Efficacy of Dehydroepiandrosterone (DHEA) to overcome the effect of ovarian ageing (DITTO): A proof of principle double blinded randomized placebo controlled trial

Keywords: Infertility, DHEA, In-vitro fertilization, Low ovarian reserve, Poor responders randomised controlled trial

Abstract

Objective: To evaluate the effect of DHEA supplementation on In-Vitro Fertilisation (IVF) outcome as assessed by ovarian response, oocyte developmental competence and live birth rates in women predicted to have poor ovarian reserve (OR). The feasibility of conducting a large trial is also assessed by evaluating the recruitment rates and compliance of the recruited participants with DHEA/placebo intake and follow- up rates.

Study design: A single centre, double blinded, placebo controlled, randomized trial was performed over two years with 60 women undergoing in-vitro fertilisation (IVF). Subjects were randomized, based on a computer-generated pseudo-random code to receive either DHEA or placebo with both capsules having similar colour, size and appearance. 60 women with poor OR based on antral follicle count or anti- Mullerian hormone thresholds undergoing IVF were recruited. They were randomised to receive DHEA 75 mg/day or placebo for at-least 12 weeks before starting ovarian stimulation. They had long protocol using hMG 300 IU/day. Data analysed by "intention to treat". Ovarian response, live birth rates and molecular markers of oocyte quality were compared between the study and control groups.

Results: The recruitment rate was 39% (60/154). A total of 52 participants (27 versus 25 in the study and placebo groups) were included in the final analysis after excluding eight. While the mean (standard deviation) DHEA levels were similar at recruitment (9.4 (5) versus 7.5 (2.4) ng/ml; $P = 0.1$), the DHEA levels at pre-stimulation were higher in the study group than in the controls (16.3 (5.8) versus 11.1 (4.5) ng/ml; $P < 0.01$). The number (median, range) of oocytes retrieved (4, 0–18 versus 4, 0–15 respectively; $P = 0.54$) and live birth rates (7/27, 26% versus 8/25, 32% respectively; RR (95% CI): 0.74 (0.22-2.48) and mRNA expression of developmental biomarkers in granulosa and cumulus cells were similar between the groups.

Conclusion: Pre-treatment DHEA supplementation, albeit statistical power in this study is low, did not improve the response to controlled ovarian hyperstimulation or oocyte quality or live birth rates during IVF treatment with long protocol in women predicted to have poor OR.

Introduction

Postponement of childbearing is a worldwide common practice because women prefer to pursue higher education and career advancement before making the decision to conceive (1). Consequently, there is a marked increase in number of women with subfertility, who seek medical interventions to overcome the involuntary childlessness incurred as a result of ovarian ageing.

Ovarian ageing, dictated by a decline in quantity and quality of oocytes within the ovaries (2,3), is accountable for the age related decline in fertility (4–7), and of the age related increase in adverse pregnancy outcomes such as miscarriage (8,9), and aneuploid pregnancies (10–12).

Women who suffer from ovarian ageing are more likely to be fast-tracked towards assisted reproductive technology (ART) but yet are still at risk of having poor treatment outcome due to diminished ovarian reserve leading to poor response to ovarian stimulation. While chronological age is a major determinant of ovarian ageing, remarkable variation across individuals in the rate of ageing process can be observed due to genetic and environmen- tal factors. The pace at which ovarian ageing occurs is determined by the rate of primordial follicle initiation, the rate of follicular growth and the rate of follicle turnover (13,14). These processes are regulated by a number of intra-ovarian autocrine and paracrine growth regulators and gonadotrophic endocrinological control which are theoretically amenable to therapeutic intervention with hormonal drugs such as Dehydroepiandrosterone (DHEA) (15,16).

DHEA, the most abundant steroid hormone in human body, is a weak androgenic steroid secreted primarily from zona glomer- ulosa of the adrenal glands, but can also be produced from both the ovaries and via peripheral conversion. As DHEA levels have been observed to decline gradually, independently of menopausal status (17), it has been proposed as an anti-ageing medication. DHEA was used for the first time as a supplement prior to ovarian stimulation in IVF patients in 2000 (18) and since then a few observational studies have reported beneficial effects in terms of improving ovarian response and treatment outcome following ART in women with reduced ovarian reserve (18–24). A recent animal study performed by our group has demonstrated a positive impact of DHEA supplementation on ovine follicular development (25). In this paper, we used the ovarian cortical autograft model (26) to determine if DHEA supplementation had an effect on the early stage of folliculogenesis in sheep, a mono-ovulatory species in which, like the human, the time taken to complete folliculogenesis takes several months. We found that DHEA supplementation can increase the

rate of primordial follicle initiation and preantral follicular growth histologically (25). The underlying mechanisms resulting in this stimulatory effect are unknown but DHEA was observed to promote granulosa cell proliferation and enhance the level of AMH protein expression in the granulosa cells (25). These findings, along with findings from androgen supplement on ovarian follicular development data in other animal species (27– 29) indicate that DHEA may potentially have a beneficial role to fight against the effects of ovarian ageing in women undergoing IVF treatment.

Despite these positive animal studies, the evidence supporting a clinical benefit in utilising DHEA adjuvant therapy in women with poor ovarian reserve is equivocal. We have recently performed a meta-analysis examining this question and of 22 studies available at the time, only 3 controlled studies were eligible for analysis, which showed no benefit of DHEA supplementation on clinical pregnancy or miscarriage rates (30). More recently a few small randomised controlled studies, albeit having some methodological weaknesses, have similarly reported that DHEA does not improve clinical outcome in women with diminished ovarian reserve undergoing IVF treatment (31–34) although it is significant that only one of these studies was placebo-controlled and even then the size of this RCT was very small with 16 participants per treatment arm (31).

Despite this equivocal experimental evidence, a recent world- wide survey has shown that 26% of IVF clinicians add DHEA as adjuvant to IVF treatment protocols in women with low ovarian reserve (35). Despite widespread use of DHEA, clinical evidence as well as knowledge regarding underlying mechanisms of DHEA on improvement of ovarian response is still limited, warranting a well designed and large randomized placebo controlled trial. We anticipated the practical difficulty of conducting a robust large trial in women with diminished ovarian reserve due to potential limitations in recruiting a large number of eligible subjects and therefore have set out to do a pilot trial (DITTO—DHEA Intervention To Treat Ovarian ageing—trial) so that we could evaluate the feasibility of conducting a large multicentre study as well. The objective of this study was to determine the effect of DHEA on the ovarian response during controlled ovarian stimulation and the developmental competence of oocytes (oocyte quality) by using molecular and clinical parameters in women predicted to have poor ovarian reserve or ovarian ageing, defined by using reported AFC and AMH thresholds (36,37).

Materials and methods

Study design, participants and setting

The study was a pilot double-blinded, randomised, placebo-controlled trial conducted to evaluate whether DHEA supplementation prior to controlled ovarian stimulation increases the number of oocytes retrieved, the response to the stimulation protocol and IVF success rates; i.e. pregnancy rates, miscarriages, in women predicted to have poor ovarian response. It was a single centre trial performed at the Nottingham University Research and Treatment Unit in Reproduction (NURTURE), a tertiary university-based IVF clinic, University of Nottingham, United Kingdom. Women aged more than 23 years, who were predicted to have diminished ovarian reserved determined by antral follicle count scan less than 10 and/or serum Anti-Mullerian hormone less than 5pmol/L undertaking either IVF or ICSI treatment at the clinic, were asked to participate in the study at the time of their initial consultation. Patients had to have a regular 21–35days menstrual cycle. The exclusion criteria were women with conditions as follow; i) obesity with BMI >35kg/M^2, ii) having only single ovary, iii) diagnosed untreated hydrosalpinx, endometrial polyp or submucous myoma at beginning of her treatment, iv) history of seizure or epilepsy, v) were not enrolled in the study before, vi) diagnosed endocrino-logical disorders for example thyroid or adrenal diseases, vii) allergy to DHEA, and viii) treated with insulin for diabetic management. Participants were divided into two arms; i) intervention arm receiving 75mg DHEA capsule, and ii) control arm receiving placebo capsule, having similar appearance, size and colour but without DHEA powder. Both groups were advised to take their intervention medicinal products (IMPs) for at least 12 weeks before the egg collection procedure (prior to and during controlled ovarian stimulation). The IMPs were supplied from the licensed pharmaceutical manufacturer (St. Mary's Pharmacy, Cardiff, UK) authorised by the Medicines and Healthcare products Regulatory Agency (MHRA). A maximum number of 20 weeks supply of IMP were provided to all subjects.

Ethical considerations

The study was approved by the NHS Research Ethics committee (East Midlands-Derby, reference number NRES 12/EM/0002), the Medicines and Healthcare products Regulatory Agency (MHRA) for using of investigational medicinal product (IMP), and the Nottingham University Hospitals Trust Research and Development department. It complied with the GCP practice and in agreement with the Declaration of Helsinki 1996, the Medicines for Human Use Regulations, Statutory Instrument 2004, and all involved UK laws and regulations. All

participants were informed regarding treatment involved and possible side effects of the IMP. They were allowed at least 24h for making their own decision after consultation with partner and family members. In addition, they were always eligible to quit the trial at any time and they were informed which allocation treatment they received when the study had been completed. Patients or patient groups were not involved in the initial stages of study design. However, while the study was being planned, about ten patients expected to have poor ovarian response have been informed about the DHEA trial at the time of IVF consultation and all of them expressed interest in taking part in the study.

Randomisation and informed consent

Women with low AMH (<5 pmol/L) and/or AFC (<10) at the time of initial evaluation (eligible participants) were given a DHEA information leaflet and invited to participate in the study. Written informed consent was signed at the time of patient initial consultation or other following appointment. Age stratified randomisation was performed by computer-based random permuted block randomisation, created by the University of Nottingham Clinical Trials Unit (CTU). Participants were issued their trial number as well as randomisation number and were issued their corresponding IMPs supply from the unblinded pharmacist according to the randomisation. They were randomised to receive either capsules of 75 mg DHEA or placebo taken orally once daily for at least 12 weeks before and during controlled ovarian stimulation until the day before egg collection. The CTU kept the randomisation code while the list of patients corresponding to allocated treatment was maintained by the pharmacy. There were plans to break the code if any participants developed a serious adverse event (SAE).

Study outcomes

The primary outcome was the number of oocytes retrieved at the time of egg collection. Biochemical pregnancy rates (positive urine pregnancy test at 2 weeks after embryo transfer), clinical pregnancy rates (positive foetal cardiac activity from ultrasonography at 6 weeks following embryo transfer), miscarriages, and live birth rates were evaluated as secondary outcomes. Other outcome data included duration of ovarian stimulation, gonadotrophin doses, serum oestradiol on hCG day, fertilisation rates, cleavage and blastocyst rates. Serum DHEA, AMH, and IGF-1 levels at the time of initial evaluation, down regulation scan, and egg collection were also evaluated. Moreover, oocyte quality and possible mechanisms of DHEA action were evaluated by determining the level of mRNA expression in cumulus and granulosa cells of ten markers including gonadotrophin (FSH and LH) receptors, pen- traxin 3 (PTX3), hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (PTGS2), BMP antagonist-Gremlin

(GREM1), and epidermal growth factor (EGF)-like signalling molecules; ampiregulin (AREG) epiregulin (EPEG) and betaregulin (BTC) using real-time PCR.

Study protocol (38)

Standard long down-regulation protocol for ovarian stimulation was used in all patients. Participants commenced GnRH agonist, either nafarelin (Synarel; Pharmacia, UK) or buserelin (Aventis Pharma, Kent, UK), approximately seven days prior to the expected first day of menstrual cycle (treatment cycle). Participants were reviewed two weeks after commencing nafarelin or buserelin to check for down regulation with ultrasound scan and blood test. After confirmation of pituitary down regulation (thin endometrium (5 mm), ovarian quiescence (follicles 10 mm), and serum oestradiol 200 pmol/L), controlled ovarian stimulation utilising 300 IU/day of Human Menopausal Gonadotrophin (HMG, Menopur, Ferring, UK) subcutaneous injection were started. Blood samples were collected at down-regulation scan visit for later AMH, DHEA, and IGF-1 assays. Follicle growth was monitored by transvaginal ultrasound scan and serum oestradiol from day 8 of stimulation. An ovulation trigger was given by subcutaneous injection of 10000 IU human chorionic gonadotrophin (hCG), Pregnyl (Organon Laboratories Ltd, Cambridge, UK) once the follicles met the criteria for ovum pick-up (OPU) procedure (≥3 leading follicles size ≥17 mm). However, if the participants developed poor ovarian response (<3 pre-ovulatory follicle response of ≥14 mm by day 12 of stimulation), the ovulation trigger was done when 1–2 lead follicle/s reached the size of ≥17 mm. IMPs and gonodotrophin injection as well as GnRH agonist were continued until the day of hCG administration. OPU was performed at 36h following hCG administration.

Extra blood samples were collected on the day of egg collection for hormonal assays (DHEA, AMH and IGF-1). Either conventional in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) was chosen for each participant on the embryologist's discretion depending on recent semen analysis results and past treatment cycle history. All media used during oocyte collection, fertilisation and embryo culture period were supplied by Vitrolife Ltd. (Warwick, UK). Follicular fluid of the largest follicle was collected and kept separately. Both serum and first follicular fluid were centrifuged at 4000 rpm for 5min. The supernatant from both serum and follicular fluid samples were store at 20C until assayed. The follicular cell pellet was also collected and snap frozen in liquid nitrogen and stored at 80C for later real-time PCR analysis. For ICSI patients, all oocytes were denuded using hyaluronic acid solution according to the standard procedure but the first cumulus oocyte complex (COC) collected from the largest follicle was kept separate from the rest. Oocyte transfer media immediately after the hyaluronic

drop of the first COC was immediately transferred to the laboratory where the media was centrifuged at high speed (>10,000rpm) for 5min. Due to the small quantity of cumulus sample, 50ml of cumulus in transfer media was diluted in a six volume (300ml) of RNAProtect agent (Qiagen, Manchester, UK) for RNA preservation, then snapped frozen in liquid nitrogen and stored a 80C for subsequent real- time PCR assays. The ICSI procedure was carried out on all metaphase II (MII) oocytes according to standard embryology practice. Fertilised embryos were cultured in microdrops under oil using G1/G2 sequential media. Media change from G1 to G2 was done on day 3 unless embryo transfer was performed. Embryo transfer with one or two embryos was performed on day 2, 3 or 5 following the day of egg collection. Extended embryo culture and blastocyst transfer were advised to the couple by the embryologist if there were 3 or more fertilised oocytes at fertilisation check. The number of embryos transferred was based on the age of the patient, quality of the available embryo/s, previous treatment history and funding criteria with national health service (NHS) funded patients were allowed only a single embryo transfer and self-funded patients could choose a maximum of two embryos for transfer. Embryo cryopreservation was done for all surplus good quality embryos (grade 1 or 2 cleavage stage embryos or grade A or B blastocysts). The study was specifically designed to include the first treatment cycle only of each participant. All women undergoing both conventional IVF and ICSI treatment were advised to self-administer progesterone pessaries (Cyclogest; Alpharma, UK) vaginally 400mg twice daily starting on the second day following egg collection until the day of urine pregnancy test or until 8 weeks of gestation, if positive pregnancy outcome.

A positive biochemical pregnancy outcome was confirmed by urine pregnancy test carried out, by a participant herself, approximately 14days following the day of embryo transfer. Positive clinical pregnancy outcome was defined by positive foetal cardiac activity at 6–7 week pregnancy scan. All positive clinical pregnancy outcome participants were contacted for their live birth outcome. The baseline characteristics, stimulation and outcome data was collected and stored in an electronic database until the randomisation code was broken. Any adverse effects reported by participants was also noted.

Hormone analysis

Determination of serum DHEA, AMH, IGF-1 and follicular DHEA and AMH was carried out by using, enzyme-linked immunosor- bent assays (ELISA) from commercial sources: The DHEA ELISA Kit (Demeditec Diagnostices, Kiel, Germany), Human anti-Mullerian Hormone (AMH) Elisa Kit (Beckman Couter, Krafeld, Germany), and IGF-1 E20 ELISA (Mediagnost, Reutlingen, Germany). All ELISA procedures were performed according to suppliers'

instructions (39–41). The intra and inter-assay coefficients of variation for all assays was less than 10% and 15% respectively.

Oestradiol (E2) levels were routinely measured during con- trolled ovarian stimulation to monitor ovarian follicular development utilising the automated ARCHITECT oestradiol chemiluminescent microparticle immunoassay (CMIA; Abbot Diagnostics, Longford, Ireland). Peak E2 value represents the E2 level that was measured in the sample obtained on the day of hCG trigger prior to egg collection. Again, the intra and inter-assay variation was less than 10%.

Real-time PCR analysis

Both granulosa cell and cumulus cell frozen pellets were thawed on ice. RNA was extracted using the RNeasy® Micro Kit (QIAGEN, Manchester, UK) according to the manufacturer's instructions. Instantaneously after, generation of cDNA libraries from resulting RNA elute was performed by using the Precision nanoScript™ Reverse Transcription kit (PrimerDesign Ltd, South- ampton, UK) according to the manufacturer's instructions. All cDNA samples were kept at 80 C until the real-time quantitative PCR (qPCR) was carried out. Real-time qPCR of mRNA expression levels of the following 10 genes; FSH receptor (FSHr), Luteinizing hormone/choriogonadotropin receptor (LHCGR), Hyaluronan syn- thase 2 (HAS2), Pentraxin-related gene (PTX3), Androgen receptor (AR), Prostaglandin-endoperoxide synthase 2 (PTGS2), Gremlin 1 (GREM1), Amphiregulin (AREG), Betacellulin (BTC), and Epiregulin (EREG), was then carried out on both granulosa and cumulus cell samples as batch when the clinical phase completed. Human- specific target primers labelled with double-dye hydrolysis probe (Taqman®) were designed and manufactured by PrimerDesign Ltd (Southampton, UK). A 15 ml real-time PCR reaction mixed of cDNA template, PrecisionFAST™ dNTP/polymerase/buffer mastermix, primer/probe mix in an opaque BrightWhite 96-well plate (Primerdesign, Southampton, UK) was performed in the Applied Biosystems 7500Fast Platform (Applied Biosystems, Life technolo- gies Ltd, Paisley, UK). The thermo-cycle program used was as follows: Enzyme activation for 2 min at 95 C followed by 50 PCR cycles consisting of denaturation for 5 s at 95 C and an annealing/ extension period of 30 s at 60 C. The target cycle threshold (Ct) values were analysed in the Microsoft Excel programme (IBM Corp, New York, USA) using the $2^{-\Delta\Delta CT}$ method outlined in (42) to calculate relative changes in gene expression compared to control (fold change), normalised against two endogenous reference genes YWHAZ and B2M. (The reference genes YWHAZ and B2M were selected in

advance utilising the geNormTM Reference Gene Selection Kit (PrimerDesign Ltd, Southampton, UK) as the most stable genes across the treatment and control.)

Statistical analysis

The study was planned as a pilot study to investigate the "proof of principle" whether DHEA supplementation prior and during ovarian stimulation will improve response and outcomes in the participants. The sample size was estimated based on the number of subjects that could be recruited over a period of 18 months. Approximately 600 women undergo IVF/ICSI treatment at NURTURE over an 18 month period. Considering 20% as predicted poor responders based on the inclusion criteria and 50% recruitment rate, the number of subjects that could be recruited during the study period was estimated as 60. The size of the effect on the primary outcome based on the data generated from this trial was expected to help to estimate the sample size for a large multicentre trial.

Statistical analysis was performed based on intention to treat analysis. Only the first treatment cycle following randomisation was analysed. Participants were randomised only once and the subsequent frozen/thawed embryo transfer cycle was not counted. If a treatment cycle was cancelled because of poor ovarian response or if there was no egg retrieved at all at egg collection, the number of oocytes (primary outcome) would be counted as zero. If participants had spontaneously conceived during the IMP treat- ment, they were advised to stop medication immediately and we assigned the number of oocytes retrieved as the highest number observed in the study cohort for primary outcome analysis. In addition, they were also included into the positive pregnancy outcome group for secondary analyses.

Statistical analyses were done using the Statistical Package for the Social Sciences (SPSS version 21, Chicago, IL). The number of oocytes were failed to demonstrate a normal distribution, therefore the data was expressed as median (interquartile) and the difference between groups were evaluated by using the Mann- Whitney U test. Differences in clinical and other proportional outcomes were tested by the chi-square test. Numerical data of stimulation, hormone and cumulus/follicular cell gene expression were analysed by either the student t-test or Mann-Whitney U test when appropriate. A two-tailed P value of <0.05 was defined to indicate a statistical significance.

Results

The participant flow diagram is shown in Fig. 1. During a 2-year recruitment period from May 2012 to May 2014, a total of 982 women underwent IVF/ICSI cycles. 702 women were assessed for eligibility and 154 of them were found eligible and invited to take part in the study. 60 women agreed to take part in the study giving the recruitment rate of 39%. One woman spontaneously conceived before taking medication while 7 participants were lost to follow up and therefore, a total of 8 participants were excluded from the study. In 52 included participants, 2 women in the treatment group spontaneously conceived after taking DHEA medication for approximately 4 and 6 weeks. All 50 participants underwent controlled ovarian hyperstimulation (COH) using long agonist protocol, nevertheless, egg collection was cancelled in 4 participants due to poor ovarian response: 2 had intrauterine insemination (IUI) treatment, 2 cycles were completely cancelled due to poor response. Embryo transfer could not be performed in 5 other cycles: 4 cycles resulted in failed fertilisation, and 1 cycle only had an immature oocyte, which was not suitable for ICSI treatment (no mature oocyte collected). All the 5 cancelled cycles due to poor response were assigned to have zero number of oocytes retrieved (primary outcome). Table 1 demonstrates baseline demographic data of the treatment and placebo groups. Mean (`Standard Deviation; SD) age of participants was 36.8 ± 3.9 and 35.2 ± 5.3 years, in the DHEA and placebo group, respectively. The mean baseline antral follicle count and serum AMH were 8.2 ± 3.2 and 3.8 ± 3.7pmol/L vs. 8 ± 2.9 and 3.6 ± 3.1pmol/L respectively, representing low ovarian reserve in both groups. A dissimilarity in causes of subfertility were observed (p value <0.05 using chi-square test) but there was no significant difference in duration of infertility and other baseline characteristics (patients' BMI, ethnicity, and baseline serum DHEA levels) between the study and control groups.

Number of oocytes retrieved and stimulation outcome

The number of oocytes retrieved was similar between both groups (median, range: 4, 0–18 vs. 4, 0–15, p=0.54; Fig. 2). In addition, doses of gonadotrophin used for ovarian stimulation and duration of stimulation were comparable so as the proportion of observed poor response (No of oocytes retrieved ≤ 4) to controlled ovarian stimulation (Table 2).

Secondary embryological and pregnancy outcome (Table 2)

In terms of embryological data, a similar fertilization rate (64.5 ± 24.9%) was observed between the treatment and control (48.0 ± 30.4%) groups although there was a favourable trend towards the treatment group (p = 0.052). Nonetheless, the numbers of blastocysts acquired

were not significantly different resulting in only 16% of grade A blastocyst transfer on day 5 in each group. In our centre, blastocyst culture was performed when patients have at least 4 cleavage embryos (3 if the cohort are all top morphological grade embryos), otherwise cleavage transfer was done instead on day 2 or 3. With regard to pregnancy outcomes, biochemical pregnancy rates, clinical pregnancy rates and miscarriages were not different between the two groups. All miscarriages, but 2 in the control group, were in the first trimester. Rate of preterm birth were 11% and 4% in both treatment and placebo group, respectively. Overall, there was one viable twin pregnancy diagnosed at 6 week scan, in the treatment group. Another participant in the treatment group was diagnosed with vanishing twin pregnancy but ended up with one singleton born. There were no congenital anomalies observed in all 15 babies born in this study.

The number of single embryo transfer (SET) in the treatment and placebo groups was 9/27 (33.3%) and 10/25 (40%) respectively and corresponding live birth rates per embryo transfer were 3/9 (33.3%) and 5/10 (50%) respectively. Live birth rates per embryo transfer with double embryo transfer (DET) in the treatment and placebo groups were 2/13 (15.4%) and 3/8 (37.5%) respectively. Logistic regression analysis showed number of embryos trans- ferred did not influence the over all live birth rates with odds ratio of DET over SET were 1.1 (95% CI 0.5-2.43; $P = 0.8$).

Hormonal levels and gene expression profiles

There was no significant difference in baseline (pretreatment) serum DHEA and AMH levels between the two groups (Table 3). In the treatment group, an increase in serum DHEA at both down regulation (DR) scan (pre-stimulation) and egg collection visits was observed (Baseline mean (SD) ng/ml: 9.4(5.0) vs. DR scan 16.4(5.8) vs. egg collection 13.0(7.0); $p < 0.05$ using repeated sample ANOVA). Curiously, a significant increase in serum DHEA over this period was also observed in the control group (Baseline 7.5 (2.4) vs. DR scan 11.1 (4.5) ng/ml; $p < 0.05$). While the mean increase in DHEA levels in the treatment group at DR scan and at egg collection compared to baseline levels were 73.4% and 48% respectively, the corresponding increase in the placebo group were 38.3% and 20% respectively. However, the mean serum DHEA levels at DR scan and egg collection time points were higher in the treatment group compared to control group. In contrast, there were no significant differences in follicular DHEA levels measured in the largest follicle at the time of egg collection between two groups (9.9 (3.8) vs. 11.5 (5.7), $p = 0.26$) (Table 3).

Mean serum AMH levels from patients in both groups followed a similar trend where the levels were significantly higher at down regulation and then reached their lowest value at the egg

collection procedure (Table 3). Neither serum AMH levels at all time points nor follicular AMH at egg collection was different between DHEA and placebo groups. Serum IGF-1 levels were not different between treatment groups and remained stable throughout the experiment. Moreover, there was no increase in peak serum E2 and endometrial thickness on the hCG day following DHEA supplementation when compared to the placebo group (Table 3).

Comparison of mRNA expression levels for target genes in cumulus (Fig. 3) and granulosa (Fig. 4) cells from ovulatory follicles revealed no differences between the treatment and control groups. For ICSI patients, there was no significant alteration in 8 cumulus mRNA expressions, including Ar, PTGS2, HAS2, PTX3, GREM1, AREG, EREG, and BTC, in DHEA group when compared to the control ($P > 0.05$ determined by Mann Whitney U test). There was a significant amplification failure of FSHr and LHr gene expression and a direct comparison could not therefore be executed. Likewise, gene expression in granulosa cells were similar between both treatment and control groups.

Adverse outcome and compliance

Adverse effects of 75mg/day DHEA supplementation were considerably low. There was no serious adverse reaction (SAR) throughout the research. Three patients in the DHEA group had reported side effects, one with non-specific gastro-intestinal (GI) disturbance (nausea) and two with androgenic side effects (acne and oily skin). Nonetheless, three patients in the control group also reported side effects with two reporting GI symptoms (nausea) and one reporting acne and oily skin. All patients with androgenic side effects did not require any further treatment and all symptoms resolved after completing their stimulation cycle. Two patients with GI disturbance (1 each from both groups) had stopped the medication after taking the trial medication for over two weeks but still continued with their controlled ovarian stimulation treat- ment. Overall, mean duration of DHEA/Placebo supplementation was 81 days (range 16–112 days).

Discussion

This is the first double blinded placebo controlled randomise controlled trial evaluating the effect of pre-treatment DHEA supplementation in women predicted to have poor response with ovarian response as the primary outcome and reporting molecular markers of oocyte quality as an outcome measure. The data from this study indicate that DHEA adjuvant treatment does not improve the ovarian response and treatment outcome as measured by the number of oocytes retrieved, clinical pregnancy rates or live birth rates during IVF treatment with long agonist protocol in women predicted to have poor ovarian response. In addition, there was no improvement in oocyte quality with DHEA treatment as a panel of 10-genes expression profile, molecular markers of oocyte quality, was not different in both cumulus cell and granulosa cell samples between DHEA treatment and control groups.

Despite a better fertilisation rate with marginal significance observed in the treatment group (64.5% vs. 48.0%; $p = 0.052$), there was no difference in either stimulation outcomes (oocyte number, days of stimulation and doses of hMG used), or clinical outcomes (pregnancy and miscarriage rates) between participants who received DHEA supplementation and who received placebo. In fact, the trend was lower pregnancy (RR 0.75; 95% CI: 0.23–2.39) and live birth rates (RR 0.74; 95% CI: 0.22–2.48) in the DHEA group compared to the control group (Table 2) albeit the difference was not statistically significant. While the results from this study agree with other recent similar RCTs (31,32), our study is the first double blinded, placebo controlled randomised trial reporting ovarian response as the primary outcome in predicted poor responders and therefore with least potential bias. Although the study reported by Kara, et al. was a larger RCT, this was not blinded and not placebo controlled as the participants in the control group were not having any IMPs (32). While Yeung, et al.'s study was blinded and placebo controlled, the sample size was small with only 32 participants in total and also the randomisation was using sealed envelopes which may have posed potential bias to blinding. Further, the defined primary outcome in this study was antral follicle count after 12 weeks of DHEA treatment in the study (43). In contrast to our study findings, another RCT published by Wiser, et al. reported a higher cumulative live birth rate over two cycles in 33 women with predicted poor response, although the number of oocytes retrieved and live birth rate in the first IVF cycle were similar between the study and control groups. In addition to being un-blinded and not placebo controlled, there were other methodological weaknesses with randomisation being done using sealed envelopes (24).

As reported (38), the study was conducted as both a proof of principle RCT with ovarian response as a surrogate for clinically important outcomes and as a pilot trial to evaluate the feasibility of conducting a large late phase multicentre RCT to test the effect of DHEA on live births. While there had been no power calculation to determine sample size because this was a planned pilot trial, the data from this trial indicate that there is lack of a positive effect of DHEA on IVF outcome and not even a trend and therefore the results do not support the concept of using DHEA as an adjunct to IVF for improving treatment outcomes in women predicted to have poor ovarian response. However, the successful recruitment, albeit the rate of recruitment being only 39%, suggests a definitive large multicentre trial would be feasible, but the lack of effect of DHEA on IVF outcome suggests it is a low priority.

The study utilised ovarian reserve tests (both AFC and AMH) to determine women who are predicted to be poor responders based on the data earlier published from our group (44). Although younger women (mean age 36 years old) were recruited, participants responded poorly to the stimulation protocol (median oocyte number = 4, percentage of patient having oocyte 4 = 52%) with an incidence of overall cycle cancellation at 20%. Interestingly, two participants in the DHEA group did spontaneously conceive during the treatment period, although the numbers are clearly too small to form any causal association with exposure to DHEA. It was also interesting that although strict criteria in the use of AMH and AFC as predictors of poor response were used, some patients in this group yielded higher number of oocytes retrieved that entered into the range of normo-responders (maximum number of oocyte retrieved=18). It is therefore possible that the ovarian reserve markers employed in this study alone were not able to detect patients with a true low ovarian reserve and this may be one of the explanations why negative finding were observed in this study. In this regard, this trial had been planned before the Bologna criteria were published, so these criteria were not used to define the inclusion criteria in this study (45).

While there may be criticism on the use of long agonist protocol for predicted poor responders in our study, instead of short agonist or antagonist protocol, there is no evidence to support the use of one particular protocol to another in women with low ovarian reserve (46–49). In fact, one recent RCT has shown an improved ovarian response with the use of long protocol compared to short agonist protocol and similar response in comparison to antagonist protocol (49). Further, this study was planned to minimally disrupt our standard clinical practice and the NURTURE fertility clinic has been using the long agonist protocol for the majority of patients with satisfactory live birth rates of 37.3%, 13.3% in women aged 38– 39 and 40–42, respectively (50).

Another major strength of the current study is that we backed up the clinical findings with molecular analysis of cumulus mRNA expression of gonadotrophin receptors (FSHr, LHr), androgen receptor (Ar), markers determining oocyte developmental competence; including HAS2, PTX3, PTGS2, GREM1, and epidermal growth factors signalling molecules (AREG, EREG and BTC) (51–54). Granulosa cell expression of a similar panel of genes was also investigated, mainly to try and understand the mechanism of the effect of DHEA on IVF treatment. There were no significant up- and down-regulation of these ligands in the DHEA group compared to placebo group, hence confirming the observed clinical effects in the study that DHEA at the particular dose and duration has no definite effect at the molecular level. In addition, neither serum (AMH, IGF-1, oestradiol) nor follicular (DHEA, AMH) hormone levels was increased in the treatment group when compared to the control and this result contrasts to other findings in which serum AMH and IGF-1 were reported elsewhere in the literature to simultaneously increase when DHEA was supplied to the patients (55). The results of this study also disagree with earlier results we have published utilizing an animal experimental model in which we found that DHEA supplementation significantly increased both serum AMH and follicular AMH protein expression (25). These differences, however, may be related to the fact that many of these effects in sheep were observed during the earlier stages of follicle development when AMH expression levels are high rather than in large ovulatory sized oestrogenic follicles in which AMH levels are known to be low (56,57). However, the lack of a clinical effect of DHEA treatment in the current experiment may also be related to the dose and treatment duration of DHEA, which may need to be altered to influence the clinical outcome in women. Further various DHEA preparations from various sources are available in the market. In our study, we used micronized DHEA to prepare the IMP capsule, so we could ensure the dose of medication prescribed is accurate.

Participants were advised to strictly take only the IMPs at least 3 months prior to commence ovarian stimulation. Even though serum DHEA levels were higher at both down regulation scan and egg collection in the treatment group, a significant increase of mean serum DHEA at down regulation was also noted in the placebo group. It is important to note that 4 out of 25 serum samples in the control had raised DHEA at both the down regulation scan and egg collection stages; and 3 out of these 4 were pregnant. While participants were instructed to take only the IMP (DHEA or Placebo as randomised), we cannot rule out the possibility of some women taking exogenous DHEA. However, serum DHEA rising spontaneously through some unknown mechanism in these placebo treated controls cannot be excluded as there is very little known concerning normal variation of DHEA during women's natural and stimulated cycles.

Thus, in addition to the pharmacological actions of DHEA (pharmacodynamics/pharmacokinetics) there is a need to identify the specific population who will be benefited and the dose and duration of intervention needed before further evaluating DHEA as an intervention in standard IVF practice.

In conclusion, pre-treatment DHEA supplementation doesn't seem to improve the ovarian response and IVF outcome as measured by the number of oocytes retrieved, clinical pregnancy rates or live birth rates during IVF treatment with long agonist protocol in women predicted to have poor ovarian response. In addition, there was no improvement in oocyte quality with DHEA treatment as a panel of 10-genes expression profile, molecular markers of oocyte quality, was not different in both cumulus cell and granulosa cell samples between the treatment and control groups. The data from the study, although statistical power is low, do not support the idea of using DHEA as an adjunct to IVF for improving treatment outcome in predicted poor responders. While there has been a great deal of attention in the use of pre-treatment DHEA in predicted poor responders recently, this practice should be restricted to as part of large RCTs.

Ethics approval

The approval of the study was granted by the NHS Research Ethics Committee (Ref number NRES:12/EM/0002), the Medicines and Healthcare products Regulatory Agency (MHRA), and the Nottingham University Hospitals Trust Research and Development department.

Clinical trial registration number

EudraCT number: 2011-002425-21,
www.clinicaltrials.gov; CTA reference: 03057/0053/001-0001.

Authors' contributions

KJ, BC, AN and WM have substantially contributed to the conception design, analysis, interpretation, drafting and revising and critical evaluation of the article. KJ, AN, MB, NRF and LP have contributed to recruitment and data collection. The final manuscript was read and approved by all authors.

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Competing interests

None.

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Table 1: Baseline characteristics of participants in both treatment and placebo groups

	DHEA n = 27	Placebo n = 25	P value
Age in years. Mean (SD)	36.8 (3.9)	35.2 (5.3)	0.198
Ethnicity **White British** **Non-white British**[¶]	 100% 0%	 84% 16%	 0.197
BMI (kg/m^2)	24.5 (4.7)	23.7 (3.3)	0.592
Antral follicle count. Mean (SD)	8.2 (3.2)	8 (2.9)	0.675
Serum AMH in pmol/L. Mean (SD)	3.8 (3.7)	3.6 (3.1)	0.844
Serum DHEA in ng/ml. Mean (SD)	9.4 (5.0)	7.5 (2.4)	0.114
Duration of infertility in months. Mean (SD)	35.4 (15.1)	36.8 (18.2)	0.758
Infertility diagnosis n (%)			0.04
Endometriosis	4 (14%)	5 (20%)	
Low ovarian reserve	5 (18%)	5 (20%)	
Male factor	2 (7.1%)	5 (20%)	
PCOS/anovulation	2 (7.1%)	0	
Tubal factor	1 (3.6%)	5 (20%)	
Unknown	14 (50%)	5 (20%)	

[¶] For placebo group, 2 (8%), 1 (4%), and 1 (4%) were Indian/Pakistani, African British, and others race, respectively

Table 2: Comparison of clinical outcomes between treatment and placebo groups

	DHEA (n = 27)	Placebo (n = 25)	RR (95% CI)	P value
Stimulation outcomes				
NO. of oocytes retrieved median (range)	4 (0-18)	4 (0-15)		0.538*
Participants with NO. of oocyte retrieved ≤4	14 (51.9%)	13 (52.0%)		0.991
Total HMG (IU) mean (SD)	3801.6 (1007.9)	3802.2 (678.9)		0.998**
Duration of stimulation (days): median (range)	12.5 (10-17)	13 (10-14)		0.810*
Cycle outcomes				
Fertilisation rate¶ (%) mean (SD)	64.5 (24.9)	48.0 (30.4)		0.052**
Blastocyst rate¶¶ (%) mean percentage (SD)	36.8 (26.6)	45.1 (32.1)		0.499**
Grade A blastocyst transfer	4/25 (16%)	4/25 (16%)		0.906
Cycle cancellation or no embryo for transfer	4/25 (16%)	7/25 (28%)		0.226
Pregnancy outcomes				
Implantation (Biochemical; urine pregnancy test positive)	10 (37%)	11 (44%)	0.75 (0.24-2.27)	0.609
Clinical Pregnancy	8 (30%)	9 (36%)	0.75 (0.23-2.39)	0.625
Miscarriage	3 (11%)	3 (12%)	0.92 (0.17-5.03)	0.920
Livebirth	7 (26%)	8 (32%)	0.74 (0.22-2.48)	0.629
Preterm (<37w)	3 (11%)	1 (4%)		0.565
Term	4 (15%)	7 (28%)		

* Mann Whitney ** Student t test

¶ N= 23 and 22 for treatment and placebo group, respectively.

¶¶ Blastocyst culture was performed only if number of cleaved embryos was at least 4 (3 if all were good morphological grade); n = 12 for each treatment group.

Table 3: Comparison of serum and follicular hormones between treatment and placebo groups

Serum hormone	Treatment mean(SD)	Control mean(SD)	P value¶
DHEA (ng/ml)			<0.01
Pretreatment	9.4 (5.0)	7.5 (2.4)	0.092
Down regulation Scan	16.3 (5.8)[a]	11.1 (4.5)[b]	<0.01
Egg collection	13.0 (7.0)[a]	9.0 (6.2)	<0.01
AMH (pmol/L)			0.405
Pretreatment	3.8 (3.7)	3.6 (3.1)	0.782
Down regulation scan	6.8 (2.8)[c]	7.0 (5.2)[d]	0.382
Egg collection	1.7 (1.7)[c]	1.8 (1.5)[d]	0.183
IGF-1 (ng/ml)			
Pretreatment	183.2 (39.9)	179.7(23.4)	0.734
Down regulation scan	181.8 (54.6)	179.7 (47.0)	0.896
Egg collection	185.1 (46.8)	179.5 (44.6)	0.697
Oestradiol on hCG day	4444 (3004)	5082 (2738)	0.463
Endometrial thickness	10.5 (3.2)	10.4 (2.6)	0.972
Follicular hormone (Mean ± SD)	Treatment	Control	P value¶¶
DHEA	9.9 (3.8)	11.5 (5.7)	0.256
AMH	6.9 (3.4)	9.2 (2.1)	0.734

¶ Repeated measure ANOVA for DHEA and AMH to evaluate p value across all time points. Independent-samples T test for serum oestradiol and follicular hormonal levels.

¶¶ Independent-samples T test after log transformation of data.

[a] Significant differences of serum DHEA were observed among time points of interest ($p<0.05$) in the treatment group.

[b] a significant difference was observed between pretreatment and down regulation scan DHEA in the control group ($p<0.05$).

[c,d] significant p values were detected between both pretreatment-down regulation and pretreatment-egg collection comparisons ($p<0.05$) in both treatment and placebo groups.

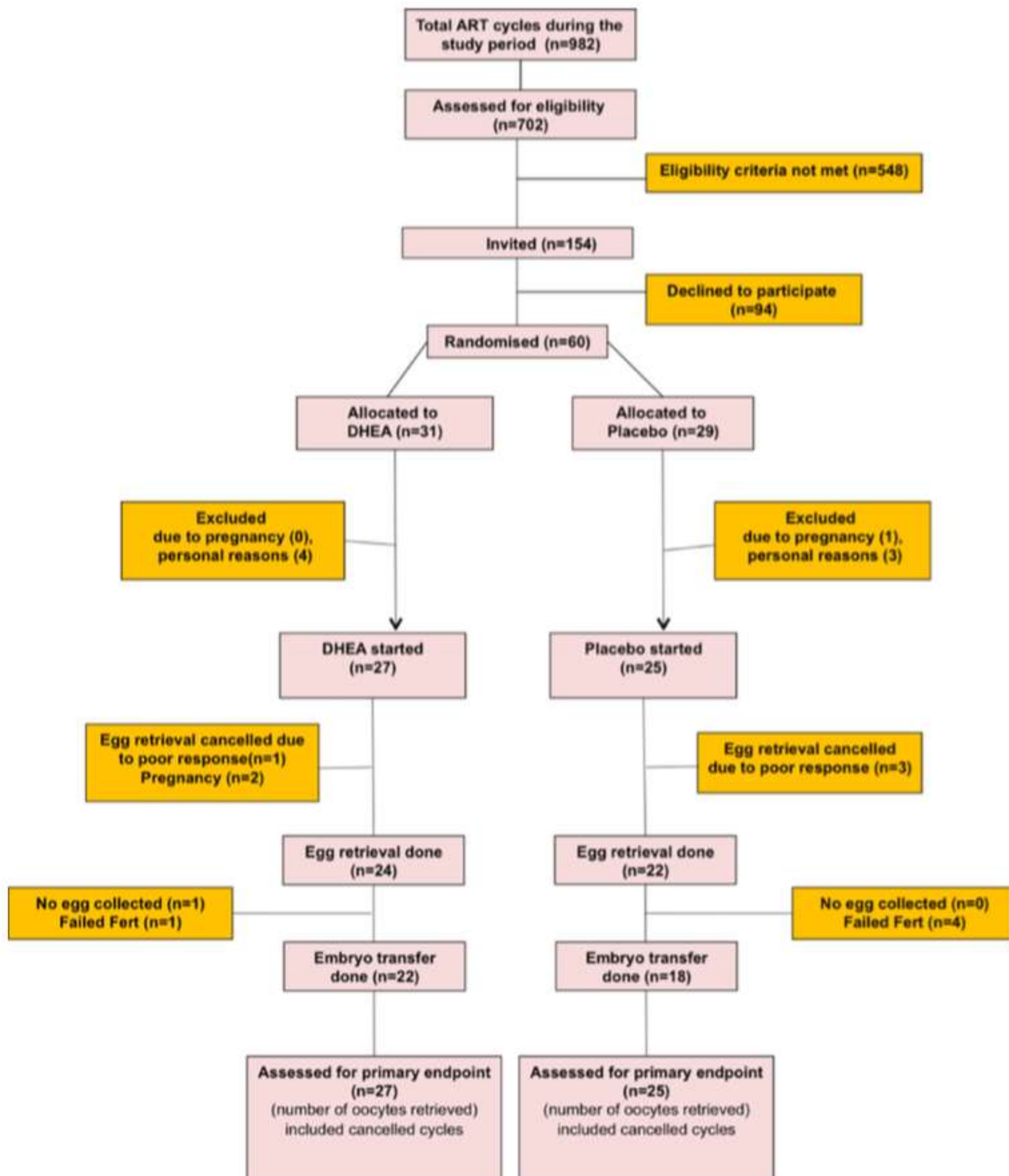

Fig 1. DITTO study flow

Fig. 2. Number of oocytes retrieved by group allocation. (X=pregnant prior to down regulation, egg retrieval not done, but coded as notional 18 oocytes for analysis).

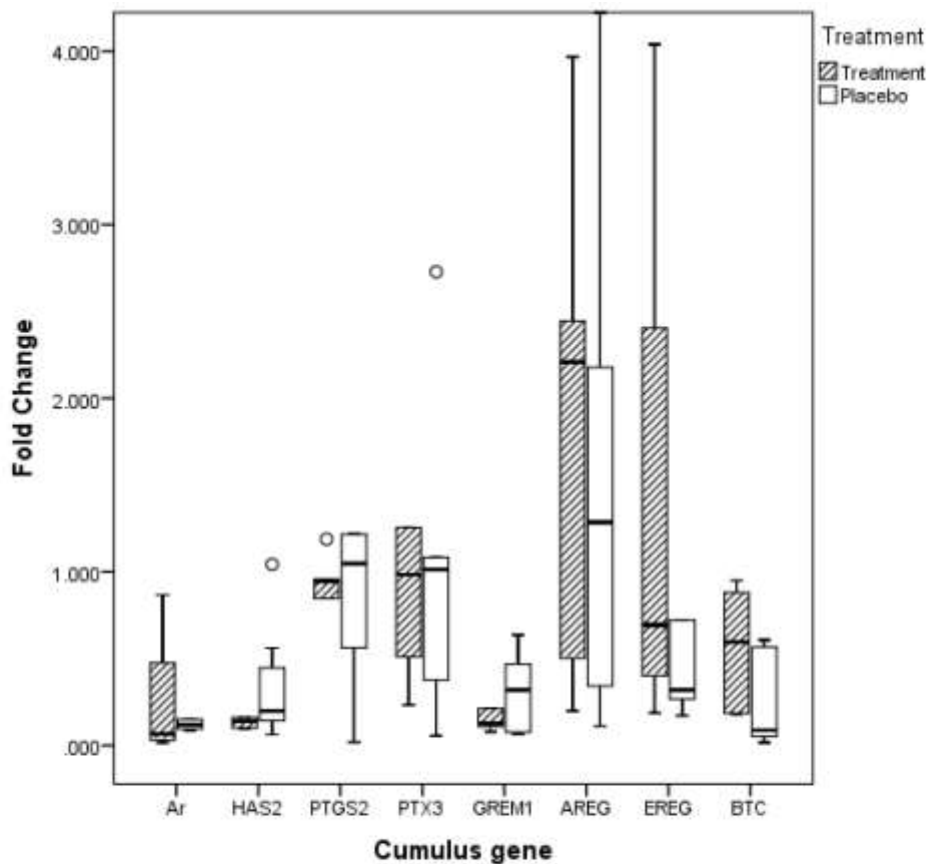

Fig. 3. Comparison of 8 cumulus gene expressions of cumulus-oocyte complexes (COCs) from the largest follicle at egg collection between treatment and control. Note: Amplification failure in all and 50% of samples were observed in LHr and FSHr, respectively. FSHr: FSH receptor; LHr: LH receptor; Ar: Androgen receptor; HAS2: Hyaluronan synthase 2; PTGS2: Prostaglandin synthase; PTX3: Pentraxin 3; GREM1: Gremlin; AREG: Ampiregulin; EREG: Epiregulin; BTC: Betaregulin.

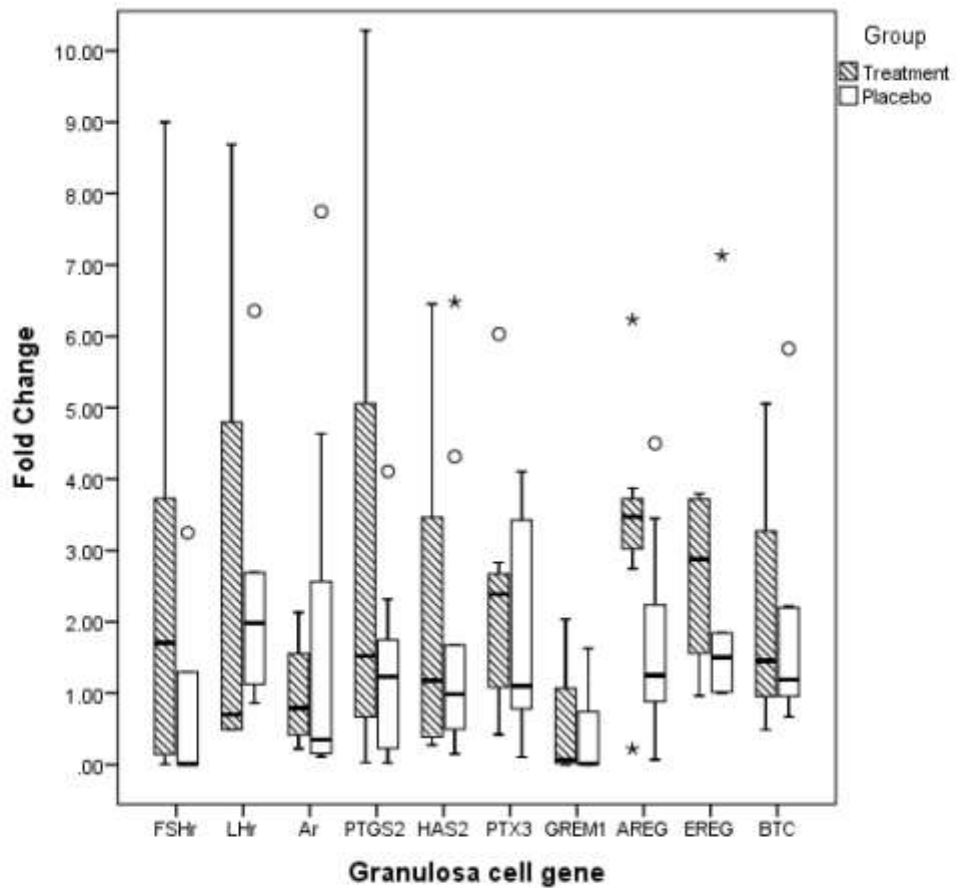

Fig. 4. Comparison of 10 granulosa gene expressions of granulosa cells from the largest follicle at egg collection between treatment and control.Note: FSHr: FSH receptor; LHr: LH receptor; Ar: Androgen receptor; HAS2: Hyaluronan synthase 2; PTGS2: Prostaglandin synthase; PTX3: Pentraxin 3; GREM1: Gremlin; AREG: Ampiregulin; EREG: Epiregulin; BTC: Betaregulin;