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What is the impact of serum and follicular fluid BMP-15 and AMH levels in ICSI-ET cycle outcomes?

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Abstract

Objective: Bone morphogenetic protein-15 (BMP-15) and anti-Müllerian hormone (AMH) are the proteins functioning during ovarian follicular growth. We aimed to investigate the impact of serum and follicular fluid (FF) AMH and BMP-15 levels on outcomes of intracytoplasmic sperm injection- embryo transfer (ICSI-ET) cycles.

Methods: This prospective, cross-sectional study was carried out with 82 patients attending ICSI-ET cycle. The women divided into two groups according to using either GnRH agonist (group1, N=38) or antagonist (group2, N=44) for pituitary down-regulation. Eight milliliter of serum samples were taken from the patients on the 3rd day of menstrual period (D3) and on the day of oocyte pick-up (OPU); and 8 mL of follicular fluid (FF) sample was collected during OPU. AMH and BMP-15 measurements were carried with ELISA method quantitatively.

Results: The mean age and body mass index of population were 30.6 ± 5.2 years and 25.5±4.2 kg/m^2 respectively. The comparison of pregnancy rates between women according to having FF AMH (35% vs 42%) and FF BMP-15 (37.8% vs 40.5%) levels under and above of the mean values revealed no significant difference. Serum AMH levels showed negative correlation with fertilization rate (FR). D3 BMP-15 level showed positive correlation with progesterone level and endometrial thickness on the day of hCG injection.

Conclusions: AMH and BMP-15 monitoring during ICSI-ET cycles for predicting cycle outcomes should be evaluated with further studies.

Introduction

Bone morphogenetic protein-15 (BMP-15) and anti-Müllerian hormone (AMH) are the members of transforming growth factor-beta superfamily. BMP-15 and its receptor are widely expressed in the mammalian female reproductive system especially in oocytes [1]. BMP-15 plays role on mammalian oocyte maturation by cumulus oophorus complex expansion [2,3]. AMH is produced by the ovarian granulosa cells and supports the follicular growth [4]. Effect of AMH and BMP-15 on estradiol and progesteron production in primary-cultured human luteinizing granulosa cells was reported [5]. BMP-15 level in follicular fluid has been demonstrated to associate with oocyte and embryo quality [6]. Researchers observed relation among cumulus cell BMP15 expression level and oocyte maturation, fertilization, and embryonic development [7]. And also follicular fluid AMH levels showed correlation with ovarian stimulation parameters and qualities of oocyte and embryo [8,9]. Researchers suggested that AMH could be a marker in the pre-treatment period for live birth chance in women with decreased ovarian reserve [4]. In this study we aimed to investigate 1-) the possible relation between follicular fluid and serum AMH and BMP-15 levels and intracytoplasmic sperm injection-embryo transfer (ICSI-ET) cycle outcomes, and 2-) the possible difference for fluid and serum AMH and BMP-15 levels according to using either gonadotropin-releasing hormone (GnRH) agonist or antagonist.

Materials and methods

This prospective, cross-sectional study was conducted with 82 women attending controlled ovarian hyperstimulation (COH)-ICSI-ET cycle in In Vitro Fertilization (IVF) Unit of Firat University Hospital between June 2012 and June 2013 after approval of local ethical committee. The women divided into two groups according to using either GnRH agonist (group1, N=38) or antagonist (group2, N=44) for pituitary down-regulation. The exclusion criterias were as follows: age >39 years, gynaecological malignancy diagnosis during evaluation, absence of sperm after testicular sperm extraction.

Controlled Ovarian Hyperstimulation and Embryo Transfer

Gynecological examination, antral follicle count, FSH-LH-estradiol-TSH-prolactin level evaluation on day 3 (D3) of menstrual period, hysterosalpingography/hysteroscopy and semen analysis were performed.

In group1; GnRH agonist leuprolide acetate (Lucrin daily®, Abbvie, Istanbul, Turkey) was started on day 21 of previous cycle and proceeded to the day of human chorionic gonadotropin (hCG) injection according to long luteal protocol (0.1 mg/day, subcutaneous from day 21 and reduction to 0.05 mg/day on stimulation). In group2;

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GnRH-antagonist ganirelix acetate (Orgalutran®, Organon, Istanbul, Turkey) or cetrorelix acetate (Cetrotide®, Merck Serono, Istanbul, Turkey) was initiated 0,25 mg daily when one of the following criteria was met: presence of at least one leading follicle >12 mm, or estradiol level exceeding 300 pg/mL. GnRH antagonist was continued to the day of hCG injection. Gonadotropin stimulation was started as 150-225 IU for normoresponders and 375-450 IU for poor responders according to each patient's basal antral follicle count, day-3 hormonal status, body mass index (BMI), and prior response. Either recombinant FSH (Puregon®, Organon, Istanbul, Turkey or Gonal-F®, Merck Serono, Istanbul, Turkey), or human menopausal gonadotropin (hMG, Merional®, IBSA Pharmaceuticals, Istanbul, Turkey), or usually a combination of two were used together.

Ovarian response was monitored by transvaginal ultrasound and serum levels of estradiol measurements. When at least three follicles with a mean diameter exceeding 17 mm were measured, 250 mcg recombinant hCG (Ovitrelle®, Merck Serono, Istanbul, Turkey) was administered. Oocytes were picked up (OPU) by transvaginal ultrasound-guided follicular puncture 35 to 36 hours after hCG administration. Embryo transfer (ET) was performed on day 3 or 5 by using a soft catheter (Wallace, Smiths Medical, Kent, UK) under guidance of transabdominal ultrasound. Luteal phase supplementation was done with vaginal progesterone gel twice a day (Crinone 8% gel, 90 mg; Merck Serono, Istanbul, Turkey) starting on the oocyte retrieval day. Beta-hCG levels were measured 12 days after ET. Fertilization rate (FR) is calculated as the ratio of two ponucleus/ mature oocyte number. Grade1 embryo quality was accepted as symmetrical blastomeres and no fragmentation. Implantation rate (IR) is calculated as gestational sac number/transferred embryo number. Clinical pregnancy is accepted as intrauterine fetal cardiac activity positivity.

ELISA

Peripheric eight milliliter venous blood samples for analysis of AMH and BMP-15 levels were taken on day 3 of menstrual period (D3) and on the day of OPU. Follicular fluid (FF) samples were taken during OPU for analysis of AMH and BMP-15 levels, too. Serum and FF samples were stored at -80ºC until run time. Enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Webster, Texas, USA) for AMH (Cusabio, catalog no= CSB-E12756h, China) and BMP-15 (East Biopharma, catalog no= CK-E90147, USA) were performed according to manufacturer's instructions. Intra and inter assay variations were <10% and <12% respectively.

Statistical analysis

The statistical analyses were performed using Statistical Package for Social Sciences version 16.0 (SPSS Inc., USA). Distribution of the continuous variables was checked by using the Kolmogorov-Smirnov One Tailed test. Student's *t*-test was used for variables with normal distribution. After testing the skewed distribution, comparisons between the groups were tested by using the Mann-Whitney *U* test. The repetitive comparisons in the same group were done with Wilcoxon signed Rank Test. To prevent the inflation of significancy, Bonferroni correction was performed (P value/ the number of comparisons being made; P<0.017 was considered as statistically significant). The X^2 test and Fisher's exact test were used to analyze nominal variables. Spearman correlation analysis and logistic regression analysis were applied to investigate the possible relation between AMH and BMP-15 levels and ICSI-ET cycle outcomes. P<0.05 was considered as statistically significant for whole comparisons.

Results

The mean age and body mass index of study population were 30.6±5.2 years and 25.5±4.2 kg/m^2 respectively. Clinical characteristics of all women in the study were presented in Table 1. Comparison of clinical parameters between groups revealed no significant difference.

The stimulation and embryology parameters of two groups were presented in Table 2. Total oocyte number and estradiol level on the day of hCG injection were significantly higher in group1 compared to those in group2. There was no significant difference between groups for the parameters of mature oocyte number, grade1 embryo number on the day of ET, rates of fertilization, implantation, pregnancy and clinical pregnancy.

There was no significant difference between groups for the parameters of D3, OPU and FF AMH and BMP-15 levels (Table 3). The comparison of pregnancy rates between women according to having FF AMH (35% *vs*. 42%) and BMP-15 (37.8% *vs.* 40.5%) levels under and above of mean values revealed no significant difference. And also,

Table 1. Clinical characteristics of all women in the study.

Characteristics	Mean ± SD / %
Age (years)	30.6 ± 5.2
Infertility type	
-Primary	69.5
-Secondary	30.5
Infertility duration (months)	60.1 ± 43
Infertility etiology	
-Unexplained	35.4
-Anovulation	22
-Male factor	25.6
-Decreased ovarian reserve	9.8
-Tubal factor	7.3
BMI (kg/m^2)	25.5 ± 4.2
D3 FSH (mIU/mL)	6.3 ± 1.9
D3 LH (mIU/mL)	5.4 ± 3.3
D3 Estradiol (pg/mL)	45.2 ± 23.5
TSH (mIU/L)	2 ± 1.9
Antral follicle count	12.6 ± 3.7

Note: BMI= body mass index; D3=day 3 of menstrual period; FSH= follicle stimulating hormone; LH=luteinizing hormone; TSH= thyroid stimulating hormone

Table 2. Stimulation and Embryology Characteristics of Groups.

Parameters	Group 1	Group 2	P value
	(N=38)	(N=44)	
Total recombinant FSH dose (IU)	1724.7 ± 144.6	1662.5 ± 155.2	NS
Total hMG dose (IU)	459.9 ± 124.5	948.9 ± 206.9	NS
Stimulation duration (days)	9.6 ± 0.2	9.8 ± 1.7	NS
Total oocyte number	14.5 ± 0.9	11.2 ± 0.9	**<0.01**
Mature oocyte number	9.42 ± 0.9	9.02 ± 0.8	NS
Two pronucleus number	6.74 ± 0.7	6.5 ± 0.6	NS
Fertilization rate(%)	70	74	NS
Grade1 embryo number on the day of ET	2.13 ± 0.3	1.93 ± 0.2	NS
Estradiol level on the day of hCG (pg/mL)	3054.2 ± 203	2161.2 ± 143.9	**<0.01**
Endometrial thickness on the day of hCG (mm)	13.78 ± 3.6	10.5 ± 0.4	NS
Progesteron level on the day oghCG (ng/mL)	0.95 ± 0.1	0.97 ± 0.1	NS
Pregnancy rate/ per cycle (%)	29	48	NS
Pregnancy rate/ per ET (%)	33	51	NS
Implantation rate (%)	18	23	NS
Clinical pregnancy rate (%)	24	32	NS

Note: FSH= follicle stimulating hormone; hMG= human menopausal gonadotrophin;

according to this classification; there was no significant difference between groups for parameters of FR and IR, too.

The comparison of AMH and BMP-15 levels between women became pregnant or not revealed no significant difference, too.

The results of correlation analysis between parameters for whole population were presented in Table 4. In both of groups, FF AMH and BMP-15 levels were significantly higher than D3 and OPU AMH and BMP-15 levels ($p<0.05$). D3 AMH level showed positive correlation with OPU and FF AMH levels ($p<0.05$). D3 and OPU AMH levels showed negative correlation with fertilization rate (FR) ($p<0.01$). In regression analysis; D3 AMH level (OR= 0.5, 95% CI= 0.010-0.091, $p<0.05$) and OPU AMH level (OR= 0.9, 95% CI= 0.025-0.070, $p<0.05$) showed minimal influence on FR.

OPU BMP-15 level showed negative correlation with FF BMP-15 level ($p<0.05$). D3 BMP-15 level showed positive correlation with progesterone level and endometrial thickness on the day of hCG injection ($p<0.05$). In regression analysis; D3 BMP-15 level showed minimal influence on progesterone level (OR=0.9, 95% CI= 0.020-0.025, $p<0.01$) and endometrial thickness (OR=0.6, 95% CI= 0.238-0.383, $p<0.01$) on the day of hCG injection.

Discussion

Our main findings with this study were as follows: 1-) We did not observe difference between GnRH agonist or antagonist users for serum and FF AMH and BMP-15 levels. 2-) Both of serum AMH levels (D3 and OPU) showed negative correlation with FR. 3-) D3 BMP-15 level showed positive correlation with progesterone level and endometrial thickness on the day of hCG injection. 4-) OPU BMP-15 levels showed negative correlation with FF BMP-15 levels.

Researchers observed the enhancing effect of culture medium enriched with BMP-15 on embryo quality in animal studies [10]. Requena *et al.,*studied FF BMP-15 levels of 90 women attended controlled ovarian stimulation for donation. They compared FF BMP-15 level among recombinant FSH, urinary FSH and hMG groups with Western blot analysis and they did not observe significant difference among groups [11]. Wu *et al.,*investigated the effect of FF BMP-15 on implantation among 207 poor responder women experiencing COH-ICSI-ET cycle. They classified their population according to having FF BMP-15 level lower than mean value and higher than mean value. They observed significantly higher IR in 97 women with high FF BMP-15 level than 110 women with low FF BMP-15 level [12]. But in our population we did not observe significant differences for IR, FR and pregnancy rate between women either having lower or higher FF BMP-15 levels than mean value. And also in our population FF BMP-15 level showed negative correlation with OPU-BMP-15 level. Genereally it was expected that increased FF BMP-15 level should increase the OPU BMP-15 level, too. But in our study we did not observe increased serum BMP-15 level on the day of OPU. This condition may be the result of production of BMP-15 from a different tissue or elimination during transition from FF to blood. Gode *et al.,*studied FF BMP-15 expression levels in 81 infertile women experiencing COH-ICSI-ET cycle. They did not observe significant difference in embryo quality among women regarding to BMP-15 expression status [13]. Wu *et al.,* reported significantly increased FF BMP-15 levels in poor responders compared to that in normoresponders [14].Researchers evaluated FF BMP-15 levels of 79 couples enrolled long luteal COH-ICSI programme due to male factor infertility. They compared FF BMP-15 levels of fertilized 60 oocytes with unfertilized 19 oocytes. FF BMP-15 level of fertilized oocytes were significantly higher than that of unfertilized oocytes [6]. Gueripel *et al.,*observed increased BMP-15 immunostaining on ovarian tissue of mouse stimulated with gonadotropins [15]. In our population; D3 BMP-15 level showed positive correlation with progesterone level on the day of hCG injection. Increased D3 BMP-15 level may be considered as a negative predictor for follicular development during COH.

The studies performed for investigating the role of FF AMH levels on ICSI-ET cycle outcome revealed different results. Some researchers observed no relation between COH outcome parameters and FF AMH level [16,17]; others observed positive correlation with FF AMH level and embryo quality [9]. The studies about dynamic change of serum AMH during COH with GnRH antagonist reported gradual decreament from stimulation start to hCG injection [18,19]. But in our study we observed gradual increament for serum AMH levels from start of stimulation to day of OPU in cycles down regulated with either GnRH agonist or antagonist. Researchers appointed significantly lower FF AMH levels in fertilized oocytes compared to that in non-fertilized oocytes [20]. Mehta *et al.,*compared FR and IR according to FF AMH level either higher or lower than median value in 132 conventional IVF-ET cycle. They observed significantly higher FR and IR in women with low FF AMH levels compared to those with high FF AMH levels [21]. Researchers observed significantly high FF AMH levels in women became pregnant after long luteal-phase down regulated ICSI-ET cycle. Chen *et al.,*analysed FF AMH levels of 64 women enrolled long luteal-phase down regulated ICSI-ET cycle. They observed higher FF AMH levels in pregnant women compared to that in non-pregnant women. And also they observed correlation between FF AMH level and IR [22]. Hattori *et al.,*investigated FF AMH levels in 58 women attending ICSI-ET cycle with mixed infertility etiology. They reported significantly high FF AMH levels in women became pregnant compared to that in non-pregnant [23]. Wunder *et al.,*observed significantly higher serum and FF AMH levels in women conceived after IVF/ICSI treatment

Table 3. Comparison of AMH ve BMP-15 levels between Group 1 and 2.

Parameters	Group1	Group2	P value
	(N= 38)	(N= 44)	
D3 AMH (ng/mL)	3.4 ± 0.9	4.4 ± 1.6	0.19
OPU AMH (ng/mL)	8 ± 1.4	10.3 ± 2.6	0.26
FF AMH (ng/mL)	558 ± 28	570 ± 28	0.05
D3 BMP 15 (ng/mL)	41.8 ± 2.3	39.2 ± 1.3	0.45
OPU BMP 15 (ng/mL)	38.2 ± 2.2	36.6 ± 1.8	0.8
FF BMP 15 (ng/mL)	278 ± 12	262 ± 7	0.16

Note: Values are presented as Mean ± SEM. AMH= anti mullerian hormone; BMP-15= bone morphogenetic protein 15; D3= day 3 of menstrual period; OPU= oocyte pick-up; FF= follicular fluid

Table 4. Correlation analysis of AMH and BMP-15 levels with clinical parameters.

Parameter	Parameter	R	P value
D3-AMH	OPU-AMH	0.32	<0.01
	FF-AMH	0.25	0.02
	Fertilization rate	-0.38	<0.01
OPU-AMH	FF-AMH	0.28	<0.01
	Fertilization rate	-0.38	<0.01
FF-AMH	Age	0.28	0.01
D3-BMP15	hCGprogesteron	0.22	0.04
	hCGendometrium	0.39	<0.01
OPU-BMP15	FF-BMP15	-0.25	0.02

Note: D3= day 3 of menstrual period; AMH= anti mullerian hormone; FF= follicular fluid; BMP-15= bone morphogenetic protein-15; hCG= human chorionic gonadotropin; OPU= oocyte pick-up

compared to those in women not conceived [24]. In our study we did not observe significant difference for FF AMH levels between women became pregnant or not. Ashrafi *et al.,* pointed out that serum AMH is not a good predictor for clinical pregnancy and live birth rates in IVF/ICSI cycles [25]. Takahashi *et al.,* observed no relation between serum AMH level and ratio of high grade embryo quality [26]. And also Mashiach *et al.,* observed no significant relation between FF AMH level and FR and cleavage rate among 22 FF sample of 11 polycystic ovary syndrome patients undergoing IVF [27]. However other researchers notified relation between serum AMH level and IR in 42 normoresponder women experiencing IVF [28]. Lee *et al.,* observed no significant difference for FF AMH levels between 43 GnRH agonist vs 44 GnRH antagonist users [29]. But in our study; FF AMH level of GnRH agonist users was significantly lower than that of GnRH antagonist users. And also in our population; FF AMH level showed influence on FR and IR. The different results among the studies may arise from population heterogeneities.

The limitations in our study were as follows: 1-) The study population was consisted of heterogenous infertility etiology. To exclude the confounding factors on oocyte and sperm quality, the population should be composed of only tubal factor infertility. 2-) BMP-15 and AMH expression levels of COC will give more detailed information about microenviroment of oocyte than FF.

In conclusion; FF AMH level may show a prognostic value for prediction of FR and IR in ICSI cycles. The negative correlation between OPU BMP-15 and FF BMP-15 levels must be evaluated with further expanded population studies.

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Declaration of interest statement

All of the authors declare that they have no conflict of interest to disclose.

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