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RESEARCH ARTICLE

STIMULATION WITH RECOMBINANT FSH/LH DURING ICSI IMPROVES SECRETION OF PROGESTERONE AND VEGF OF LUTEINIZED GRANULOSA CELLS COMPARED TO RECOMBINANT FSH ALONE IN PATIENTS OVER 35

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ABSTRACT

Objective: With this study we wanted to understand, why stimulation with recombinant FSH/LH could be advantageous for patients over 35 years during an in-vitro-fertilisation treatment over recombinant FSH stimulation alone. **Design Prospective case-control study**: Setting Recruitment of patients at the VivaNeo Fertility Centre Wiesbaden, Germany. Measurements of the parameters at University Medical Centre of the Johannes Gutenberg University Mainz, Gynaecological Endocrinology and Reproductive Medicine, Germany. April 2012 until April 2013 **Patients**: After obtaining a declaration of consent 78 patients between 35 and 40 years, without endocrine disorders as low LH, aberrant menstrual cycle or low ovarian reserve, planed for intracytoplasmic sperm injection (ICSI) treatment with antagonist protocol were selected. They were divided in two stimulation groups of which 39 were stimulated with recombinant follicle-stimulating hormone plus recombinant luteinizing hormone (FSH/LH, Pergoveris®) and the other with recombinant FSH (Gonal®). **Interventions**: During oocyte pick-up granulosa-cells were obtained from the cumulus-cell complex and from follicular fluid. They were seeded separately and cultured for 48 hours. At average 16, 24 and 40 hours media change took place and secretion of progesterone, inhibin-A, vascular endothelial growth factor (VEGF), endothelin-1 were measured several times. After 48 hours culture was terminated and cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) were detected out of the cell-lysate. **Main Outcome Measure**: A granulosa cell culture could successfully be established. Secretion rate of progesterone, VEGF and cGMP in luteinized human granulosa cells is increased in after stimulation with recombinant FSH/LH in comparison to FSH alone. **Results**: Vitality and concentration of luteinized human granulosa cells were stable during 48 hours of culture. Secretion rates of progesterone, VEGF and endothelin-1 showed higher tendency after stimulation with recombinant FSH/LH. For inhibin-A an opposite secretion rate was observed. For cAMP no differences were found, but cGMP content favoured the combined stimulation. **Conclusion**: Beside the fact we did not find clear significance, we think that stimulation with recombinant FSH/LH can improve different functions of human granulosa cells during in-vitro culture. Perhaps this can lead to an explanation, why this stimulation could be beneficial for the outcome of in-vitro-fertilisation.

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INTRODUCTION

During early follicle maturation granulosa cells evolve from the follicular epithelial cells surrounding the primary follicle. Later endogen gonadotropins FSH and LH control their development and steroid hormone secretion. After ovulation granulosa cells form with immigrating theca cells the corpus luteum, which secretesunder the influence of gonadotropins substantial hormones of luteal phase (Jaffe, 2017). The interaction between oocytes and granulosa cells takes place through paracrine and intercellular communication (Canipari, 2021). In this cross-talk cGMP was observed affecting meiotic arrest and oocyte selection (Liu, 2013 and Chen, 2007). And cAMP would mediateas a second messenger the effects of gonadotropin (Andrieu, 2009).

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In hibin A antagonizes the effect of FSH and influences proliferation and apoptosis of the granulosa cells (Chen, 2007 and Anderson, 1998). VEGF and endothelin-1 are involved in neoangiogenesis in the follicular oocyte complex as well as the development of corpus luteum (Lu, 2019; Sudik, 1996). Production of progesterone in the corpus luteum makes a significant contribution for maintaining pregnancy and is therefore dependent on the functionality of the granulosa cells (Wiltbank, 2012 and Mihm, 2011). As the complex of oocytes, granulosa and theca cells in the maturing follicle is influenced by gonadotropins, the choice of a suitable stimulation treatment for In-vitro-Fertilisation becomes crucial. It is assumed that success rates of IVF treatment in older patients can be increased by using recombinant FSH/LH (Bosch, 2011; Gómez-Palomares, 2006; Lisi, 2005; Paterson, 2012; Barberi,, 2012). Granulosa cells can provide insights into the processes of oocyte maturation and corpus luteum.

They are easily accessible during in vitro fertilization andcan be obtaineddirectly from follicular fluid or after the oocyte-cumulus complex has been separated. This cell biological work measured the secretion rate of progesterone, inhibin-A, VEGF, endothelin-1 as well as the concentration ofcAMP and cGMP in cell lysates of cultured granulosa cells after stimulation with recombinant FSH/LH compared torecombinant FSH alone in patients over 35 years. The granulosa cells were obtained during oocyte pick up and were cultured for 48 hours. Detectable differences could provide an explanation for described improvements through the stimulation with FSH/LHin older patients.

MATERIAL AND METHODS

All principles of the Helsinki Declaration and its annexes were complied with planning andprocessing. Evaluation of the data were carried out anonymously. The investigations werecarried out on excess therapy material at the fertility centre of the University medical centre Mainz and the Vivaneo fertility centre Wiesbaden in 2012and 2013. A written consent form for the use of excess biological material for research purposes was obtained from each patient. Due to the study design, it was not necessary to obtain an ethic vote. Two groups with 39 patients each were formed. All patients were treated in an antagonist protocol for intracytoplasmic sperm injection because of male infertility. Patients with hormonal disorders or low ovarian reserve were excluded. Only patients between 35 and 40 years and with a comparable number of oocytes were selected. Patients of the first group were stimulated with recombinant FSH(Gonal®) and their median age were 37,2 years. In the second group stimulation was undertaken with recombinant FSH/LH (Pergoveris®) and the mean age was 36,7 years. All patients were stimulated with comparable total doses and triggered with 250 µg recombinant human chorion gonadotropin (Ovitrelle®) 36 hours before oocyte collection.
During follicle aspiration, the oocyte-cumulus complex was identified, transferred to nutrient medium and kept in the incubator until further treatment. The remaining follicular fluid was transferred to culture vessels and also stored in the incubator. After denudation the obtained remaining liquid was similarly incubated.

By separating erythrocytes from the follicular punctate and using cell strainer a single cell solution could be prepared from remaining granulosa cells and was hereinafter referred as FFfraction. The medium obtained after denudation contained already single cellsand was subsequently termed CC fraction. After washing several times with PBS 450.000 granulosa cells from the FF fraction and 25.000 from the CC fraction could be applied to each well of a 24-well culture plate and incubation started. The cell culture dishes were previously coated with 10 µg/ml fibronectin solution. Culture of the granulosa cells was carried out in DMEM/Hams´s F12 Medium. After 16, 24 and 40 hours, medium was changed and an automated hormone immunoassay from Beckman Coulter from the supernatants was used to measure progesterone and inhibin A. VEGF and endothelin-1 were determined using a manual ELISA from R&D Systems. At the end of the culture, cells were enzymatically detached, washed and after ultra-centrifugation, the obtained pellets were cryopreserved at -80 °C. For measurements lysates were subsequently prepared from the cryopreserved pellets and the total protein content of each fraction was measured using the Pierce BCA Protein Assay Kit. Out of protein fraction, cAMP and cGMP was determined with R&D Systems ELISA. Total protein content and measurements of progesterone, inhibin A, VEGF and endothelin-1 at time of medium change displayed the results as secretion rates per hour and mg protein. Statistical evaluation was carried out in MS Excel using the Student's t-test. A value of ≤ 0.05 was recognized as statistically noticeable.

RESULTS

Comparative morphological assessment revealed no differences betweengranulosa cells derived from cumulus cellcomplex (CC) and from follicular punctate (FF). 25.000 granulosa cells/micro well from cumulus cell complex (CC) and 450.000 granulosa cells/micro well from follicular punctate fluid (FF) were seeded after separation of debris and erythrocytes.

Progesterone: Secretion of progesterone was measured 39 times in each fraction. After 16 hours highest rates were found in the CC group after FSH/LH treatment with 2532,15 ng/ml compared to 1214,05 ng/ml after FSH alone. Inthe follicular fluid differences with 1230, 19 ng/ml to 1145,94 ng/ml were not so pronounced. Clear increasement was detected after 24 hours with 3037,85 ng/ml after FSH/LH stimulation in CC fraction. For FSH stimulation slight rise could be observed with 1670,87 ng/ml. Similarly but at lower level it was seen for the FF fraction. After 40 hours increase for the FSH/LH group slowed down to 3769,15 ng/ml respectively 2491,75 ng/ml. This could also be observed for the secretion rate of progesterone after FSH stimulation with 1787,65 ng/ml and 2580,07 ng/ml. In the FSH/LH stimulation group of cumulus complex cells results at 40 hours were significantly higher than for FSH alone (p=0,039). Figure 3 demonstrate mean values of the secretion rates.

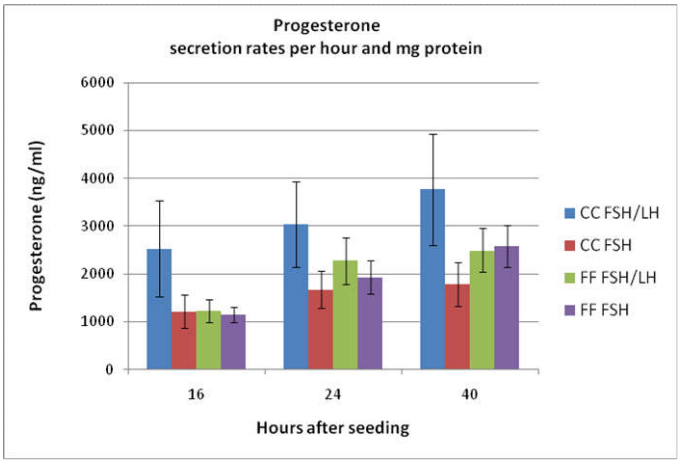


Figure 3 Average values of progesterone in the supernatants of cultivated human granulosa cells. After stimulation with recombinant FSH/LH orrecombinant FSH, ovulation was triggered with recombinant human chorion gonadotropin (HCG) and cells were collected from the cumulus-cell complex (CC) and from the follicular-punctate fluid (FF) and seeded out. 39 measurementscycles were undertaken for each group after 16, 24 and 40 hours of culture.

Inhibin-A: Inhibin-A measurements were undertaken 12 times for each group. Mean levels are demonstrated in figure 4.Over all secretion was detected at higher degree in follicular

fluid derived granulosa cells with 1024,43 ng/ml respectively 906,64 ng/ml at 16 hours, 453,72 and 618,66 ng/ml at 24 hours and 1293,90 and 1141,47 ng/ml after 40 hours of culture. No statistical relevant results were found between the different stimulation protocols. For granulosa cells measurements were distinctly lower with 68,99 ng/ml and 82,00 ng/ml at 16 hours. At 24 hours with 59,13 ng/l to 83,13 ng/ml and at 40 hours 87,56 and 131,73 ng/ml. For these cells,although at lower level, inhibin-A tends to be higher secreted after FSH stimulation.

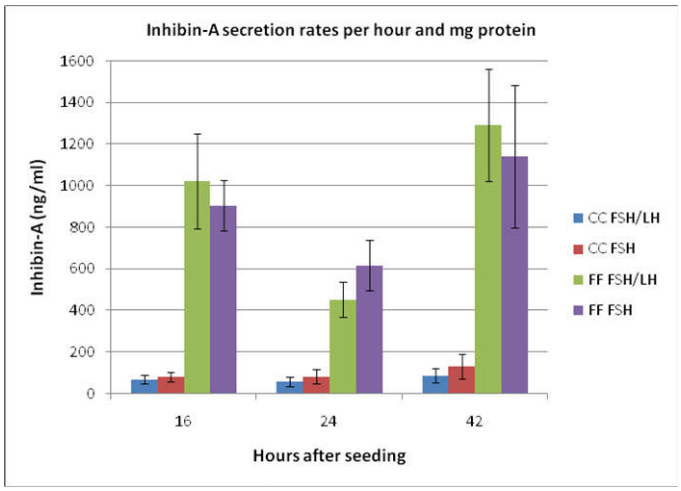
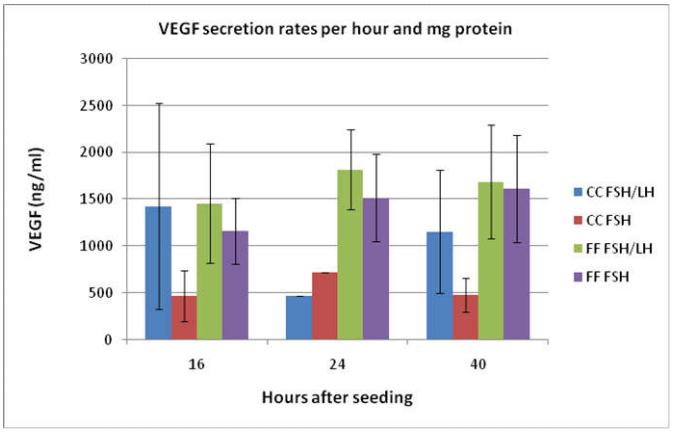


Figure 4 Average values of inhibin-A in the supernatants of cultivated human granulosa cells. After stimulation with recombinant FSH/LH or recombinant FSH, ovulation was triggered with recombinant human chorion gonadotropin (HCG) and cells were collected from the cumulus-cell complex (CC) and from the follicular-punctate fluid (FF). 12 test cycles were undertaken for each group after 16, 24 and 40 hours of culture.

VEGF: For VEGF 20 test runs were undertaken. The averages are demonstrated in figure 5. After 16 hours 5 of them in the CC group delivered usable results with a mean level of 1427,19 after FSH/LH stimulation and 466,83 ng/ml, after FSH alone, which was not significant. Granulosa cells isolated from follicular fluid showed in 4 test runs a mean levels of 1457,47 ng/ml after FSH/LH and 1162,11 ng/ml in the FSH alone stimulation group. At 24 hours of culture from the CC group after FSH/LH stimulation only in 2 of 20 trials and after FSH alone even 1 of 20, results were able to detect with 470,09 ng/ml respectively 717,49 ng/ml. In the FF group 4 test runs for FSH/LH stimulation and 5 for FSH alone showed a mean secretion rate of 1817,15 ng/ml and 1517,99 ng/ml. After 40 hours of culture results were found in 6 (CC after FSH/LH), 5 (CC after FSH), 4 (FF after FSH/LH) and 4 (FF after FSH) test runs, with 1157,61 ng/ml, 478,17 ng/ml, 1687,49 ng/ml and 1613,78 ng/ml. A lower VEGF secretion was remarkably in the FSH stimulation alone in comparison to FSH/LH among culture time. Exceptional after 24 hours relationship was not as expected, which could be explained by the low amount of test runs with reasonable results. Figure 5 Average values of VEGF in the supernatants of cultivated human granulosa cells. After stimulation with recombinant FSH/LH or recombinant FSH, ovulation was triggered with recombinant human chorion gonadotropin (HCG) and cells were collected from the cumulus-cell complex (CC) and follicular-punctate fluid (FF). 20test cycles were undertaken for each group after 16, 24 and 40 hours of culture.



Endothelin-1: Endothelin-1 was only after 16 hours over the detectability limit. In further course we were not able to measure Endothelin-1,although 6 test runs were undertaken. Almost only from cumulus-cell-complex derived granulosa cells secretion rates were found and favoured FSH/LH stimulation with 10,41 pg/ml versus 5,28 pg/ml. In case of measurable levels ofthe FF-group they also tended towards FSH/LH stimulation with 1,53 pg/ml versus not detectable as seen in Figure 6.

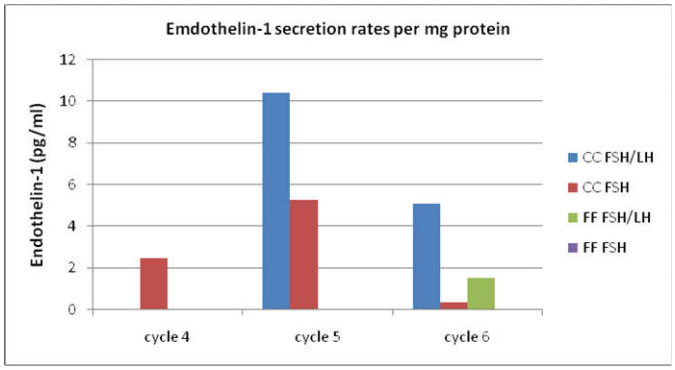


Figure 6 Average values of endothelin in the supernatants of cultivated human granulosa cells after 16 hours culture. After stimulation with recombinant FSH/LH or recombinant FSH, ovulation was triggered with recombinant human chorion gonadotropin (HCG) and cells were collected from the cumulus-cell complex (CC) and follicular-punctate fluid (FF). 6 test cycles were undertaken for each group after 16, 24 and 40 hours. Endothelin-1 was only measurable after 16 h during test cycle 4, 5 and 6. At later time points endothelin could not be detected.

cAMP, cGMP: cAMP and cGMP were measured directly from the cell lysates at the end point of culture and concentration calculated as pmol per ml. cAMPand cGMP displayed in the cumulus-cell-complex clear higher levels than out of granulosa cells from follicle puncture fluid. For cAMP we hardly found any differences between the stimulations. But cGMP showed higher levels after FSH/LH medication.This effect was not significant, but evident in both fractions; seen in Figure 7 b.

DISCUSSION

As there were no discernible morphologically differences after different stimulations during culture, we assumed that our results were due to molecular biological processes within the granulosa cells and their cellular environment.

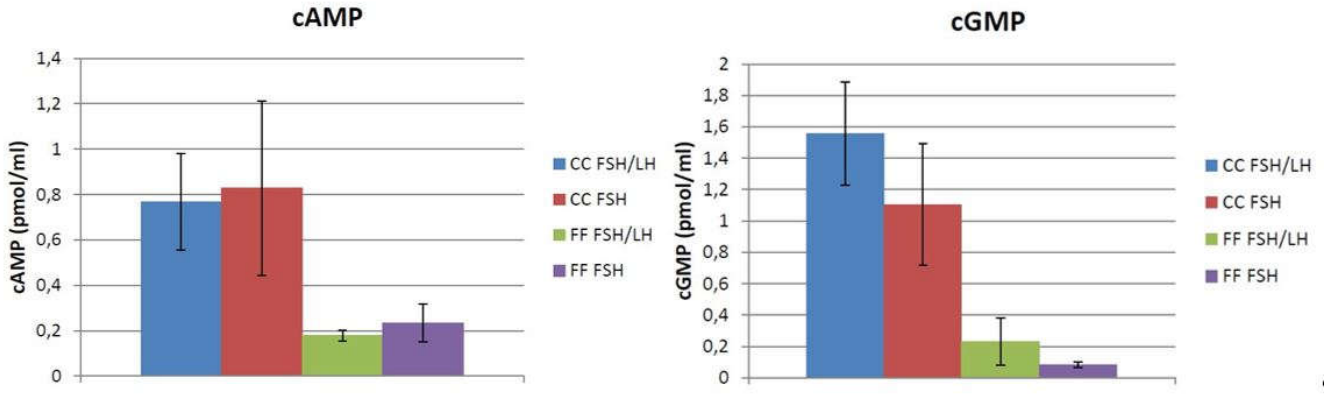


Figure 7. Average values of cAMP(a) and cGMP (b) from granulosa cell lysates after 40 hours of culture. Cells were obtained from the cumulus-cell complex (CC) and follicular-punctate fluid (FF) and were out of stimulation protocol with recombinant FSH/LH and recombinant FSH. 45 single measurements were undertaken for each group

Barberiet al[15]found that progesterone in follicular fluid was more pronounced after FSH/LH than after FSH treatment alone. We could confirm this effect, which we attributed to an increased secretion rate of granulosa cells. This was displayed at all measuring points and obvious in the cumulus-cell-complex. Increased progesterone levels in luteal phase were favourable criterions for pregnancy and life birth [15]and an expression of competent luteinized granulosa cells [16,17]. An important mechanism for this can be the increased absorption of cholesterol in mitochondria [18], by upregulation ofStAR (STARD1) (Steroidogenic acute regulatory protein), making cholesterol available for steroid biosynthesis[19,20]. Further investigations [5] showed that stimulation of the LH receptor increases the intracellular cAMP level and thereby upregulates protein kinase A (PKA), which activates the StAR protein. Our study could not detect an increased cAMP level, which may be cause by the used detection technology [21].Furthermore the addition of LH stimulation could modify prostaglandin F2α binding to its receptors and thus influences the regulation of StAR protein [9].

DISCUSSION

As there were no discernible morphologically differences after different stimulations during culture, we assumed that our results were due to molecular biological processes within the granulosa cells and their cellular environment. Barberiet al[15]found that progesterone in follicular fluid was more pronounced after FSH/LH than after FSH treatment alone. We could confirm this effect, which we attributed to an increased secretion rate of granulosa cells. This was displayed at all measuring points and obvious in the cumulus-cell-complex. Increased progesterone levels in luteal phase were favourable criterions for pregnancy and life birth [15] and an expression of competent luteinized granulosa cells [16,17]. An important mechanism for this can be the increased absorption of cholesterol in mitochondria [18], by upregulation of StAR (STARD1) (Steroidogenic acute regulatory protein), making cholesterol available for steroid biosynthesis [19,20]. Further investigations [5] showed that stimulation of the LH receptor increases the intracellular cAMP level and thereby upregulates protein kinase A (PKA), which activates the StAR protein. Our study could not detect an increased cAMP level, which may be cause by the used detection technology [21].

Furthermore the addition of LH stimulation could modify prostaglandin F2α binding to its receptors and thus influences the regulation of StAR protein [9]. In our investigation Inhibin A tends to higher levels in the cumulus-cell-complex after FSH stimulation alone. This could be an explanation for better follicle recruitment after FSH/LH [22,23]. However, the differences between the two fractions were striking. Assuming the suppressive effect of Inhibin A, our observations could be explained by maturation of the oocyte-cumulus-complex in the dominant follicle during LH surge [4, 6, 24, 25]. Increased secretion of VEGF could favour proliferation of cytoplasmic cell extensions of granulosa cells in maturing follicles[26, 27]. On the other hand, neoangiogenesisis relevant for vascular formation in the area of maturing follicles as well as the corpus luteum, which supports the supply of proteoglycans and lipids [26, 28]. In cell biological studies [19], however, it has so far only been possible to show for FSH that VEGF synthesis is induced via the PI3K/AKT/mTORsignalling pathway. The meaning of LH in this context is not yet fully understood.

In addition to its known vasoconstrictive effects, endothelin-1 has already been described within the ovarian endothelin-renin-angiotensin system [29] influencing follicular maturation and the corpus luteum [8]. Increasing endothelin-1 secretion, angiogenesis, cell migration as well apoptotic processes [30]are influenced within the maturing follicle and corpus luteum. Our investigation supports these arguments, but was compromised by the low detection rate, as it was only possible to measure endothelin-1 after 16 hours and not in all test rounds. To understand higher rates in the cumulus-cell-complex further investigations are needed. cAMPcontent of granulosa cells was slightly higher after FSH stimulation, but significantly in the cumulus-cell-complex.For cGMP FSH/LH stimulation revealed higher contents but at similar distinct levels and distribution between both fractions. The assumed improvement of FSH/LH stimulation correlates with other observations[3] thatcAMPisassociated with follicle arrest and cGMP improvement offollicular maturation and recruitment [31]. Our results coincided with these investigations, even though the differences were small. To our knowledge, this is one of the first cell biological studies which showed biochemical differences in human granulosa cells after recombinant FSH/LH and FSH stimulation in elder patients undergoing In-vitro-Fertilisation. Especially Progesterone and VEGF as product of competent granulosa cells could give

insights in the developing follicle during stimulation. Conclusions must be drawn with caution, as the investigated hormones, growth factors and second-messengers are integrated into a highly complex system of molecular processes. Therefore isolated consideration keeps difficult.

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