Effects of dehydroepiandrosterone on \textit{in vivo} ovine follicular development

A. Narkwichean\textsuperscript{1,2,*}, K. Jayaprakasan\textsuperscript{1,3}, W.E. Maalouf\textsuperscript{1}, J.H. Hernandez-Medrano\textsuperscript{1}, C. Pincott-Allen\textsuperscript{1}, and B.K. Campbell\textsuperscript{1}

\textsuperscript{1}Division of Obstetrics and Gynaecology, School of Clinical Sciences, University of Nottingham, Nottingham NG7 2UH, UK \textsuperscript{2}Department of Obstetrics and Gynaecology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand \textsuperscript{3}Reproductive Medicine and Fertility Unit, Royal Derby Hospital, Derby, UK

*Correspondence address. E-mail: mzxan@nottingham.ac.uk, amarin.narkwichean@gmail.com

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\textbf{STUDY QUESTION:} What are the effects of exposure of ovarian tissue to dehydroepiandrosterone (DHEA) supplementation in \textit{vivo}?

\textbf{SUMMARY ANSWER:} DHEA exposure stimulates initiation of primordial follicles and development of gonadotrophin-responsive preantral/early antral follicles possibly mediated through promoting granulosa cell proliferation and enhancing anti-Mullerian hormone (AMH) expression.

\textbf{WHAT IS KNOWN ALREADY?} Ovarian ageing is a cause of subfertility and is associated with poor outcomes of IVF treatment and premature menopause. A few clinical studies have shown that DHEA can improve ovarian response and increase the chances of pregnancy after IVF treatment in women with a diminished ovarian reserve (DOR) suggesting DHEA may help to overcome the effect of ovarian ageing. However, there are no data about how DHEA acts on ovarian folliculogenesis.

\textbf{STUDY DESIGN, SIZE AND DURATION:} A cortical autograft experimental model was conducted in six female sheep aged at least 24 months. The period of DHEA treatment in the animals lasted for 10 weeks.

\textbf{PARTICIPANTS/MATERIALS, SETTING, METHODS:} All the animals were subjected to unilateral oophorectomy. Half of the ovary was fixed for histological analysis as a time-zero control. The remaining tissue was used to isolate patches of ovarian cortex which were autografted back onto the ovarian pedicle. The grafting procedure eradicated all growing follicles and synchronized early follicular development. After a 10-week treatment period with DHEA implants, the ewes were sacrificed and the graft and remaining ovary were harvested. Histological and immunohistochemistry (IHC) findings, accompanied with serum hormonal profiles were compared to determine the effect on the follicle population.

\textbf{MAIN RESULTS AND THE ROLE OF CHANCE:} Higher proportions of the follicle population in the remaining ovary were observed to be in the antral stage after DHEA treatment. The observation coincided with an increase in the rate of primordial follicle initiation and preantral follicle development in cortical grafts and the remaining ovarian tissue, respectively. The IHC results indicated that DHEA increased the expression of both the proliferation marker (KI-67) in granulosa cells and the follicular AMH expression at the preantral and early antral follicle stages.

\textbf{LIMITATIONS, REASONS FOR CAUTION:} The experimental design compared follicle populations before and after DHEA treatment within individual animals to allow changes over time to be detected against a background of high inter-animal variation. However, since no controls without DHEA were included, we cannot say what would have happened over time in its absence, and it is possible that other factors may have resulted in the changes in follicle development observed during the experiment.

\textbf{WIDER IMPLICATIONS OF THE FINDING:} Our data supports the idea that DHEA might be a useful therapy to delay the effects of ovarian ageing. Therefore, it may have a role as an adjunct during IVF to improve ovarian response in women with DOR and as a treatment for premature ovarian insufficiency.

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\textbf{Key words:} dehydroepiandrosterone / ovarian follicular development (folliculogenesis) / diminished ovarian reserve / \textit{in vitro} fertilization
Introduction

The well-established observation of age-related decline in female fertility (Menken et al., 1986; Templeton et al., 1996) is primarily due to ovarian ageing, which dictates a decrease in both quantity and quality of oocytes within the ovaries (Gougeon et al., 1994; Gougeon, 1998; Broekmans et al., 2006). Consequences of ovarian ageing also include an age-related increase in adverse reproductive outcomes, including miscarriage and pregnancies affected with aneuploidy, and ultimately long-term health implications during IVF [relative risk 1.87, 95% confidence interval (CI) 1.67, P = 0.001] (Narkwichean et al., 2013). This meta-analysis is limited by a small number of study participants included from only three controlled studies including a small randomized controlled trial (RCT) (Barad et al., 2007; Gleicher et al., 2010a; Wiser et al., 2010). For that reason, more clinical data are needed to decide if DHEA has a beneficial effect in assisted reproductive technology. Moreover, despite its use in more than 25% of fertility clinics worldwide (Leong and Patrizio, 2010), neither the mechanism nor the effect of DHEA on ovarian folliculogenesis is clearly known.

Unlike estrogens, the roles of androgens in female reproductive function are relatively poorly understood (Walters et al., 2008) and this is particularly true for androgens produced by the adrenal axis. Currently, the proposed mechanism and effect of DHEA are based on evidence from other gonadal androgens, e.g. testosterone, androstenedione and dihydrotestosterone which are known to act directly via the androgen receptor (AR). In polyovular mice, AR knock-out studies indicate a stimulatory role of androgens on follicular growth and development, particularly at the preantral stages (Walters et al., 2008). Regarding DHEA itself, controlled studies have demonstrated that oral DHEA supplementation increases serum insulin-like growth factor-1 concentrations (Casson et al., 1998), which is known to have a positive effect on follicular development and oocyte quality. However, it is not clear whether DHEA also exerts its actions through direct interaction with the AR or after conversion to more potent androgens such as testosterone. The effect of DHEA treatment on ovarian function is therefore unclear and further evaluation of the role of DHEA on ovarian ageing is required.

The objective of this study is therefore to test the hypothesis that DHEA influences ovarian folliculogenesis by enhancing preantral and small antral follicle development. It was planned to test this hypothesis using a sheep (as a monovulatory model species for the human) model in which one ovary has been removed and replaced with autografts of ovarian cortex. This, so-called, normograft procedure allows destruction of the normal follicular hierarchy and synchronization of primordial follicle initiation (Campbell et al., 2004) without endocrine disturbance (BK Campbell unpublished results). This, therefore, serves as an ideal model to examine potential effects of DHEA treatment on the rate and pattern of preantral follicular development in both the cortical patches and the remaining contra-lateral ovary, which retains the normal follicular hierarchy.

Materials and Methods

Experimental animals and hormone preparations

The research used 6 Grey-face (Scottish Blackface-Border Leicester cross) ewes that were at least 24 months old at the time of the first operation and the experiment lasted for 10 weeks during the non-breeding season in Edinburgh, Scotland (April–July). The animals were sheltered indoor at the Marshall building, University of Edinburgh under natural lighting and maintenance diet consisting of concentrates and hay ad libitum throughout the period of experiment from April 2011 to July 2011. Experiments were conducted with approval from the UK Home Office and in accordance with The Animal (Scientific Procedures) Act 1986 (2013) (UK). After surgery, the animals were treated prophylactically with antibiotics (Clamoxil 3 ml IM once a day for 3 days, SmithKline Beecham, Surrey, UK).

DHEA single rod implants (length 5 cm, diameter 3.35 mm) were prepared by filling SILASTIC tubing (dimethylsiloxane/methylvinylsiloxane copolymer Catalogue no. 508-011; Dow Corning, Midland, MI, USA) with DHEA powder (EMD Chemicals Inc., Darmstadt, Germany). Implants were subcutaneously administered at the anterior axilla under local anaesthetic. The first implant was inserted 7 days after the hemi-oophorectomy and an additional rod was inserted a month later. Both implants were left in place for the period of the experiment.

Ovarian cortical autograft (‘normograft’) model

In addition to allowing disruption of the normal follicular hierarchy and synchronization of primordial follicle initiation without endocrine disturbance (see above), the normograft model allows within animal comparison of follicle number by utilizing the follicle population in ovarian tissue recovered at time-zero as an internal control. This controls between animal variation and minimizes the number of experimental animals, in accordance with Home Office animal research guidelines (The Animal (Scientific Procedures) Act, 1986, 1 January 2013). Surgical preparation of the model involved unilateral oophorectomy by mid-ventral laparotomy. The left ovary was completely removed and transferred to a laminar flow hood in Leibovitz L-15 medium (Gibco, Paisley, UK). The ovarian vessels were transfixed and ligated with a
5/0 silk suture, the ends of which served to identify the preferred site of the autograft transplantation.

The left ovary was hemisected and one-half ovary was fixed immediately in 4% paraformaldehyde to act as a time-zero controls for histology. Four to five 1-mm thick ovarian cortical patches (size 16–25 mm²) were prepared from the remaining half ovary avoiding large follicles and hilar regions. The slices were estimated to weigh <50 mg. All slices were immediately autografted to the original ovarian pedicle and attached either side of the blood vessels using three 6.0 Prolene sutures as previously described (Campbell et al., 2004).

Ten weeks after insertion of the first DHEA implants, under terminal general anaesthesia, both the right ovary and left ovarian graft were recovered following mid-ventral laparotomy. In addition, prior to excision of ovarian tissue, 5 ml samples of blood from both the left and right utero-ovarian vein were collected with the aid of indwelling venous catheters. The right ovary and left ovarian grafts were fixed immediately in 4% paraformaldehyde and processed for histological analysis.

Blood sampling and hormone assays

During the 10-week period of DHEA implantation, jugular venous blood was collected by venepuncture twice a week (every 3–4 days). All blood collected, including ovarian venous blood, was centrifuged at 3500 rpm, 4°C for 10–15 min and the plasma was stored at −20°C until assay. Measurements of FSH (Campbell et al., 2004), LH (Campbell et al., 2004), androstenedione (A4) (Campbell et al., 2012) and inhibin A (Onions et al., 2009) levels were done using previously described radioimmunoassay (RIA) protocols. DHEA and anti-Mullerian hormone (AMH) measurements were performed by enzyme-linked immunosorbent assays (ELISAs) from commercial sources. The DHEA ELISA Kit (Demeditec Diagnostics, Kiel, Germany) and Human AMH ELISA Kit (CUSABIO, Wuhan, China) were used according to the suppliers’ instructions. The intra- and inter-assay coefficients of variation for all assays were <10 and 15%, respectively.

Histological analysis

All ovarian tissue was fixed overnight in 4% paraformaldehyde in Dulbecco’s phosphate buffered saline 0.01 M, pH 7.6 (Sigma-Aldrich, Dorset, UK) and transferred to 70% ethanol until processing. Blocks of 3 mm paraffin-embedded ovarian tissue sections were prepared. Serial sections of 5 μm were cut for each block and the sectioned tissue was then transferred to either ground edge 90° frosted end slides for Haematoxylin–Eosin (H&E) staining or Superfrost™ (Thermo Scientific, Braunschweig, Germany) slides for immunohistochemistry (IHC).

Standard H&E staining was done on sections of all ovaries from time-zero, final time and cortical graft tissue at final time. Stained sections were examined on a standard compound microscope Olympus CH2 (Olympus, Japan) and were photographed with a DMRB microscope (Leica Microsystems UK, Milton Keynes, Hertfordshire, UK). Follicles were classified as primordial, tertiary, secondary, preantral, early antral and antral according to criteria previously described by Lundy et al. (1999) and McNatty et al. (1999, 2000). The follicular diameter of 2.5 mm was used as a cut-off value to define (late) antral follicles since follicles at this size become gonadotrophin-dependent (Campbell et al., 2003). Clearly degenerative follicles with few layers of degenerate granulosa cells, pyknotic nuclei and a shrunken oocyte were few and were not counted. In regard to ovarian cortical grafts, the numbers were determined from the summation of follicles from at least three sections or until the entire graft had been sectioned. Because difference in sizes among ovarian tissue from time-zero, time-end and cortical graft, the absolute number of follicles in each class is not reported. Data are shown as follicles in each class as a percentage of the total number of follicles counted.

IHC studies of AMH (Campbell et al., 2012) and Ki-67, a marker of cellular proliferation (Onions et al., 2013) were carried out on ovarian sections comparing between time-zero and final time utilizing the automated Bond-Max™ IHC system (Vision BioSystems Ltd, Newcastle-Upon Tyne, UK). Protein Ki-67 is expressed in the cellular nucleus during all active cell cycle phases (G1, S, G2 and even mitosis) but is absent from the G0 phase (Scholzen and Gerdes, 2000).

The primary antibody for AMH-IHC was purified mouse antihuman AMH monoclonal antibody (Clone 5/6; Serotec) prepared in 1:40 working dilution. While mouse anti-Ki67 monoclonal antibody (Vector Laboratories, Peterborough, UK) diluted 1:100 in BondTM diluent (Vision BioSystems Ltd) was used for Ki-67 staining. To validate the staining protocol, normal sheep ovarian tissues enriched of preantral, small and large antral follicles were used as negative and positive tissue controls. Negative staining controls were done by omitting the primary antibody.

Image analysis

Both time-zero and final time sections were evaluated under a DMRB microscope (Leica Microsystems UK) in an anonymous fashion (coded slides). Time 200 (×200) magnification was used to observe preantral and antral follicles and higher magnification (×400) for smaller follicles. Each follicle image from all slides was captured and saved using Volocity software (Improvion® PerkinElmer, Cambnidge, UK). Negative or positive staining was determined independently by two investigators (A.N and C.P.A) who were blinded to the source of the sections.

Staining was assessed in all but atretic follicles having pyknotic nuclear material of granulosa cells present in the antrum. Follicles were classified according to developmental stage described earlier in H&E staining section. For AMH IHC, the staining intensity was determined by two following methods: (i) criteria described by Stubbs et al. (2005) which categorized follicles into scores 0, no staining; 1+, very little staining; 2+, moderate staining; 3+, intense staining, and (ii) using CellSens Dimension software version 1.6 (Olympus Software Imaging System, Germany) to more objectively measure darkness intensity of images after conversion into greyscale pictures. The earlier classification was used to calculate the percentage of staining which was considered as overall showing expression follicles, score 1–3; and strong showing expression follicles, score 2–3; respectively. In the later method, 8–12 small circle areas of granulosa cells were randomly measured and calculated for the mean intensity in every slide; a numerical score ranging from 0 to 255 for the strongest positive to negative, respectively. Both means percentage of positive AMH staining and AMH intensity were compared between time-zero and time-end tissue.

Regarding Ki-67, slides were observed under a Nikon Eclipse 90i microscope and images were taken, again using Volocity software (Improvion® PerkinElmer) after being controlled for similar white balance background. All positive stained and counterstained granulosa cells in each image were counted. Positive stained cells were classified as (i) positive (+ + +), weakly positive (+ + ) and negative (−). The percentages of cells expressing (total) positive Ki-67 (+ + and ++ +) and expressing strongly positive (+ + only) from a total number of granulosa cells, so-called ‘labelling index (LBI)’, were then calculated in each follicle from all sections.

Statistical analysis

All statistical analyses were carried out using statistical package SPSS version 19.0 (IBM Corp., New York, USA). Data concerning all hormonal levels were expressed as mean ± standard error (SEM) and statistical analyses were performed by repeated-sample ANOVA with logarithmic transformation. Regarding the H&E histological analysis, either χ² or Fisher’s exact test was used to compare percentage of follicle in each class as appropriate.

In terms of IHC, percentages of positive AMH stain in each developmental class were estimated beginning from primary to large antral follicle. Significant
difference between time-zero and time-end tissue was determined by multiple logistic regression analysis (adjusted for developmental class of follicle). Additionally, mean AMH intensity scores (mean ± SD) and Ki-67 LBI between two groups before and after DHEA treatment were compared by using a factorial ANOVA in which variations among animals and follicular classes were taken into account. The overall P-value of treatment effect was calculated and the significance of individual comparisons determined by post-hoc pairwise comparisons using the least significant difference (LSD) method.

Results

Hormonal assays

Baseline pretreatment FSH and LH concentrations (mean ± SEM) in these ewes were 2.22 ± 0.30 and 0.47 ± 0.18 ng/ml, respectively.

![Figure 1](image1.png)

**Figure 1** Jugular venous concentrations of gonadotrophins (A) and androgens (B; DHEA = dehydroepiandrosterone, A4 = androstenedione) measured by RIA or ELISA, respectively, over the days from the cortical autograft (first operation) to the end of the experiment. Values are mean ± SEM (n = 6). Arrows represent the first (Day 7) and second (Day 37) DHEA implantation. RIA, radioimmunoassay; ELISA, enzyme linked immunosorbant assay.

After hemi-ovariectomy and ovarian cortical graft re-transplantation, both serum gonadotrophin concentrations were stable throughout the 10-week period of experiment (FSH 2.14 ± 0.38 ng/ml, P = 0.5; LH 0.4 ± 0.18 ng/ml, P = 0.1; Fig. 1A). DHEA concentration before treatment was 0.43 ± 0.18 ng/ml. After drug administration, DHEA levels progressively increased and peaked at around week 6–7 after treatment. Despite levels gradually falling after this peak towards the final week, post-treatment concentrations were significantly higher at every time-point than the pre-administration level (2.65 ± 1.52 ng/ml after DHEA treatment, P < 0.05; Fig. 1B). In contrast, A4 levels were low and remained stable throughout the period (0.17 ± 0.33 ng/ml). No significant difference to the baseline A4 was detected at any time-point (P = 0.07; Fig. 1B).

In contrast, the two markers of ovarian reserve, inhibin A and AMH, exhibited divergent patterns after DHEA treatment. Thus, inhibin A declined with days after implantation and significant differences were detected between before and after treatment (baseline 263 ± 32.5 pg/ml versus 10 weeks post-treatment 139 ± 11.2 pg/ml; P < 0.05 using repeated-sample ANOVA with post-hoc analysis). On the other hand, there was a significant 2-fold increase in jugular AMH concentrations at 2 months after surgery (baseline 43.5 ± 18.34 ng/ml versus Day-62 112 ± 32.1 ng/ml; P < 0.05) but the levels subsequently declined to the pretreatment level by the end of the experimental period (Fig. 2). Wide variations in AMH and inhibin A levels in utero-ovarian venous blood were observed among individual sheep. As

![Figure 2](image2.png)

**Figure 2** Jugular venous concentrations of inhibin A (A) and AMH (B) measured by ELISA in relation to days from cortical autograft (first operation). Values are mean ± SEM (n = 6). See description in text. *P < 0.05 determined by repeated-sample ANOVA with post hoc analysis. ELISA, enzyme linked immunosorbant assay.
expected, AMH and inhibin A levels were lower in samples taken from left ovarian veins (graft) than the right ovarian veins (intact exposed ovary) and these levels were in turn lower than the jugular venous samples on the same day (data not shown). Inhibin A levels were directly correlated with number of growing follicles in histological sections. For instance, correlation coefficients \( (r) \) between inhibin A levels and number of all growing follicles and small antral follicles were \( r = 0.88 \) \((P < 0.05)\) and \( r = 0.89 \) \((P < 0.05)\), respectively. AMH concentrations in right ovarian vessels, however, failed to demonstrate a significant correlation with number of either growing preantral/antral or small antral follicles \((P = 0.98\) and 0.79, respectively).

**Ovarian histology and IHC**

**Histology**

The mean total follicular counts in time-zero, time-end and graft tissue were 1224, 1996 and 222 follicles, respectively. A normal follicular hierarchy, in which primordial follicles represent a majority of the population and proportions progressively decline at increasing stages of development, was demonstrated in ovarian tissue from both time-zero controls and in the whole ovary following DHEA treatment. Nonetheless, there was a significant difference in overall follicular distributions between time-zero and time-end \((P < 0.001)\) determined by \( \chi^2 \) analysis with lower proportions of primordial and preantral follicles \((\text{primordial } 57 \text{ versus } 63\%, \ P < 0.001; \text{preantral } 5.5 \text{ versus } 8.2\%, \ P < 0.05)\) and higher proportion of large antral follicles \((3.9 \text{ versus } 1.5\%, \ P < 0.001; \text{Fig. 3})\) following DHEA treatment.

Within cortical autografts, as expected, a marked deviation from the normal hierarchy of follicle distribution was noted relative to time-zero controls \((P < 0.001)\) with complete absence of the antral follicle pool. Relative to control tissue, the proportions of primary and secondary follicles in cortical grafts were increased while there was a significant reduction in the cohort of primordial follicles \((\text{primordial } 63 \text{ versus } 45\%, \ P < 0.001)\) and an increase in the proportion of primary \((10 \text{ versus } 39\%, \ P < 0.001)\) and secondary \((5.6 \text{ versus } 11.3\%, \ P < 0.05)\) follicles in cortical tissue following DHEA treatment.

**Expression of AMH**

AMH was expressed exclusively in the cytoplasm of granulosa cells. Patterns of expression in terms of percentages and intensity of staining were quite similar before and after DHEA treatment with expression being first detected at the primary/tertiary stage, increasing to a peak in small antral follicle \((<2.5 \text{ mm})\), and gradually declining in large antral follicles \((\text{Fig. 4 and Table I})\). When assessed by either numerical scoring or measurement of greyscale intensity, DHEA treatment resulted in an increase in AMH protein expression within preantral and small antral follicles. Proportions of follicles exhibiting strong expression of AMH protein were greater in both preantral and small antral follicles following DHEA treatment \((\text{Fig. 4})\) with multiple logistic regression analyses indicate that there were approximately a four times more likely to observe strongly positive AMH stained follicles following DHEA treatment \((\text{OR } 3.93, 95\% \text{ CI } 1.18-13.07, P < 0.05)\) when compared with time-zero control. However, there was no significant difference in the percentages of follicles showing any expression \((\text{OR } 0.53, 95\% \text{ CI } 0.05-5.56, P = 0.6)\) \((\text{Fig. 4})\).

Concerning AMH intensity, factorial ANOVA analysis shows that follicular class is considered as an independent factors of AMH greyscale

![Figure 3](image-url)
intensity \((P < 0.001)\) while there was no variation of the measurements among the animals \((P = 0.87)\). Moreover, no interaction among timing (before and after DHEA treatment), animal and follicular class was observed \((P > 0.05)\). Time-end ovaries had significantly lower (more intensely stained) mean greyscale intensity of AMH staining than controls \((109 \pm 63.9 \text{ versus } 134 \pm 67.1; P < 0.05)\) in which the post-hoc pairwise comparisons across all follicular classes demonstrate significant differences in both small antral and preantral follicles \((73 \pm 50.0 \text{ versus } 100 \pm 60.2, P < 0.05)\) using LSD method; Table I).

### Table I

<table>
<thead>
<tr>
<th>Class</th>
<th>Time-zero (n)</th>
<th>Time-end (n)</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total follicles</td>
<td>134 \pm 67.1 (51)</td>
<td>109 \pm 63.9 (77)</td>
<td>0.02</td>
</tr>
<tr>
<td>Preantral</td>
<td>187 \pm 32.4 (14)</td>
<td>136 \pm 42.0 (19)</td>
<td>0.02</td>
</tr>
<tr>
<td>Small antral&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100 \pm 60.2 (32)</td>
<td>73 \pm 50.0 (45)</td>
<td>0.02</td>
</tr>
<tr>
<td>Large antral&lt;sup&gt;c&lt;/sup&gt;</td>
<td>201 \pm 8.2 (4)</td>
<td>192 \pm 25.1 (13)</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Values range from 0 (darkest, most intense staining) to 255 (brightest, least staining).<sup>a</sup>CellSens dimension software version 1.6 (Olympus Software Imaging System).<sup>b</sup>Overall P-values for total follicles were calculated by Factorial ANOVA followed by post-hoc pairwise comparisons in each follicular class (LSD method), \(P < 0.05\) = significant.<sup>c</sup>Each follicle was randomly taken at least two to four pictures at high-power magnification \((\times 200)\). Each picture was measured for at least eight areas by the computer and calculated for mean follicular intensity.

### Discussion

This is the first study to evaluate the effect of DHEA treatment on early follicle development in vivo and overall the findings indicate that DHEA treatment results in an increase in the antral follicle population, possibly through increases in both the rate of primordial follicle initiation and the rate of preantral follicle development. Further, our findings suggest that in addition to an increase in follicle number, at least part of the underlying...
mechanism resulting in these stimulatory effects may be mediated by an increase in intra-follicular AMH expression.

DHEA treatment had a stimulatory effect on both primordial follicle initiation in ovarian autografts in which the follicular hierarchy had been lost and an increase in the proportion of small antral follicles in the remaining ovary in which the follicular hierarchy had been maintained. As this stimulatory effect occurred in the absence of any change in peripheral FSH concentrations, it is arguable that the stimulation in follicle development observed can be attributed to DHEA treatment. This hypothesis is supported by the fact that previous investigations in untreated pre-pubertal lambs subjected to the normograft procedure did not display any change in the follicular hierarchy (BK Campbell unpublished observations). However, further experiments utilizing contemporaneous placebo treated controls are required to fully confirm this result.

The finding of a possible stimulatory effect of DHEA on follicle development is supported by the fact that ARs are present in mammalian ovarian tissue at almost all stages of follicular development with higher levels of expression in granulosa cells of preantral and small antral follicles (Hillier et al., 1997; Juengel et al., 2006). Likewise, AR knock-out mice have reduced ovulation rates that can be overcome by hyperstimulation with gonadotrophins. Moreover, the heterozygous AR mutation demonstrates age-related reduction in fertility or eventually develops premature ovarian failure (Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007). All of these findings signify that normal preantral follicular growth requires AR-mediated androgen functions and abnormality in the androgen axis can result in accelerated ovarian ageing. In contrast, high levels of extra-ovarian androgens (predominantly DHEA) are present in polycystic ovary syndrome (PCOS) women who typically have enlarged ovaries containing higher number of ‘cystic’ antral follicles as well as increasing in stromal tissue volume (Ehrmann, 2005; Speroff and Fritz, 2005).

The results of immunohistochemical analysis of markers of cellular proliferation (Ki-67) and AMH provide further insights into the action of DHEA in the ovarian tissue. Thus, DHEA increased granulosa cell proliferation, both quantitatively and qualitatively (intensity) and this effect was more obvious in small antral follicles (Table II). In addition, DHEA stimulated AMH expression within preantral and small antral follicles. In most species AMH is most highly expressed in these early antral follicles (Figs 2 and 5; Weenen et al. 2004; Campbell et al., 2012) and the gradual rise in average serum AMH levels following DHEA treatment can most likely be attributed to an increase in both follicle number and AMH expression within individual follicles. This interpretation is supported by the study of Gleichner et al. (2010b) in women with DOR, in which the authors found a significant increase in AMH after DHEA supplementation before IVF treatment.

<table>
<thead>
<tr>
<th>Class</th>
<th>Total LBI</th>
<th>Time-zero</th>
<th>Time-end</th>
<th>P-valuea</th>
<th>Strongly expressed LBI</th>
<th>Time-zero</th>
<th>Time-end</th>
<th>P-valuea</th>
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<tr>
<td>Total Follicles</td>
<td></td>
<td>2.6 ± 0.4</td>
<td>10.6 ± 1.8</td>
<td>0.02</td>
<td>0.4 ± 0.1</td>
<td>3.6 ± 0.7</td>
<td>&lt; 0.001</td>
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<tr>
<td>Preantral</td>
<td>5.8 ± 1.3</td>
<td>8.6 ± 2.6</td>
<td>0.51</td>
<td></td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Small Antral</td>
<td>2.4 ± 0.4</td>
<td>17.2 ± 3.8</td>
<td>&lt;0.001</td>
<td></td>
<td>0.6 ± 0.1</td>
<td>5.9 ± 1.5</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Large Antral</td>
<td>0.2 ± 0.1</td>
<td>2.4 ± 0.7</td>
<td>0.57</td>
<td></td>
<td>0.2 ± 0.1</td>
<td>2.4 ± 0.7</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error of the mean (SEM).

Total LBI, index of positive and weakly positive cells; strongly expressed LBI, index of (strong) positive cells only.

*aOverall P-values for total follicles were calculated by Factorial ANOVA followed by pairwise comparisons using LSD method on each class of follicle, P-value < 0.05 = significant.
The role of AMH in follicular development is poorly understood but data from a range of species indicate that it may be a key intraovarian factor. In rodents, AMH appears to act both as a regulator of primordial follicle initiation and as a modulator of the response of follicles to FSH (Durlinger et al., 2002a,b). Similarly in sheep, in vitro AMH was found to modulate the sensitivity of ovarian somatic cells to gonadotrophic stimulation and in vivo, although no clear effects on primordial follicle initiation were found, immunization against AMH boosted the preantral and small antral follicle population and resulted in an increase in ovulation rate (Campbell et al., 2012). Finally, in women elevated AMH levels have been observed in PCOS women (Franks et al., 2008). It is, therefore, likely that at least part of the mechanism whereby DHEA may stimulate gonadotrophin-responsive follicle development is mediated by regulating follicular AMH expression.

In conclusion, our findings suggest that DHEA exposure stimulates early follicular growth during the preantral and early antral gonadotrophin-responsive stages of follicle development. Further, our results suggest that these effects of DHEA on follicle development may be mediated in part by modulation of the level of AMH expression during these stages of follicle development in addition to stimulating follicle number per se. Overall these findings suggest a mechanism whereby DHEA treatment may be potentially useful clinically as a means to increase the number of gonadotrophin-responsive follicles for ovarian stimulation. Thus, DHEA may be a useful therapy to delay the effects of ovarian ageing and as an adjunct during IVF to improve ovarian response in women with DOR and as a treatment for premature ovarian insufficiency although a positive effect of DHEA treatment on oocyte response in women with DOR and as a treatment for premature ovarian ageing and as an adjunct during IVF to improve ovarian re-

Conflict of interest
None declared.

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