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Infertility and ovarian follicle reserve depletion are associated with dysregulation of the FSH and LH receptor density in human antral follicles

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ABSTRACT

The low take-home baby rate in older women in Australia (5.8%) undergoing IVF (5.8%) is linked to the depletion of the ovarian reserve of primordial follicles. Oocyte depletion causes an irreversible change to ovarian function. We found that the young patient FSH receptor and LH receptor expression profile on the granulosa cells collected from different size follicles were similar to the expression profile reported in natural cycles in women and sheep. This was reversed in the older patients with poor ovarian reserve. The strong correlation of BMPR1B and FSH receptor density in the young was not present in the older women; whereas, the LH receptor and BMPR1B correlation was weak in the young but was strongly correlated in the older women. The reduced fertilisation and pregnancy rate was associated with a lower LH receptor density and a lack of essential down-regulation of the FSH and LH receptor. The mechanism regulating FSH and LH receptor expression appears to function independently, *in vivo*, from the dose of FSH gonadotrophin, rather than in response to it. Restoring an optimum receptor density may improve oocyte quality and the pregnancy rate in older women.

1. Introduction

As women age, the reserve of primordial follicles is depleted, and the quality of oocytes, fertilisation, and pregnancy rate are reduced. Following their initial recruitment from the ovarian reserve, activated primordial follicles grow and differentiate into pre-antral and small antral follicles (McGee and Hsueh, 2000). From the onset of puberty, cyclic fluctuations in follicle stimulating hormone (FSH) secretion from the anterior pituitary reach a threshold point sufficient to rescue a cohort of small antral follicles and initiate cyclic follicle recruitment (McGee and Hsueh, 2000). The number of antral follicles selected for dominance and ovulation is largely dependent on the regulatory action and the density of FSH receptors and LH receptors on the granulosa cell surface (Hillier, 2001; Baird, 1987; Baerwald et al., 2012).

When the FSH level falls, the growth of the smaller follicles is reduced, and only the follicles with sufficient FSH and LH receptors continue to develop further because of their enhanced capacity to convert androstenedione to oestrogen for growth (Loumaye et al., 2003). As the ovarian primordial follicle reserve declines, the rate of cyclic recruitment of follicles diminishes (Baerwald et al., 2012; Almog et al., 2011). The number of these small antral follicles at the beginning of each cycle is representative of the ovarian reserve of primordial follicles that remain in the ovary.

Older patients, typically, have a slower follicle growth rate and a reduced number of granulosa cells per follicle (Santoro et al., 2003). Other ovarian age related changes are associated with increased mitochondrial deletions in granulosa cells and reduced FSH receptor mRNA expression, which have been linked with infertility (González-Fernández et al., 2010; Seifer et al., 2002; Cai et al., 2007). Reduced receptor density may directly contribute to poor

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oocyte quality by increasing the number of chromosomal errors (Maman et al., 2012; Handyside et al., 2012).

In this study, the aim was to comprehensively profile the expression of granulosal FSH receptor and LH receptor protein in a range of patients of different ages and stages of ovarian primordial follicle depletion, who were receiving treatment for infertility. An average of ~8000 granulosa cells per follicle was collected from follicles ranging in size from 4 to 27 mm. Antibody labelling and flow cytometry were used to evaluate the receptor density. Previous studies of receptor expression have been confined to expression at the mRNA level, which may not be a reliable indicator of the level of translated 'mature' receptor protein expressed on the cell surface (Jeppesen et al., 2012; Pidoux et al., 2007; Ascoli et al., 2002). The changes observed in receptor density may explain the adverse impact that ovarian reserve depletion has on fertility as women age.

2. Materials and methods

2.1. Patients

A total of 415 follicles were collected from 56 patients undergoing standard fertility treatment with PIVET Medical Centre Perth, Western Australia, and are presented in Table 1. Patients were aged between 23 and 45 years, and follicles were collected irrespective of previous aetiology, but limited to exclude unusual medical conditions, hormonal dysfunction, and polycystic ovarian syndrome.

2.2. Human IVF: ovarian stimulation, follicular fluid, and oocyte

Patient treatment consisted of two types of gonadotrophin releasing hormone-LH suppression (Puregon or Gonal F) in conjunction with commercially prepared recombinant human FSH, from cycle day 2 for ~10 days as previously described (Regan et al., 2016). Ovulation was triggered with 10 000 IU HCG, and the collection of granulosa cells and oocyte retrieval was 36 h later by transvaginal oocyte aspiration (Regan et al., 2016). *In vivo* human studies are more complex and therefore variables such as BMI have been minimised in this study (Table 1). We initially used the merino sheep model in a natural cycle (Regan et al., 2015) to establish a comparative data set with the human model during IVF treatment. The sheep and women both showed prerequisite down-regulation at the two critical follicle sizes that are equivalent to the size at the time of follicle selection and follicle maturation. Indeed, the administration of the artificial LH surge to induce maturation appears not to alter the receptor expression in the young patient with a good ovarian reserve based on the similarities between the models and demonstrated in Fig. 5.

Furthermore, analysis of the effect of rFSH dose on the receptor expression in a homogeneous group of patients with all variables controlled (ovarian reserve, age, follicle size, BMI, and AMH) was not significantly different (Fig. 6, $p = 0.7$).

2.3. Ovarian reserve measured by the antral follicle count

Patients received daily FSH according to a long established algorithm based on the patient's profile of age and ovarian reserve in order to predict the FSH dose required to stimulate multiple preovulatory follicles, as reported previously (Yovich et al., 2012). Ovarian reserve was measured indirectly by the antral follicle count and was defined as the number of follicles between 2 and 10 mm in size that are present in total on ~ day 2–5 of a preliminary assessment cycle (Hansen et al., 2011). The patients were divided into group levels from A to E; good to poor ovarian reserve, respectively based on the algorithm, as described previously (Regan et al., 2016), and a well-established clinical practice of patient treatment (Yovich et al., 2012): Poly cystic ovarian syndrome (PCOS) patients were excluded from the study group based on the Rotterdam criteria, initially prepared in 2003, and updated to reflect the advances in ultrasound technology (Lujan et al., 2013): specifically; per ovary > 24 follicles, along with other criteria (Lujan et al., 2013). In the current study the combined ovary follicle total corresponded to Group A+ = 30–39 small follicles; group A = 20–29 small follicles; group B = 13–19 small follicles; group C = 9–12 small follicles; group D = 5–8 small follicles; group E = ≤ 4 small follicles.

2.4. Collection of granulosa cells

The diameter of the follicle was calculated using ultrasonography as described previously (Regan et al., 2016). Flushing of the follicle by the clinician (Quinn's Advantage with Hepes, Sage Media, Pasadena, California) removed the loosely attached layers of granulosa cells. The cumulus ovarian complex was removed from the sample by the embryologist. The follicular fluid and flush was then layered onto a ficoll density gradient (555 485; BD Biosciences, Perth, Australia) and centrifuged to isolate the granulosa cells (Regan et al., 2016). Pure follicular fluid were analysed for oestrogen and progesterone using a random access immunoassay system (Siemens Medical Solutions, Bayswater, Victoria, Australia).

2.5. Immunolabelling of granulosa cells

Aliquots of suspended granulosa cells (1×10^6 cells in 100 μl) were immunolabelled using a double-indirect method as previously described (Regan et al., 2016). The antibodies have been used previously in human studies (Regan et al., 2016; Abir et al., 2008; Haÿ et al., 2004), including flow cytometry analyses (Regan et al., 2016; Regan et al., 2015; Gao et al., 2007; Whiteman et al., 1991). Briefly, the cells were incubated with affinity purified goat polyclonal antibody for the FSH receptor (sc-7798), LH receptor (sc-

Table 1
Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE Year	IVF Patient	Total Follicle	BMI	Ovarian Reserve Group Follicles Collected						Fertility %			
				A+	A	B	C	D	E	Failed Fertilisation	Not Pregnant	Pregnant	Live Birth
23–30	11	95	24.1 ± 4	31	64					0	36	**64	43
35–45	34	232	24.8 ± 5			88	21	99	24	9	52	**39	18
*39–45	19	131	23.9 ± 5			42	5	66	18	17	72	11	6

Ovarian reserve measured indirectly by the antral follicle count (AFC). Antral follicle count is the number of follicles between 2 and 10 mm on day 2–5 of a cycle: A+ = 30–39 follicles; A = 20–29; B = 13–19; C = 9–12; D = 5–8; E = ≤4. Follicle count is based on the combined total from both ovaries. *Subgroup of older patients. **1 Ectopic pregnancy. Frozen embryo transfers cycles included.