbation, sperm aspiration from the testes by fine needle aspiration (FNA), or from testicular biopsy [14].Frozen sperm were used in special cases .ICSI was performed 3-5h after oocyte aspiration as described by [15]. Assessment of fertilization was done about 18 hour after ICSI .Then embryo quality was assessed by recording the size and number of blastomeres, defect in the cytoplasm and zona pellucida, and the fragmentation percentage [16].

High grade embryo G1 were those having all these characteristics: either 4-6 cells on day 2 or 8-10 cells on day 3 of development ,symmetrical blastomeres ,colorless cytoplasm with moderate granulation with no inclusions ,less than 15% fragmentation, clear perivitelline space, and absence of zona pellucida dysmorphology.

Low quality embryos are those lacking any one of these characteristics [17]. Embryo transfer was done on day 2 and 3 of embryonic development and rarely on blastocyst stage. Mostly two or three embryos were transferred depending on the law, recommendation of the couple and the quality of the embryos.

Support of the luteal phase was performed by injecting 1500 IU hCG immediately after oocyte retrieval and repeated seven days later for those who are not at risk of ovarian hyper stimulation syndrome (OHSS). In addition vaginal administration of 200mg of micronized progesterone, three times a day ,was started in the evening after oocyte pickup [18]. For each patient ,measurement of serum Activin A (pg/ml), Fibrillin-3 (ng/ml), and Follistatin (ng/ml) was done at the day of ovum pickup also the concentrations of those hormones were determined in follicular fluid after ovum pickup.

The samples were stored at -20º C. Then the levels of these hormones were measured using quantitative sandwich enzyme immunoassay technique as described by ELISA technical guide and protocol. The number of oocytes retrieved, the number of mature metaphase II oocyte, and the numbers of good quality embryos for each patient were recorded.

Statistical Analysis Method

The results of current work were analyzed using R project for statistical computing version 3.2.1 with R Studio interface.

Numeric variables were expressed as mean ±SE. Nominal variables were expressed as numbers and percentages. Comparison of variables was done using Fisher exact test and two sample t-tests with Satterthwaite approximation of the degrees of freedom which allows separates variance estimations per group. The non-parametric Spearman rank correlation coefficient was calculated for each combination of a hormone level (mid-cycle in serum or follicular fluid) and the oocyte, MII oocyte or embryo numbers [19].

Results

ICSI Outcome in Relation to the Type of COS Protocol, Oocytes and Embryos

The number and percentage of women who were pregnant or not is shown in table I in relation to stimulation protocol. Two types of COS protocols were used :the long GnRH-a used for 114 patients (57%) it resulted in a PR of 21.1%.The second protocol was the short GnRH-ant protocol ,used for 86 patients (43%) with a PR of 22.1%. There was no statistically significant association between stimulation protocol and pregnancy in this study, and the long and the short protocol resulted in very similar pregnancy frequencies (long: 21.1%, short: 22.1%).The average of sum of the PR in long and short ovulation induction protocols following ICSI cycle for all the patients in the study was 21.5%. Pregnancy rate in Janpalpijn IVF center was 40% while in Kamal Al-Samarei IVF center it was 19.4%. In table I, tabulated the number of total oocytes, MII oocytes, total embryos and embryos per grade was compared between the pregnant versus the non- pregnant women. There was a significant difference (p=0.045) between the MII oocytes of pregnant and non-pregnant women, with the pregnant women having a mean number of 7.07±0.6 MII oocytes and the non-pregnant women having a mean number of 5.68±0.33 MII oocytes.

There was a significant difference (p=0.041) between the G1 embryos of pregnant and non-pregnant women, with the pregnant women having a mean number of 2.07±0.22 G1 embryos and the non-pregnant women having a mean number of 1.56±0.11 G1 embryos. There were no significant differences between pregnant and non-pregnant women regarding the total number of oocytes (P=0.131), the total embryo numbers (P=0.190) and the G2 embryo numbers (P=0.099).

Table 1: Number of pregnant and non-pregnant women per ovulation stimulation protocol with the total oocytes, MII oocytes, total embryos, G1 and G2 embryos following ICSI procedure

Variable		Non-pregnant	Pregnant	Statistical Comparison Status
Ovarian Stimulation	Long	90/114 (78.9%)	24/114 (21.1%)	OR=1.063 (0.506, 2.213), p=0.864
	Short	67/86 (77.9%)	19/86 (22.1%)	
Total Oocytes		8.77 ±0.41	10.26±0.88	p=0.131
MII Oocytes		5.68±0.33	7.07±0.6	p=0.045
Total embryos		2.58±0.1	2.81±0.17	p=0.190
G1 embryos		1.56±0.11	2.07±0.22	p=0.041
G2 embryos		1.03±0.1	0.74±0.14	p=0.099

Values are expressed as mean + SE
Fisher exact test and Satterthwaite t-test were used

The Number of Oocytes Retrieved, the Number of Metaphase II Oocytes, and Quality of Embryo Transferred with the Pregnancy Rate and the Level of Each Hormone on the Day of Ovum Pick Up

In order to find the relation between, hormones levels at mid-cycle in serum and follicular fluid on one hand and on the other hand the number of oocytes, metaphase II oocytes, and embryos. The hormone levels together with the oocytes and embryo counts are shown in tables II and III per stimulation protocol and pregnancy status. Table II shows the total number of the retrieved oocytes, total MII, and good MII in relation to the mid cycle serum levels of activin-A, firillin-3, and follistatin hormones. The total oocytes number was higher in pregnant than non-pregnant women for both the long and short protocols .The number of total oocyte nearly similar in both protocols .While the total number of metaphase II oocytes and the morphologically good MII are higher in

pregnant than in non-pregnant women in both the short and long protocols which reached statistical significant with PR. The total number of embryos and G1 embryos are shown in table II with the levels of the three hormones in serum. The total and G1 embryos number are higher in pregnant than non-pregnant for both types of COS protocols. Table III shows the number of total oocytes retrieved, MII oocytes, good MII, total embryos and G1 embryos in relation to the concentration of each of the hormones in follicular fluid. The correlation of these variables with the hormone levels are discussed below.

The Correlation between the Oocytes, Embryos and Hormone Levels

In Table IV, overall the correlation coefficients between the hormones levels and the oocytes, MII oocytes and embryo numbers were quite low, with a maximum correlation coefficient of -0.29 (MII oocytes total versus follistatin level in follicular fluid).Most correlation coefficients were very close to zero, indicating no correlation. Both the mid-cycle level in serum and follicular fluid of activin-A show a negative correlation of around -0.2 with the total number of embryos and the number of G1 embryos. Increased activin-A was thus correlated with decreased total and G1 embryo numbers. The follicular fluid fibrillin-3 was positively correlated (r =0.24) with the G2 embryos numbers, and very little (r =0.16) correlated with the total embryo numbers. While the follicular fluid follistatin was negatively correlated with the total number of oocytes (r = -0.26) and the number of MII oocytes (r = -0.29).

Table II: The number of total oocyte, total metaphase II oocytes, good metaphase II oocytes retrieved, total embryos, grade 1 embryos, and the mid-cycle hormones levels shown per stimulation method and pregnancy status

Ovarian stimulation method	Pregnancy state	Total oocytes retrieved	Total metaphase II oocytes	Good metaphase II oocytes	Total embryos	Grade 1 Embryos	Mid-cycle Activin-A	Mid-cycle Fibrillin-3	Mid-cycle Follistatin
Long	Non Pregnant	8.9 (0.54)	5.61 (0.4)	3.73 (0.42)	2.6 (0.13)	1.88 (0.15)	11.99 (1.72)	0.59 (0.05)	0.72 (0.07)
	Pregnant	10.04(0.98)	7.08 (0.86)	5.33 (1.02)	3.08 (0.22)	2.25 (0.29)	21.25 (4.48)	0.49 (0.06)	0.67 (0.15)
Short	Non pregnant	8.6 (0.63)	5.77 (0.56)	4.89 (0.59)	2.54 (0.16)	1.14 (0.15)	39.5 (6.54)	0.57 (0.05)	0.79 (0.09)
	Pregnant	10.53(1.58)	7.05 (0.83)	6.37 (0.85)	2.47 (0.23)	1.84 (0.34)	32.99 (11.38)	0.7 (0.11)	0.44 (0.08)

Table III: The number of total oocytes, total metaphase II oocytes, good metaphase II oocytes retrieved, total embryos and grade 1 embryos, the follicular fluid hormone levels shown per stimulation method and pregnancy status

Ovarian stimulation method	Pregnancy state	Total oocytes retrieved	Total metaphase II oocytes	Good metaphase II oocytes	Total embryos	Grade 1 Embryos	Activin A follicular fluid	Fibrillin-3 follicular fluid	Follistatin follicular fluid
Long	Non pregnant	8.9 (0.54)	5.61 (0.4)	3.73 (0.42)	2.6 (0.13)	1.88 (0.15)	24.88 (2.81)	4.17 (0.48)	8.26 (1.38)
	Pregnant	10.04(0.98)	7.08 (0.86)	5.33 (1.02)	3.08 (0.22)	2.25 (0.29)	42.68 (8.84)	4.64 (0.8)	3.68 (1.14)
Short	Non Pregnant	8.6 (0.63)	5.77 (0.56)	4.89 (0.59)	2.54 (0.16)	1.14 (0.15)	46.91 (7.75)	2.93 (0.42)	11.02 (1.83
	Pregnant	10.53(1.58)	7.05 (0.83)	6.37 (0.85)	2.47 (0.23)	1.84 (0.34)	35.23 (8.94)	1.91 (0.32)	8.05 (1.89)

Table IV: Spearman rank correlation coefficients for the relation of the hormone levels and the oocyte, MII oocyte and embryo numbers

	Oocytes total	MII Oocytes	MII Oocytes good	Total embryos	G1 embryos	G2 embryos	Mid-cycle Activin-A	Mid-cycle Fibrillin-3	Mid-cycle Follistatin	Activin A follicular fluid	Fibrillin follicular fluid	Follistatin follicular fluid
Oocytes total		0.80	0.56	0.38	0.27	-0.02	-0.03	-0.08	0.01	-0.12	-0.08	0.26
MII Oocytes	0.80		0.67	0.51	0.32	0.06	-0.01	-0.04	0.00	-0.06	-0.09	0.29
MII Oocytes good	0.56	0.67		0.30	0.19	0.04	0.09	0.03	0.04	0.06	-0.02	0.16
Total embryos	0.38	0.51	0.30		0.62	0.21	-0.26	-0.13	0.07	-0.19	-0.16	0.06
G1 embryos	0.27	0.32	0.19	0.62		-0.60	-0.25	-0.03	0.04	-0.23	-0.04	-0.06
G2 embryos	-0.02	0.06	0.04	0.21	-0.60		0.03	-0.08	0.05	0.07	0.24	0.03
Mid-cycle Activin-A	-0.03	-0.01	0.09	-0.26	-0.25	0.03		-0.04	-0.21	0.52	-0.25	0.29
Mid-cycle Fibrillin-3	-0.08	-0.04	0.03	-0.13	-0.03	-0.08	-0.04		-0.07	0.03	-0.17	0.02
Mid-cycle Follistatin	0.01	0.00	0.04	0.07	0.04	0.05	-0.21	-0.07		-0.19	-0.38	0.61
Activin A follicular fluid	-0.12	-0.06	0.06	-0.19	-0.23	0.07	0.52	0.03	-0.19		-0.20	0.31
Fibrillin follicular fluid	0.08	0.09	0.02	0.16	-0.04	0.24	0.25	-0.17	-0.38	0.20		0.45
Follistatin follicular fluid	-0.26	-0.29	-0.16	-0.06	-0.06	0.03	-0.29	0.02	0.61	-0.31	-0.45	

The Correlations between the Parameters using Linear Regression

In table V, the variables total oocytes, MII oocytes, good MII oocytes, total embryos and G1 embryos have a significant p-value indicating a significant linear regression slope and thus a significant relation between these variables. Follistatin in follicular fluid is the only hormone level which has a significant p-value in the linear regression.

It has a significant slope in the linear regression with total oocytes, MII oocytes and good MII oocytes. The mid-cycle serum follistatin was not significant in the linear regression with total oocytes, MII oocytes and good MII oocytes. Thus in the given data set the mid-cycle and follicular fluid hormone levels (except follistatin follicular fluid) appear not to be good predictors in a linear regression of the total oocytes , MII oocytes (good), total embryos and grades of embryos. The mid-cycle activin A has a significant linear slope in regression versus activin a follicular fluid and follistatin follicular fluid levels. The mid-cycle follistatin has a significant linear slope in regression versus fibrillin-3 follicular fluid and follistatin follicular fluid levels.

The mid-cycle activin a follicular fluid has a significant linear slope in regression versus fibrillin-3 follicular fluid and follistatin follicular fluid levels. And fibrillin-3 follicular fluid has a significant linear slope in regression versus follistatin follicular fluid levels. In Table VI the relation between the mid-cycle and follicular fluid hormone levels is further assessed separately per stimulation and pregnancy state.

Table V: A matrix showing the p-value of the linear regression for any pair of variables. The linear regression has an intercept and a slope. The p-value of the slope is shown in the table

	Oocytes total	MII Oocytes	MII Oocytes good	Total embryos	G1 embryos	G2 embryos	Mid-cycle Activin-A	Mid-cycle Fibrillin-3	Mid-cycle Follistatin	Activin A follicular fluid	Fibrillin follicular fluid	Follistatin follicular fluid
Total Oocytes		0.000	0.000	0.000	0.009	0.379	0.958	0.262	0.830	0.696	0.200	0.023
MII Oocytes	0.000		0.000	0.000	0.001	0.131	0.587	0.281	0.986	0.980	0.292	0.012
MII Oocytes good	0.000	0.000		0.000	0.033	0.307	0.071	0.829	0.707	0.416	0.973	0.043

Total embryos	0.000	0.000	0.000		0.000	0.000	0.062	0.203	0.501	0.541	0.118	0.827
G1 embryos	0.009	0.001	0.033	0.000		0.000	0.078	0.873	0.829	0.201	0.840	0.838
G2 embryos	0.379	0.131	0.307	0.000	0.000		0.917	0.248	0.672	0.430	0.161	0.873
Mid-cycle Activin-A	0.958	0.587	0.071	0.062	0.078	0.917		0.081	0.313	0.000	0.416	0.025
Mid-cycle Fibrillin-3	0.262	0.281	0.829	0.203	0.873	0.248	0.081		0.673	0.899	0.080	0.636
Mid-cycle Follistatin	0.830	0.986	0.707	0.501	0.829	0.672	0.313	0.673		0.253	0.000	0.003
Activin A follicular fluid	0.696	0.980	0.416	0.541	0.201	0.430	0.000	0.899	0.253		0.044	0.001
Fibrillin follicular fluid	0.200	0.292	0.973	0.118	0.840	0.161	0.416	0.080	0.000	0.044		0.000
Follistatin follicular fluid	0.023	0.012	0.043	0.827	0.838	0.873	0.025	0.636	0.003	0.001	0.000	

Significant p-values ($p < 0.05$) are shown in red

Correlation Serum Level and Follicular Fluid Level for Each Hormone in Mid-cycle Phase for the Pregnant and Non-pregnant for Both Short and Long Protocols

In Table VI the Spearman rank correlation was provided between the mid-cycle phase and the follicular fluid hormone levels per combination of stimulation protocol and pregnancy status. In the correlation there seems to be two emerging patterns in the correlation of a hormone in the serum (mid-cycle) and the same hormone in the follicular fluid. For both activin A and fibrilin-3, there is a high or relatively high correlation within the pregnant subject and low till very low correlation in the non-pregnant subjects, independent of the stimulation protocol. A different pattern was observed for follistatin when the stimulation pattern does affect the correlation between serum and follicular fluid levels, regardless of the pregnancy status. The follistatin levels have a relatively high correlation of around 0.67 in the long stimulation and a low correlation of around 0.3 in the short stimulation subjects.

Table VI: Correlation (Spearman rank) between the mid-cycle phase and the follicular fluid hormone levels provided per combination of stimulation and pregnancy status

Stimulation	Pregnancy status	Hormone	Activin A follicular fluid	Fibrillin follicular fluid	Follistatin follicular fluid
Long GnRH agonist	Pregnant	Mid-cycle Activin-A	0.80	0.47	-0.45
		Mid-cycle Fibrillin-3	-0.26	-0.60	0.48
		Mid-cycle Follistatin	-0.30	-0.73	0.67
	Non-pregnant	Mid-cycle Activin-A	0.15	0.37	-0.33
		Mid-cycle Fibrillin-3	-0.14	-0.04	0.00
		Mid-cycle Follistatin	-0.23	-0.50	0.68
Short GnRH agonist	Pregnant	Mid-cycle Activin-A	0.76	0.41	-0.69
		Mid-cycle Fibrillin-3	0.35	0.50	-0.41
		Mid-cycle Follistatin	-0.16	-0.04	0.30
	Non-pregnant	Mid-cycle Activin-A	0.50	0.00	-0.50
		Mid-cycle Fibrillin-3	0.29	-0.09	-0.23
		Mid-cycle Follistatin	-0.20	0.07	0.27

Discussion

The result of present study clearly indicated that total oocytes number was higher in pregnant than non-pregnant women for both the long and short protocols. However it was not reached the statistical significant difference. While the total number of metaphase II oocytes and good MII oocytes were significantly higher in pregnant than in non-pregnant women in both the short and long protocols. The same observation was reported by [20]. Other study [21] showed a strong relationship between the number of egg and the live birth rate in fresh ICSI cycle which rose with increasing number of eggs up to ~15, plateaued between 15-20, and steadily declined beyond 20 eggs. The data of this work found that low in oocytes number coupled with high gonadotrophin dose in conventional ovarian stimulation method can employ lower oocyte quality.

Furthermore, endometrial quality can also be hampered in high-dose stimulation protocols. As the granulosa cells in antral follicle stimulated by FSH increasingly express inhibin, a physiological antagonist of activin [22].This might have a deleterious effect on the maturation of the oocyte. Thus milder form of stimulation resulted in a better oocytes quality, and embryos with lower incidence of chromosomal aneuploidies although it result in lower oocytes number [23].But on the other hand ,higher number of oocytes simply allows for better selection of quality embryos from a larger cohort of available embryos [24].

This study showed significant difference between the G1 embryos of pregnant and non-pregnant women in this study. But the total embryo numbers and the G2 embryo numbers were not statistically different. Embryo morphology is one of the factors that may influence the outcomes of ICSI treatment which is the most common and useful tool in selecting the best embryo for transfer. The decision of embryo selection depends on useful tools such as pronuclear morphology, early cleavage, blastomere morphology, and blastocyst grading. It has been shown that the embryo characteristic was an independent predictor of live birth in ICSI cycles [25].

Other studies showed a strong association between embryo quality from one side and implantation and live birth rates from the other side ,both with cleavage and blastocyst embryos [24,26]. The other factor that may interfere with PR was the program of ovarian stimulation that done to improve the ICSI outcome which resulted in retrieving multiple oocytes, but at the same time it affect oocyte and embryo quality ,luteal phase endocrinology ,and endometrial receptivity [26].

As the use of GnRH-Ag in the COS protocol may affect the process of follicular development, luteinization, steroidogenesis, and apoptosis in the ovary, thus it in turn affect the number and quality of oocytes [27]. It has been emphasized that environmental factor may also influence the outcome of IVF programs e.g. smoking and obesity have a deleterious effect on oocyte development due

to the increased oxidative stress in the oocyte microenvironment [28]. In addition to the mentioned factors, abnormal sperm DNA is a possible cause of poor quality embryos obtained after ICSI besides the laboratory incubation environment and techniques [29].

The levels of activin A, follistatin and fibrillin-3 in FF are far exceeding those in serum; this is due to the physiological production of these hormones in the ovarian tissue first and then can be measured through the blood serum. This study showed that follistatin in FF has a significant negative correlation with total oocytes, MII oocytes and good MII oocytes.

Thus follistatin concentration in FF could be used as a predictor for successful ICSI outcome. This observation can be explained by the direct action of follistatin on activins as the granulosa cells in antral follicles and luteinized granulosa cells are the main sites that express follistatin mRNA, whereas other structures, such as the theca cells, stroma and oocytes, appear to be devoid of follistatin mRNA. Thus the role of the follistatin in ovarian function was related to the manner by which its function through interactions with activins [30] because activin A is a promoter of oocyte maturation in vitro [31].

The activin a concentration in serum and FF at mid-cycle in the present study revealed a negative correlation with the total number of embryos and the number of G1 embryos. Increased activin A is correlated with decreased total and G1 embryo numbers. Researchers found that activin A during the later stage of embryo development ,but not the early cleavage stage can enhance development of embryo by increasing hatching rates and affecting expression levels of genes related to hatching and implantation [32].

In another study has been found that both activin A and follistatin were elevated controversy in the FF of older women suggests that elevated follistatin production compensates for the increased FF levels of activin A, thus potentially conferring a protective effect against activin [33]. In this study for both activin A and fibrilin-3, there is a high or relatively high correlation within the pregnant subject and low till very low correlation in the non-pregnant subjects, independent of the stimulation protocol. And activin A follicular fluid has a significant correlation with fibrillin-3 follicular fluid and follistatin follicular fluid levels.

This may indicate a role of these hormones on the endometrium receptivity and implantation rate as the follicular fluid fibrillin-3 was positively correlated with the G2 embryos numbers, and very little correlated with the total embryo numbers .But also may have a role on advanced stage in oocyte maturation and embryo development. So far, no other studies have particularly investigated the correlation between all these hormones collectively in ICSI cycle to compare these results.

It has been recorded that fibrillins perform regulatory functions by binding and sequestering growth factors [10].Because many members of the TGF-ß super family are involved in the development and function of the ovary [34]. Thus both hormones may facilitate the mechanism of implantation through preparing the factors involved in uterine receptivity. Whereas the low concentration of activin A at the mid-cycle with higher amount at follicular fluid with follistatin follicular fluid contributed to the neutralization action of follistatin on activin in distant target tissue.

This data showed mid-cycle serum follistatin has a significant correlation with fibrillin-3 follicular fluid and follistatin follicular fluid levels. And fibrillin-3 follicular fluid has a significant linear correlation with follistatin follicular fluid levels. Interestingly both fibrillin-3 and follistatin share common TGF-ß binding domains and both FBN-3 and FS regulate the activity of members of the TGF-ß super family [35].

A different pattern was observed for follistatin when the stimulation pattern does affect the correlation between serum and follicular fluid levels, regardless of the pregnancy status this might be due to the effect of the pattern of pituitary suppression in the two protocols. The supra-physiological levels of FSH in COS and the pituitary suppression lead to change in the local hormonal milieu in the ovary which affects the number and quality of the oocytes and embryos and the implantation rates which in turn affect the PR .Thus the use of milder form of ovarian stimulation may improve the IVF outcome.

Acknowledgments

The authors are grateful to Professor Dr. Nabeel Kadhim Abdul-Sahib the President of Al-Nahrain University for his financial support and the staff of Janpalfijn IVF Center in Ghent-Belgium to facilitate all the requirements to complete this work. A special thanks to Mr. W. Wouter for doing the statistical analysis.

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