Original Investigation

Ovarian stimulation drugs alter the metabolite content of the growing follicle: in-vivo spectroscopic evaluation of follicle fluid
Güngör and Güngör. Ovarian stimulation drugs alter the follicle

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Abstract
Objective: By using magnetic resonance spectroscopy (MRS) to determine metabolite content of growing nfollicle in patients with polycystic ovary syndrome (PCOS) receiving recombinant FSH, clomiphene citrate (CC) or aromatase inhibitor (AI) for ovarian stimulation.
Material and Methods: 30 patients diagnosed with PCOS and infertility and scheduled for ovarian stimulation were divided into 3 equal groups according to the drugs they took as follows: rFSH (n: 10), or CC (n: 10), or AI (n = 10). Five fertile cases were determined as the control group. When the follicle diameters reached 16-18 mm in each group, patients were directed to the MRS and the metabolite content of a dominant follicle was analyzed. N-acetylaspartate (NAA), lactate (Lac), creatine (Cr), and choline (Cho) metabolite levels determined in the spectrum were measured in ppm.
Results: Approximately 3-fold decrease in dominant follicle Cho content was found in patients receiving CC compared to control subjects. Similarly, the dominant follicle Cho intensities of patients given rFSH and AI were noted to be significantly higher than those who received CC. Only dominant follicle lactate levels of the patients who received CC were found to be significantly higher than the other groups. Cr peak intensities of patients receiving CC were found to be approximately 3 times less than control subjects. Cr signal intensity was significantly higher in patients receiving rFSH or AI than in patients receiving clomiphene. While 2 patients became pregnant in the CC group, 3 patients in the AI group and 5 patients in the rFSH group became pregnant. The main metabolites detected in patients who conceived in each group were Cho and Cr. In cases who could not conceive, while lactate and lipid signals increased, Cho and Cr signals decreased.
Conclusion: Unlike CC, ovulation stimulation with rFSH or AI does not alter dominant follicle metabolite content. Developmental capacity of growing egg can be determined non-invasively with MRS.

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Keywords: MR spectroscopy, rFSH, Aromatase inhibitor, Clomiphene citrate, Dominant follicle

Introduction

Drugs such as recombinant follicle stimulating hormone (rFSH), clomiphene citrate (CC) and aromatase inhibitor (AI), which we use for ovarian stimulation, have all the features necessary for the selection, growth and numerical increase of eggs. However, sometimes these drugs may lead to the formation and selection of poor quality oocytes, resulting in poor quality of embryos to be obtained (1-4). Letrazole, an aromatase inhibitor, has a reversible effect and can block estrogen synthesis from androgens (4). While rFSH directly promotes follicle development CC and AI stimulate follicle growing indirectly by increasing endogenous follicle stimulating hormone (FSH) release. Both the Letrozole and CC provide adequate follicle development when used for 5 days between 3rd and 7th days of the cycle. However, in addition to the number of retrieved oocytes, both the quality of oocytes and the pregnancy rates obtained with these drugs are not the same. The main reason for this difference between Induction Drugs may be changes in follicle metabolism and development depending on the drug dose and mechanism of action. Both the agonistic and antagonistic effects of CC on estrogen receptors may affect follicle developmental capacity independently of endogenous FSH levels. Likewise, aromatase enzyme inhibition effect of letrazole may distturb estrogen production capacity of granulosa cells that may lead to unfavorable consequences on follicle developmental potential (1-4). In addition, use of these drugs may also affect the morphological and receptive properties of the endometrium (1,2). However, there are not enough clinical data regarding the impact of these drugs on developing follicle metabolite composition. We therefore have to act on the basis of our clinical and embryological data and experience rather than on molecular data when deciding on which group of drug we will use.

It is not possible to make a clear comment about follicle quality based on images obtained during folliculometry. For this reason, there is a need for a method to evaluate the developmental potential of the follicle noninvasively during the ovulation stimulation cycles. A study by Wallace et al (5) reported a positive correlation between follicle fluid metabolite content and oocyte developmental capacity. However, in the study mentioned, the follicle liquid must be taken out in order to be evaluated in terms of content. Therefore, an oocyte pick-up (OPU) is required, which is an invasive procedure. In addition, since the nuclear magnetic resonance (NMR) process will be performed after OPU, there is no possibility of interfering with the developing egg in vivo. To eliminate all these disadvantages and to evaluate egg development in vivo, the only non-invasive method we have is magnetic resonance spectroscopy (MRS).

It has been reported that MRS can detect metabolite content in biological fluids with great accuracy. With the help of this method, we can record the metabolic changes in a living cell using conventional magnetic resonance imaging. Primary advantage of this technique is non-invasive nature and cost. MRS can measure various intrafollicular molecules that are present in a ppm levels. Although the method was originally developed for brain lesions, its use in reproductive organs such as endometrium, myometrium, and ovary has been reported by many authors (5-8). This technique also allows comparison of follicular fluid metabolite levels in different treatment regimens in vivo and non-invasively. Although there are many studies investigating the metabolite concentrations in the reproductive organs (5-10) there are no published studies investigating the effects of ovarian stimulation drugs on the follicular

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fluid metabolite compositon. Therefore, the primary outcome of the study was to investigate whether rFSH, AI, and CC have an effect on metabolite content of growing follicle in women with polycystic ovary syndrome (PCOS) undergoing ovarian stimulation. The secondary outcome is to compare the possible relationship between the metabolite intensities obtained from the dominant follicle and clinical pregnancy rate.

Material and Methods

The study was conducted in the BAU Medical Park Göztepe Hospital, Clinic of IVF. Thirty-five women (30 PCOS and 5 fertile control) between the ages of 19 and 37 years with a body mass index (BMI) of 18–29 were enrolled the study. A total of 30 primary infertile and anovulatory women with PCOS were were divided into to three groups consisting of women on antagonist protocol with rFSH (n:10) or PCOS women receiving CC (n:10) or PCOS women receiving aromatase inhibitor (n=10). Five fertile women with similar age and BMI were accepted as control group. They were not given any drugs for ovarian stimulation. The women in control group had at least one or more child. They had no history of clinical or laboratory findings of PCOS. Women in each treatment group should met the inclusion criteria for PCOS that was diagnosed when existence of at least two of the following three features (11): [1] amenorrhea or oligomenorrhea with chronic anovulation, [2] clinical and/or biochemical evidence of hyperandrogenism, and [3] ultrasonographic appearance of PCOS (2). Participants with the history of current or previous use of metformin, tubal or male factor infertility, evidence of any uterine, ovarian or peritoneal diseases such as leiomyoma, benign ovarian cyst, endometrioma or endometriosis potentially affecting the ovarian microenvironment were also excluded. This study has institutional review board approval. PCOS participants on antagonist protocol were daily given rFSH starting from the 3th day of the menstrual cycle. Dose adjustments were performed according to body mass index and ovarian response. The rFSH dose used ranged from 100 units to 187.5 units. Follicle development was monitored by using transvaginal ultrasonography and serum estradiol concentrations. As soon as one of the follicles reached 14 mm in diameter, the GnRH antagonist was started and this treatment was continued until hCG was administered. Oocyte maturation was triggered with recombinant hCG (Ovitrelle, Merck–Serono, 250 mg, Modugno, Iatly). As soon as the follicle diameters reached 16-18 mm in each group, patients were directed to the MR spectroscopy and the metabolite content of a dominant follicle was analyzed. N-acetylaspartate (NAA), lactate (Lac), creatine (Cr), and choline (Cho) metabolite levels determined in the spectrum were measured in ppm (10,12).

PCOS subjects on CC group were treated with clomiphene citrate (Serophene, Serono, Rome, Italy) at dosage of 150 mg/day for 5 days beginning from the 3rd day of spontaneous or progesterone-induced withdrawal bleeding. Patients in the clomiphene group were selected from CC-resistant PCOS patients. CC resistant defined as failure to ovulate after six months of treatment at an appropriate dose, the patient is regarded as resistant to CC. In our study, patients who could not achieve ovulation despite using 150 mg/day clomiphene were considered CC resistant. PCOS subjects on aromatase inhibitor group were treated with letrazole (Femara, Novartis, Istanbul, Turkey) at dosage of 2.5 mg/day for 5 days beginning from the 3rd day of a spontaneous or progesterone-induced menstrual bleeding. Follicle development was monitored using transvaginal ultrasound. Women on CC or letrazole also underwent spetroscopy analysis of one dominant follicle as soon as the detection of a follicle with a mean diameter of at least 16-18 mm at TV-USG examination. Women on CC or letrazole group underwent either timed intercourse or intrauterine insemination. Fertile women on their natural cycle underwent spectroscopy when the dominant follicle with a mean diameter of at least 16-18 mm is detected.

Spectroscopy analysis of dominant follicle

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Axial and coronal plane T1-weighted images of dominant follicle with 5 mm thick sections were achieved by using 1.5 T MRI. Single voxel MR spectroscopy procedure was applied with a short and long TE. Because signals from other follicles may obscure the metabolite content of dominant follicle voxel should be placed in the appropriate area so that it is possible to get clear information about the content of follicle fluid. We therefore carried out great effort to exclude the other neighboring in the area of voxel, while determinig the voxel placement. Finally, the voxel was placed just in the middle of the dominant follicle. Thus, the signal records of the neighboring intestines were not obtained. The metabolite ratios of acquired signals from dominant follicle were determined using Magnetic Resonance User Interface software. N-acetylaspartate (NAA), lactate (Lac), creatine (Cr), and choline (Cho) peaks of each group was analysed. Detailed analysis of spectroscopy method can be found elsewhere (6,7,8,9,12). The primary outcome of our study is to reveal the effect of rFSH, CC and AI treatment on dominant follicle metabolite levels. The secondary outcome is to compare the possible relationship between the metabolite intensities obtained from the dominant follicle and clinical pregnancy rate. All metabolite peaks were analyzed quantitatively and compared with the results of the either timed intercourse or intrauterine insemination or embryo transfer. Clinical pregnancy rate was defined as evidence of a gestational sac confirmed by ultrasound examination.

Statistical analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The normality distribution of data was tested with the use of the Kolmogorov-Smirnov test, and all variables were skewed normally. The continuous variables were analyzed by means of analysis of variance test with posthoc Tukey procedure and Mann-Whitney U test. A p value of <.05 was considered to be significant. The results are expressed as mean±SD.

Results

The clinical and demographic features of four groups of subjects were similar (Table 1). Three participants were excluded from study due to inadequate follicle growing in one fertile control and bad spectral image in two women receiving letrazole. The remaining 32 subjects in control and treatment groups had good spectral image. Compared to fertile group significantly decreased Cho signal was found in PCOS group taking CC. Approximately three-fold decline in Cho peak intensity was found in PCOS subjects compared the Cho peak intensity of control participants (2.11±3.10 ppm *vs.* 0.68±1.14 ppm, p<.01). Likewise, the Cho peak intensity of women receiving rFSH or letrazole were higher than those from Cho signal intensity of women receiving CC. The Cho peak intensity of fertile control, patients receiving rFSH or letrazole were similar.

The lactate signal intensity of patients receiving CC was significantly higher than that of lactate signal of fertile control (1.82±1.72 vs. 0.82±2.44, p<0.02) or patients receiving rFSH (1.82±1.72 vs. 0.95±4.50, p<0.03) or letrazole(1.82±1.72 vs. 0.93±4.77, p<0.01). Almost 2-fold increase in lactate signal was detected after CC treatment compared to rFSH, letrazole or fertile groups. On the other hand, lactate signal intensities of fertile control, rFSH, and letrazole groups were similar to each other. Follicle fluid Cr signal intensity of fertile group was approximately 3 times higher than Cr signal of patients receiving CC (2.41±1.32 vs. 0.81 ±3.14, p<0.01). Likewise, Cr signal intensity of women receiving rFSH or letrazole was significantly higher than those in the Cr intensity of women receiving CC (p <0.01 and p <0.01 respectively). No significant difference was found between the fertile control, rFSH, and letrazole groups in terms of Cr metabolite. NAA content of fertile and treatment groups were found to similar (Table 1 and Figure 1).

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When a comparison was made between FF metabolite signal intensities and pregnancy rates, the following results were obtained. While clinical pregnancy was detected in 2 of 10 patients in the CC group, pregnancy could not be achieved in the remaining 8 cases. While the main metabolites were Cho and Cr in two cases who got pregnant, lactate peak was in the foreground in those who could not conceive. While pregnancy was achieved in 3 of 10 cases in the AI group, pregnancy was not detected in 7 cases. While high Cho signal was detected in pregnant patients, weak Cho signal and high lipid signal were detected in those who could not conceive. While 5 out of 10 patients in the rFSH group had clinical pregnancy, the remaining 5 cases did not become pregnant. High Cho and low lactate signal were typical findings in patients who became pregnant in this group. Weak Cho and Cr signal was striking in patients who could not conceive.

Discussion

If we have ability to determine the best quality oocyte before OPU we could improve pregnancy rates. Although morphological analysis of oocyte is the basic method for determining embryo quality, it does not always correctly detect reproductive outcome (13,14). Metabolite composition of developing follicle is an indicator of whether an embryo will complete its developmental process and reach to the cleavage stage or not (5,15). Although drugs used for ovarian stimulation improve maturation of oocytes adverse effects of these drugs on natural selection steps can cause retrieving of developmentally incompetent oocytes. In good agreement with this, it has been reported that few of the embryos collected in the OPU can complete their developmental stages in a healthy way and gain implantation potential (16). Fortunately, we have a new and non-invasive method to test the effects of ovarian stimulation drugs on the metabolite profiling of developing follicle. MRS is an advanced technology that enables us to determine the metabolite content in all biological fluid layers in detail. This non-invasive method has been used in the evaluation of reproductive tissues including ovary and endometrium.

Because follicle fluid has nutritive effect on growing follicle (17) determining the metabolites in this fluid non-invasively provides preliminary information about the metabolite content of developing follcle. Studies have demonstrated that measurement of Cho and lactate metabolites in follicle fluid with nuclear magnetic resonance spectroscopy correlates with oocyte developmental capacity (5,15). The metabolite composition of the cumulus oocyte complex in in vitro maturation cycles is detailed by Uhde et al (18). After 23 hours of follow-up, approximately 369 different metabolites were isolated in maturation medium. The metabolites whose levels significantly increase during follicle growing are amino acid, saccharide, lipids, UDP-glucose, lactate, pyruvate and sphingomyelin. The two metabolites with the most significant increase in levels are creatinine and lactate. During 23 hours of maturation period, a 516-fold increase in Cr and a 1227-fold increase in lactate are detected. Lipids have a crtical role in energy production during the oocyte maturation and the establishment of a developmentally mature oocyte (18). The transport of CCderive pyruvate with lipids into the oocyte is critical for ATP production by the oocyte (19). Uhde et al (18) showed that during the oocyte maturation there was a significant increase of concentrations of both pyruvate and lactate in the medium, while glucose concentrations decreased. One possible reason for decrease in glucose levels in follicle fluid is increased glycolysis in cumulus cells during maturation period.

In the current study we investigated, for the first time that, the effects of rFSH, CC, and AI on dominant follicle metabolite content. The main result we obtained from this study was that the metabolite signals of growing follicle were quite different in the patients using each drug even though the follicle size was sufficient in the ultrasonographic examination. The metabolite composition of dominant follicles of women receiving AI or rFSH were not different from those of fertile patients. On the other hand, the metabolite signals of patient receiving CC

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were different from that of fertile control, rFSH or AI groups. If we need to do detailed analysis, spectroscopic evaluation of control and treatment groups showed the existence of Cho, Cr, NAA and Lac signals. However, the Cho, Cr, and Lac signals obtained from subjects were given CC were significantly different from the signals of rFSH and AI groups. Of the four measured metabolites, only NAA signals were similar between the groups. Additionally, the intensity of Lac signal of dominant follicle was elevated in the CC group compared with rFSH or AI group. Administarion of CC for ovarian stimulation disturbed the release of Cho and Cr that basic metabolites produced by the developing follicle. The mechanism of impaired metabolite signal in patients using CC is not clear. This adverse effect may be due to the estrogen receptor blocking effect of CC. As we know, CC shows negative impact on the endometrium (1). Despite high ovulation rates low clinical pregnancy rates in women were given CC may be due to failed follicle development. Both AI and CC stimulate follicle development by increasing FSH levels. However, AI reduces estrogen levels by blocking the aromatase enzyme and leads to an increase in FSH levels. CC increases FSH levels by binding to estrogen receptors. In fact, although the mechanisms of action are similar, they contain a fundamental difference. Since CC binds to all estrogen receptors, it also binds to estrogen receptors in cumulus cells. Thus, while CC contributes to follicle development via FSH, it also negatively affects follicle development as it also blocks estrogen receptors on the cumulus cells. Since AI does not block estrogen receptors, it is a natural finding that AI have different follicular growth patterns with CC.

The increase in FF lactate levels may have both negative and positive effects on follicle development. High levels of follicle fluid lactate may lead to anaerobic gylicolysis and impair healthy oocyte development in women on CC (6-8). Concordantly, exposure of developing mouse oocyte to high fat diet results in failed oocyte nuclear maturation (20). Likewise, decreased levels of Cho metabolite in women on CC can be sign of defective follicle development. Cr signal is an important indicator of energy metabolism of living cells (6-8). Low Cr signal in women receiving CC may be another indicator of impaired follicle maturation and can be associated with poor follicle morphology. On the other hand, the increase in FF-lactate levels in the CC group should not be interpreted as a absolute pathological state. Increase in FF-lactate levels is also detected in natural follicle development processes. Especially in in vitro maturation cycles, during the first 8 hours of follow-up, a very significant increase in lactate levels is detected in the maturation medium. It has been reported that the lactate concentration in the maturation medium increased 667-fold during in vitro maturation (18). What is important here is whether the oocyte has enzymes responsible for carrying lactate rather than the increase in lactate levels. Herubel et al (21) showed that oocytes have great amount of monocarboxylic acid transporters that enable the transport of lactate between cumulus cells and oocyte. If the increase in lactate levels during follicle development exceeds the transport acapacity of the amount of monocarboxylic acid transporters, the rate of cell growth and proliferation will slow down. Since the high lactate increase seen in the CC group is above the carrying capacity of the follicle, the cumulus cells will try to balance the lactate increase by slowing the cell proliferation. Since Cho is a marker of cell membrane turnover, the amount of Cho passing into follicular fluid will also decrease due to decreased cumulus cell proilferation.

Conclusion

We can predict the developmental capacity of egg before OPU by determining of more specific and accurate metabolites from growing follicle with spectroscopy. This would help the choice the best quality embryo and would aid in overcoming the immature and incompetent oocyte selection. Thus, the selection of oocytes that have completed their developmental process before embryo transfer is possible and the transfer decision is made

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accordingly. This decision may be in the form of approval or cancellation of the embryo transfer.

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Table 1. Demographic characteristics and metabolites ratios of treatment and control groups

	rFSH	**Clomiphene**	**Letrazole**	**Fertile control**
Mean age (yrs)	29.1 ± 6.31	28.3 ±1.40	28.6± 0.21	29.2±2.45
BMI (kg/m2)	27.4±5.14	26.5±5.32	27.1±2.11	27.5±3.30
Infertility duration	7.11±1.33	6.74±2.10	6.91±2.03	**NA**
Etiology of infertility	PCOS	PCOS	PCOS	**NA**
Mean follicle size (mm)	18.4 (±0.3)	17..4 (±0.3)	18.2 (±2.6)	16.8 (±1.2)
Method	IVF/ICSI	IUI or TIC	IUI or TIC	NA
Dominant follicle metabolite composition				
1- NAA	1.21 ±2.12	1.01 ±1.20	1.33 ±5.02	1.05 ±2.01
2- Lactate	0.95±4.50	1.82±1.72*	0.93±4.77	0.82±2.44
3- Choline	1.96±2.24	0.57±6.44*	1.89±0.37	2.27±4.20
4- Creatine	1.87 ±2.14	0.81 ±3.14*	1.85 ±2.10	2.41 ±1.32
*: Compared to CC group there were statistically significant difference between the fertile control, rFSH, and letrazole groups in terms of Cho, Lac, and Cr metabolites				

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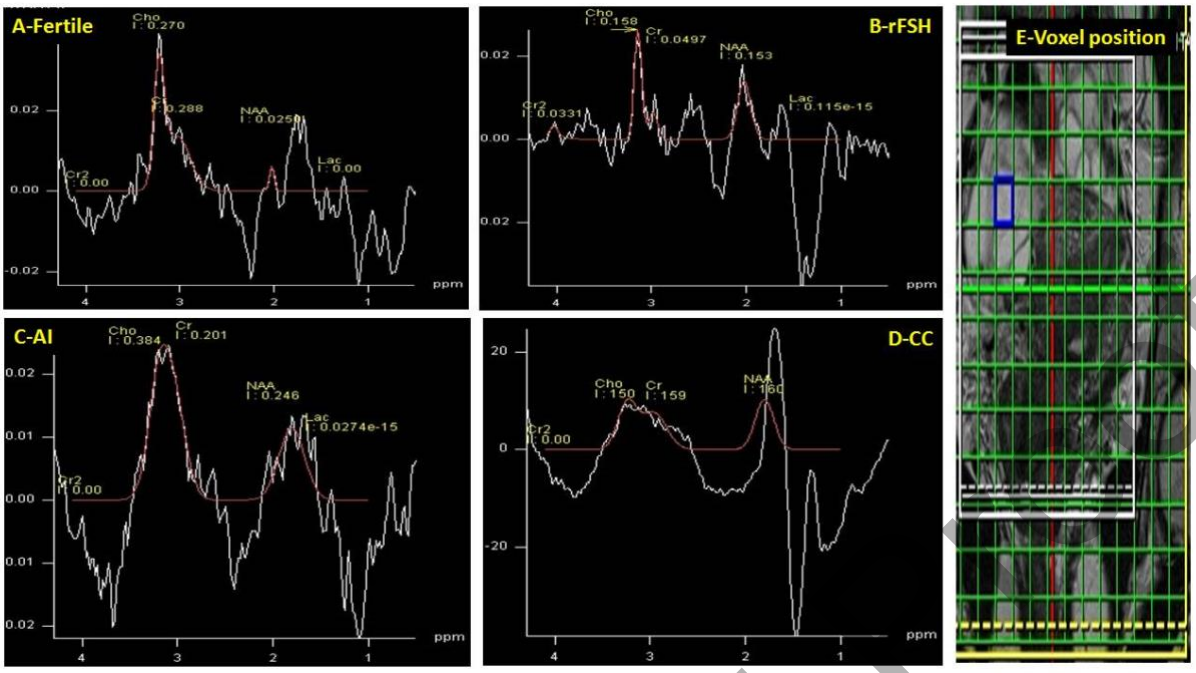

Figure 1. Spectroscopy analysis of fertile group (A) and participants receiving rFSH (B), aromatase inhibitor (C) or clomiphene citrate (D). Approximately four-fold decrease in Cho signal was detected in PCOS group receving CC (D). Cho signal of subjects taking rFSH or AI were significantly higher than in the CC group. Signals from ovary and other pelvic structures may obscure the metabolite resonance of dominant follicle. Hence, the voxel was placed at the center of dominant follicle to avoid any signal contamination from neighboring tissues (E). NAA; N-acetylaspartate, Lac; Lactate, Cr; Creatine, Cho; Choline; AI; Aromatase inhibitor, CC; Clomiphene citrate.

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