

Concentration of R-spondin 2 in the Follicular Fluid is Correlated with Implantation Rate, Estrogen and Amphiregulin, in Iraqi Women Undergo ICSI

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Abstract: ***Objective:** to find out if there is any correlation between the concentration of R - spondin2, Amphiregulin, bone morphogenic protein - 15 in serum or follicular fluid, and serum estrogen before hCG administration (E2 before ICSI) and to investigate if there is a role of R - spondin2 in implantation rate of women who undergo ICSI **Methods:** A prospective study was conducted between the first of August 2015 to the third of April 2016, E2 was measured before hCG administration by vidas instrument using estrogen biomerieux kits for 45 women age ranged 18 - 42 years who will undergo ICSI and match the inclusion criteria, follicular fluid and serum were collected from those patients in the day of oocyte retrieval and then the concentration of R-spondin - 2, Amphiregulin, and bone morphogenic protein - 15 in follicular fluid and serum were measured using ELISA kit. **Results:** the concentration of F. R - spondin 2, S. R - spondin 2 and F. AREG correlated positively with the concentration of E2 before ICS. The concentration of F. R - spondin 2, and F. AREG and E2 before ICSI. correlated positively with implantation rate. there was no difference in the SEM of the concentration of F. R - spondin 2, S. R - spondin 2 and F. AREG, S. AREG, F. BMP - 15, S. BMP - 15 between pregnant and non - pregnant women. **Conclusion:** data of the present study show that R - spondin2 directly affected by the concentration of E2 and has a positive role in implantation rate that may forbid the early miscarriages after embryo transfer*

Keywords: R-spondin - 2, follicle development, Amphiregulin, Bone morphogenic protein - 15, ICSI

1. Introduction

Assisted Reproductive Techniques are methods used to achieve pregnancy by artificial or partially artificial means and used primarily for infertility treatment, Intracytoplasmic Sperm Injection (ICSI) is a procedure in which a single sperm is injected directly into an egg cytoplasm. ⁽¹⁾ the inner environment of oocytes has been investigated by some studies and found that the paracrine factors that is secreted by the oocytes itself has an important role in regulation the maturation process and these factors mostly protein in nature and can be measured in the follicular fluid ⁽²⁾. R - spondin2 is one of the members of The R - spondin protein family which is described as a group of four distinct secreted proteins (RSpo1 - 4), and Their ligand - type activities mimic those of the canonical wingless ligand (Wnt) ⁽³⁾. R - spondin2 is highly expressed in the ovary , developed follicles and mature oocyte, treatment with R-spondin - 2 agonist promote the early follicle development in human from primary follicle to the secondary stage and the effect of R - spondin2 on follicle growth mimic the effect of FSH but in independent cellular pathway, studies demonstrate that ⁽⁴⁾: treatment with R - spondin - 2 could promote the development of human early follicles in patients with FSH low responders. Amphiregulin, is a protein that is a member of the epidermal growth factor family. ⁽⁵⁾ which interacts with the Epidermal growth factor receptor to promote the growth of normal epithelial cells ⁽⁶⁾. Estradiol and progesterone mostly induce amphiregulin expression to mediate ductal development of the mammary glands and

play an essential role for mammary ductal development ⁽⁷⁾. There is important role of Amphiregulin in oocyte maturation ⁽⁸⁾; Luteinizing hormone stimulates ovarian somatic cells to induce hormone release for oocyte maturation, Since the ovulation occurred under the influence of LH hormone by acting on its target cells (somatic granulosa and theca cell) to induce hormone released for oocyte maturation, the oocyte itself lack LH receptors, studies show that there is compensation mechanism promote the oocyte to secret some factors affecting maturation including Amgpiregulin ⁽⁹⁾. Bone morphogenetic protein - 15 is a protein member of the transforming growth factor - β superfamily, It is a paracrine signaling molecule exclusively expressed in the ovaries ⁽¹⁰⁾. and involved in folliculogenesis, oocyte and follicular development by regulation of the sensitivity of granulosa cells to follicle - stimulating hormone (FSH) action, the determination of the number of eggs that are ovulated, prevention of granulosa cell apoptosis ⁽¹¹⁾. Serum estrogen levels increase gradually during multiple follicular maturation, probably reflecting the number of developing follicles during controlled ovarian stimulation, it synthesis is directly related to follicular size ⁽¹²⁾, along with ultrasound, estrogen levels are used to determine the optimal timing for the administration of human chorionic gonadotropin hormone injections (hCG) to complete the oocyte maturation and trigger ovulation ⁽¹³⁾

2. Subject and Methods

A prospective study was conducted between the first of August 2015 to the third of April 2016. Over than three hundred women were interviewed, one hundred fifty were selected because they were matching the inclusion criteria, the others were rejected because they were match the exclusion criteria (mentioned below). Those women were interviewed using structural questionnaire to determine the following: history examination, type of infertility, duration of infertility, presence of other diseases, renal disease, thyroid disease, PCOS. Only Forty five women aged between (18 - 42) years undergoing IVF/ICSI treatment were selected and chosen for the final results because of the obstacles that occurred in this prospective study which was as follows:

- Contaminated F. F with blood no=60
- Women have hyper stimulation syndrome no=10
- Patient who is their husbands did not have sperms in the testicle biopsy
- No=10
- Patients did not cooperate and refused to participate in the study no=20
- Others no= 5

Inclusion criteria: Male factor infertility, Tubal –factor infertility, unexplained Infertility.

Exclusion criteria: Polycystic ovarian syndrome, Endometriosis, Diminished ovarian reserve, Patients with renal disease, Patients with thyroid disease (hypo, hyper). The concentration of estrogen E2 was measured in serum for each patient a day before the hCG injections administration to complete oocyte maturation and trigger ovulation (E2 before ICSI) using the vidas instrument (biomeroux kit), and The level of R - spondin2, Amphiregulin, and BMP - 15 were measured and evaluated in the serum and follicular fluid obtained from all women who participate in this study in the day of oocyte pickup using using an Enzyme Linked Immunosorbent Assay (ELISA) Kits. R - spondin2 concentration evaluated by using (human R - spondin - 2 ELISA kit; catalog number CSB - EL02055IHU, Cusabio, China), Amphiregulin using (human Amphiregulin ELISA kit; catalog number CSB - E04496h, Cusabio, China), Bone morphogenic protein - 15 by using (human BMP - 15 ELISA kit; catalog number MBS - 261936, MyBioSource, USA), using the device (Biotek ELISA 216360 USA). Patients were enrolled in this study were received two kinds of protocols of IVF/ICSI cycle: Long protocol , no=7 patients with Short protocol, no =38 patients. Implantation rate were measured using the formula:

Implantation rate= No. of gestational sac found × 100
The total number of embryo transferred

Standards curve was plotted for each of the parameters between absorbance (optical density) versus a prepared set of different standard concentration for each of the R - spondin - 2, Amphiregulin and BMP - 15 , the unknown concentration of patients' blood and follicular fluid is calculated. Data were summarized, presented and analyzed using statistical package for social sciences (SPSS) version

23. Numeric variables were expressed as mean + standard error (MSE), while nominal variables were expressed as number and percentage. Independent sample student t - test was used to compare mean of numeric variables between any two groups. **Pearson's correlation coefficient** was used to evaluate correlation between numeric variables. P - value was considered significant when it was equal or less than 0.05

3. Results

3.1 Correlations with E2 before ICSI in all patients

There is a significant positive correlation between the concentration of (F. R - spondin2), (S. R - spondin2), and (F. Amphiregulin) with S. E2 before ICSI (r=0.309, p=0.039) (r=0.409, p=0.05). (r=0.383, p=0.009) respectively. No correlation were found between S. Amphiregulin, (S. BMP - 15), (F. BMP - 15) and E2 before ICSI (p>0.05). Table (1), figure (1), (2) and (3)

Table 1: Correlation of some variables with E2 before ICSI in all study cases

Parameter	S. E2 before ICSI		interpretation
	r	p	
S. R - spondin2 (ng/ml)	0.409	0.005	significant
F. R - spondin2 (ng/ml)	0.309	0.039	significant
S. Amphiregulin (pg/ml)	0.074	0.629	Not significant
F. Amphiregulin (pg/ml)	0.383	0.009	significant
S. BMP - 15 (ng/ml)	- 0.220	0.147	Not significant
F. BMP - 15 (ng/ml)	- 0.184	0.226	Not significant

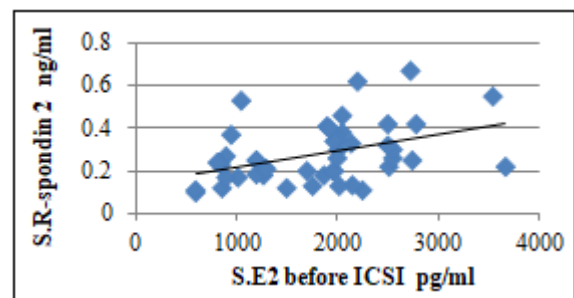


Figure 1: Positive correlation between S. R - spondin2 and S. E2 before ICSI

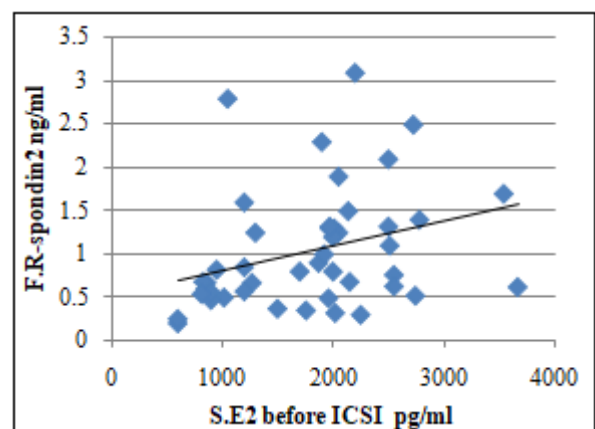


Figure 2: Positive correlation between F. R - spondin2 and S. E2 before ICSI

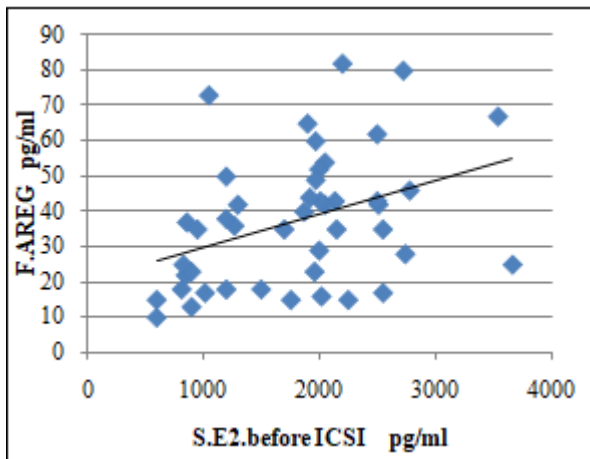


Figure 3: Positive correlation between F. AREG and S. E2 before ICSI

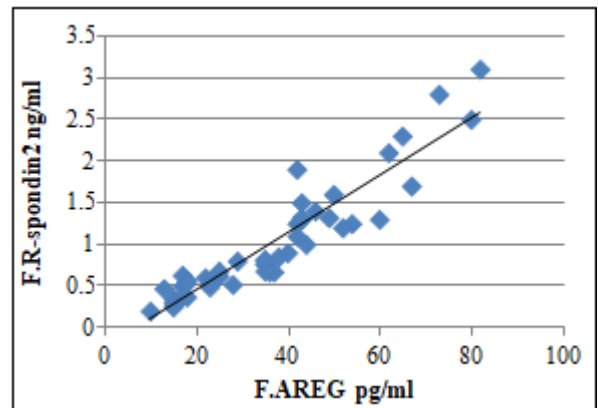


Figure 5: Positive correlation between F. R - spondin 2 and F. AREG

3.1 Correlations of parameters with each others in all patients

S. R - spondin 2 , FF Amphiregulin ($r=0.811$, $p= 0.000$), F. R - spondin 2 positively correlate with F AREG ($r=0.930$, $p= 0.000$). Figure (4) and Figure (5).

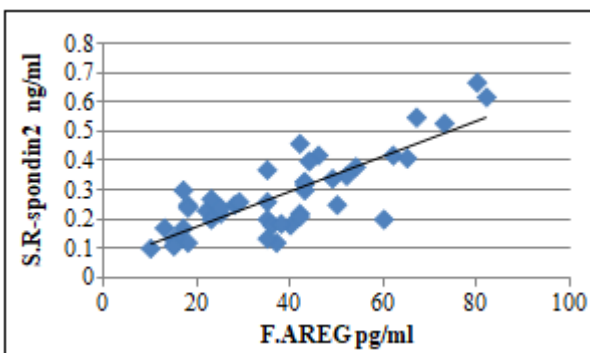


Figure 4: Positive correlation between S. R - spondin 2 and F. AREG

3.2 Correlations of the measured parameters with implantation rate in all patients

There is a significant positive correlation between the concentration of (F. R - spondin2), and (F. Amphiregulin), with implantation rate ($r=0.360$, $p\leq 0.05$), ($r=0.391$, $p\leq 0.05$) respectively. No correlation were found between S. R - spondin 2, S. AREG, S. BMP - 15, F. BMP - 15 and implantation rate ($p>0.05$). Table (2)

Table 2: Correlation of the parameters with implantation rate in all study cases

Parameter	Implantation rate		Interpretation
	r	p	
S. R - spondin2 (ng/ml)	0.226	0.136	Not significant
F. R - spondin2 (ng/ml)	0.360	0.015	Significant
S. Amphiregulin (pg/ml)	- 0.047	0.762	Not significant
F. Amphiregulin (pg/ml)	0.391	0.008	Significant
S. BMP - 15 (ng/ml)	- 0.061	0.688	Not significant
F. BMP - 15 (ng/ml)	- 0.011	0.945	Not significant
S. E ₂ before ICSI (pg/ml)	0.7	0.02	Significant

3.3 Comparison of mean follicular and serum parameters measured in this study between pregnant and non pregnant women

In the current study, a comparison between the mean of each parameter between pregnant and non pregnant as showed in the table (3). No significant difference between pregnant and non pregnant group were found ($p>0.05$).

Table 3: Comparison of mean follicular and serum parameters measured in this study between pregnant and non pregnant group

Parameter	In all patients N=45 Mean±SE	Non - pregnant N=33 Mean±SE	Pregnant N=12 Mean±SE	P value	Interpretation
S. R - spondin2 (ng/ml)	0.28±0.02	0.27±0.02	0.3±0.04	0.513	Not significant
F. R - spondin2 (ng/ml)	1.04±0.1	0.97±0.12	1.23±0.2	0.283	Not significant
S. Amphiregulin (pg/ml)	6.08±0.19	6.06±0.19	6.12±0.5	0.918	Not significant
F. Amphiregulin (pg/ml)	37.27±2.78	35.52±3.24	42.08±5.38	0.308	Not significant
S. BMP - 15 (pg/ml)	55.18±18.72	60.7±25.53	40.0±3.86	0.428	Not significant
F. BMP - 15 (pg/ml)	112.85±24.3	117.99±32.95	98.72±12.15	0.586	Not significant

4. Discussion

4.1 Parameters estimation

The ovarian follicle is the functional unit of the ovary, in which the somatic components (thecal and granulosa cells),

and germ (oocyte) are closely related and interdependent⁽¹⁴⁾. The complete development of the oocyte within the follicular structure requires continuous two - way communication between the oocyte and cumulus cells that surround it (cumulus - oocyte complex), as well as other

somatic cells included in the follicle, such as theca and the granulosa cells⁽¹⁵⁾. The functionality and action of these cells are dependent on some factors derived from the oocyte, capable of acting directly in the coordinated processes of follicular maturation through a paracrine signaling⁽¹⁶⁾.

In the current study; the concentration of R - spondin 2, AREG, BMP - 15, is measured as a protein in serum and follicular fluid of women undergone ICSI procedure were measured. There was a significant correlation between R - spondin2, AREG, and E2 before ICSI, implantation rate. BMP - 15 did not show any correlation correlated. In previous study Concentration of R - spondin 2 in the follicular fluid is correlated with oocyte number and metaphase II oocytes women undergo ICSI⁽¹⁷⁾.

Other study⁽⁴⁾ found that the cultured ovarian explants from prepubertal mice containing preantral follicles treated with R - spondin2 the primary follicles developed to the secondary stage in effect similar to follicle stimulating hormone, while R - spondin agonist treatment of immune - deficient mice grafted with human cortical fragments stimulated the development of primary follicles to the secondary stage, while neutralized endogenous R - spondin2 in some ovarian explants when incubated with affinity - purified R - spondin2 antibodies lead to decreased basal ovarian weights, suggesting that the oocyte - derived R - spondin2 is a paracrine factor essential for primary follicle development, and R - spondin agonists could provide a new treatment regimen for infertile women with low responses to the traditional gonadotropin therapy (4).

In the current study Amphiregulin (AREG) correlated positively with S. E2 before ICSI. Some studies measured the concentration of AREG in the follicular fluid and found the level of AREG is increased in the follicular fluid and associated with human oocyte maturation suggesting an important role in optimal human oocyte maturation⁽¹⁸⁾.

Other study suggested that the oocyte competence (defined as the ability of the oocyte to complete maturation, undergo successful fertilization and reach the blastocyst stage) is linked to granulosa cell AREG secretion and the levels of AREG in follicular fluid reflects the mode of triggering ovulation.⁽¹⁹⁾

Other study found that the supplementation of the maturation medium, with AREG significantly improves the maturation rate, fertilization rate, and pregnancy rate of human GV oocytes in vitro.⁽²⁰⁾

Bone morphogenetic protein 15 (BMP15), have been implicated as essential for follicular development, some studies found that high level of FF BMP - 15 has a potential role of in the prediction of the IVF outcome where⁽²¹⁾. Another study found that the BMP - 15 level in FF appears to be a potential factor in predicting oocyte quality and subsequent embryo development⁽²²⁾ in the current study there is no correlation between bone morphogenetic protein 15 with E2 before ICSI or implantation rate.

4.3 AREG, BMP15 and Steroidogenesis

The current study found that there is a significant positive correlation between the concentration of R - spondin2 in the follicular fluid and serum with S. E2 before ICSI, and there is a significant positive correlation between the concentration of Amphiregulin in the follicular fluid with E2 before ICSI. There is no correlation between the concentration of BMP - 15 in the follicular fluid and serum with serum E2 before ICSI ($p > 0.05$).

In the current study: a significant positive correlations between follicular R - spondin 2, F. AREG, and implantation rate while BMP - 15 did not correlated with implantation rate. Studies show that there is certain mechanism that leads to production of steroids from granulosa cell, in animal show that AREG is involved in steroidogenesis in the ovary of mouse under the stimulatory effect of LH, but not in the testis⁽²³⁾. Steroidogenesis is a complex process that involves multiple enzymatic reactions and in the ovaries, progesterone is synthesized from cholesterol in the mitochondria of granulosa cells; Once free cholesterol has been transported to the mitochondria, it is transported from the outer to the inner mitochondrial membrane by the Steroidogenic Acute Regulatory protein (StAR) which is well recognized as the key regulatory protein involved in the rate - limiting step of steroidogenesis, Cholesterol is catalyzed to pregnenolone by the cholesterol side - chain cleavage enzyme complex at the inner mitochondrial membrane; Pregnenolone is then transferred to the cytoplasm and catalyzed to progesterone by 3 β - hydroxysteroid dehydrogenase⁽²⁴⁾.

Another study demonstrate that AREG mediated by hCG induce StAR expression and progesterone production in human granulosa cells, providing a novel evidence for the role of AREG in the regulation of steroidogenesis and progesterone production in human granulosa cells⁽²⁵⁾, while BMP15 Suppresses Progesterone Production by Down - Regulating StAR in Human Granulosa Cells⁽²⁶⁾, suggest that oocyte may play a critical role in the regulation of progesterone to prevent premature luteinization during the late stage of follicle development.

In humans, the LH surge stimulates multiple intra - follicular activities and triggers ovulation⁽²⁷⁾. At the time of ovulation, a series of morphological transitions and tissue remodeling cause the ruptured ovarian follicle to develop into the corpus luteum, a temporary endocrine structure that secretes progesterone⁽²⁸⁾. The release of progesterone targets and prepares the reproductive tract for initiation of fertilization and maintenance of early pregnancy⁽²⁹⁾. Premature luteinization refers to an elevation of serum progesterone levels on (or before) the day of hCG administration in patients undergoing controlled ovarian stimulation⁽³⁰⁾. The premature rise of progesterone can shift the implantation window (synchronization between embryonic development and endometrial receptivity), which may hamper embryo implantation and decrease the pregnancy rate⁽³¹⁾. During the antral follicle stage, one of the most important physiological functions is the prevention of premature luteinization, which maintains follicular growth and somatic cell proliferation⁽³²⁾. Progesterone and estrogen synthesis is very important

factor affecting implantation rate, pregnancy rate and successful rate reflected by live birth, in the current study the correlation between R - spondin2, AREG and implantation and E2 before ICSI may be explained by its tight connection by steroidogenesis which is a prerequisite for endometrial development and embryo implantation. Adequate progesterone exposure in addition to preceding estrogen priming is essential for transformation of the endometrium to a receptive phase, In fertility proven women, endogenous production of progesterone from the corpus luteum is sufficient to support the embryo implantation in a natural ovulatory cycle However, the women undergoing ET are often subfertile or infertile, and their corpus luteal progesterone production may be insufficient during their natural cycles. Inadequate progesterone production during the luteal phase or early pregnancy period can result in implantation failure or spontaneous abortion, Therefore, luteal phase progesterone supplementation may be needed if frozen embryo is transferred during a natural cycle to an infertile women⁽³³⁾

5. Conclusion

R -spondin 2 and correlated positively with implantation rate, Serum Estrogen level and AREG in what it seems to be a cooperative manner and suggesting a positive role in uterine receptivity of embryo, maybe by affecting the estrogen and progesterone level, leading to forbid the early miscarriages after embryo transfer , while estrogen may play an important role in the synthesis of R - spondin 2 and regulate it receptors, however extensive future studies should be done for definitive understanding of the exact regulatory mechanism that lead to a deeper explanation for the correlation between both estrogen and AREG with R - spondin 2.

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