Metabolism and karyotype analysis of oocytes from patients with polycystic ovary syndrome

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BACKGROUND: Polycystic ovary syndrome (PCOS) is associated with metabolic disturbances which include impaired insulin signalling and glucose metabolism in ovarian follicles. The oocyte is metabolically dependent upon its follicle environment during development, but it is unclear whether PCOS or polycystic ovarian (PCO) morphology alone affect oocyte metabolism and energy-demanding processes such as meiosis.

METHODS: Immature human oocytes were donated by PCOS (n = 14), PCO (n = 14) and control (n = 46) patients attending the assisted conception programme at Leeds Teaching Hospitals NHS Trust. Oocytes were cultured individually and carbohydrate metabolism was assessed during overnight in vitro maturation (IVM). Meiotic status was assessed and oocyte intracellular nicotinamide adenine dinucleotide phosphate (NAD(P)H) content and mitochondria activity were measured prior to karyotype analysis by multifluor in situ hybridization.

RESULTS: Patient aetiology had no significant effect on oocyte maturation potential or incidence of numerical chromosome abnormalities (44%), although PCOS and PCO oocytes were more likely to suffer predivision. Group G chromosomes were most likely to be involved in non-disjunction and predivision. PCOS was associated with increased glucose consumption (2.06 ± 0.43 and 0.54 ± 0.12 pmol/h for PCOS and control oocytes, respectively) and increased pyruvate consumption (18.4 ± 1.2 and 13.9 ± 0.9 pmol/h for PCOS and control oocytes, respectively) during IVM. Prior prescription of metformin significantly attenuated pyruvate consumption by maturing oocytes (8.5 ± 1.8 pmol/h) from PCOS patients. Oocytes from PCO patients had intermediate metabolism profiles. Higher pyruvate turnover was associated with abnormal oocyte karyotypes (13.4 ± 1.9 and 19.9 ± 2.1 pmol/h for normal versus abnormal oocytes, respectively). Similarly, oocyte NAD(P)H content was 1.35-fold higher in abnormal oocytes.

CONCLUSIONS: The chromosomal constitution of in vitro matured oocytes from PCOS is similar to that of controls, but aspects of oocyte metabolism are perturbed by PCOS. Elevated pyruvate consumption was associated with abnormal oocyte karyotype.

Key words: aneuploidy / metabolism / metformin / oocyte maturation / polycystic ovaries

Introduction

Polycystic ovary syndrome (PCOS) affects up to 15–20% of women of child-bearing age (Azziz et al., 2004; Balen and Michelmore, 2002; Balen et al., 2009) and is a major cause of patient referral to assisted conception clinics. PCOS is a heterogeneous condition, in which patients typically present with a combination of menstrual disturbance (oligomenorrhoea/anovulation), hyperandrogenism and polycystic ovaries (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004a, b). There may also be associated metabolic disturbances. Various candidate genes have been implicated in the manifestation of PCOS (Menke and Strauss, 2007; Goodarzi et al., 2008; Lee et al., 2008; Park et al., 2008; Shah et al., 2008), and hypotheses implicating epigenetic influences have also been proposed to account for the syndrome (Hickey et al., 2006; Li and Huang, 2008).

PCOS is very often associated with obesity, the metabolic syndrome, glucose intolerance and insulin resistance in many tissues, including those in the ovary. PCOS-associated anovulation may be alleviated by reducing body weight and to a lesser extent by using compounds, such as metformin, that increase insulin sensitivity, although these preparations are not as successful as was initially hoped (Tang et al., 2006a; Balen, 2007).

Although PCOS is well-defined, women who present with polycystic ovaries but no other characteristics of the syndrome are classified...
as having polycystic ovarian (PCO) morphology (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004a, b; Balen et al., 2005). Indeed, PCOS is characterized by PCO morphology combined with hyperandrogenaemia and anovulation. Furthermore, patients presenting with PCO but without other symptoms of the full syndrome may still have disturbances in insulin and glucose metabolism (Norman et al., 1995). Hyperandrogenaemia is thought to influence oocyte gene expression (Wood et al., 2007), but the effects of systemic changes in glucose and insulin balance and the downstream effects on oocyte gene expression are unclear. There are several extra-ovarian aspects to the pathophysiology of PCOS yet ovarian dysfunction is central. Polycystic ovaries are commonly detected by ultrasound or other forms of pelvic imaging, with estimates of the prevalence in the general population being in the order of 20–33% (Michelmore et al., 1999; Balen et al., 2003). However, not all women with polycystic ovaries demonstrate the clinical and biochemical features which define the full syndrome of PCOS (Polson et al., 1988). Furthermore, there is considerable heterogeneity of symptoms and signs among women with PCOS and for an individual these may change over time.

During assisted conception, the numbers of antral follicles and oocyte yields collected after controlled ovarian stimulation are typically higher in PCOS and in women with polycystic ovaries compared with other causes of subfertility (MacDougall et al., 1993). However, fertilization rates tend to be lower in PCOS patients (MacDougall et al., 1993), indicating that reduced oocyte quality may be a manifestation of this syndrome. Furthermore, poorer pregnancy outcomes after assisted conception have been linked to PCOS (Balen et al., 1993; Glueck et al., 2000), which are primarily thought to be associated with obesity, hyperandrogenaemia and insulin resistance (Balen et al., 2006; Cocksedge et al., 2008). The contribution of chromosome aberrations to the lower pregnancy rates in patients with PCOS is unclear.

During follicle growth and development in vivo, the growing and maturing oocyte obtains nutrients and energy substrates from the reservoir provided by the follicular environment. The bidirectional communication between the oocyte and follicle is vital for the development of a fertile gamete (Simon et al., 1997; Sugiyura et al., 2005; Harris and Picton, 2007). The nutrients, energy metabolites and growth factors required by the oocyte are most frequently sourced from granulosa cells via follicular fluid (Leese and Barton, 1985; Leese and Lenton, 1990; Harris et al., 2005), as well as by direct cell–cell transfer between the oocyte and somatic compartment (Eppig et al., 2005). Studies of granulosa cell function from IVF patients with PCOS have established that follicular glucose metabolism is altered, with cultured cells showing a significant reduction in insulin-stimulated glucose consumption (Rice et al., 2005). It is likely that the metabolic lesion in the follicle precipitates an altered metabolic milieu throughout oogenesis, which may have downstream consequences for oocyte energy generation and/or could result in alteration of the metabolic profile of the oocyte enclosed within the follicle. It is unclear whether nutrient turnover by individual oocytes is altered by PCOS or whether the compromised metabolic status of the follicular somatic cells has any downstream effects on other aspects of oocyte function.

Many energy-consuming processes occur within the oocyte around the time of ovulation, including chromosome congression, segregation and separation, as well as polar body (PB) formation. An adequate ATP supply in the oocyte is essential to support these vital processes. Oocyte ATP is predominantly derived from the mitochondria and, as such, adequate mitochondrial number and function are paramount to fulfill the requirement for ATP. In this context, it has previously been suggested that there might be a link between declining mitochondria function and reduced meiotic integrity in oocytes (Schon et al., 2000). In support of this idea, reduced expression of genes encoding oxidative phosphorylation components has been observed in women with PCOS (Skov et al., 2007), a phenomenon which is alleviated by agents which indirectly increase insulin sensitivity (Skov et al., 2008).

An additional factor associated with the fidelity of oocyte meiosis in PCOS is the recent detection of altered expression of key genes associated with chromosome alignment and segregation (Wood et al., 2007).

Chromosome counts are thought to be similar between PCOS and non-PCOS oocytes (Sengoku et al., 1997), however, many chromosome abnormalities can only be detected by full karyotype analysis. The development of techniques such as spectral karyotyping and multifluor in situ hybridization whole chromosome painting (mFISH WCP) and their application to whole oocyte evaluations (Marquez et al., 1998; Clyde et al., 2001) mean that it is now possible to accurately karyotype oocytes from subfertile patients with different aetiologies. Common chromosome abnormalities in human oocytes include whole chromosome losses and/or gains (Clyde et al., 2001, 2003; Snitiz et al., 2007) as well as premature separation of sister chromatids (PSSC; also known as predivision) (Dailey et al., 1996). In human oocytes, chromosome abnormalities are common (Clyde et al., 2003) and can be a cause of aneuploidy in the embryo (Dailey et al., 1996). Furthermore, chromosome losses or gains can be induced as a result of in vitro culture (Dailey et al., 1996; Rosenbusch and Schneider, 2000; Rosenbusch et al., 2008); increasing maternal age (Dailey et al., 1996); and, in mouse, as a result of over-stimulation with FSH (Roberts et al., 2005). Chromosome abnormalities can occur singly or in various combinations. An oocyte with a loss of one chromosome but gain of another will still have a chromosome count of 23, even though serious abnormalities are present. In order to accurately gauge whether PCOS predisposes oocytes to chromosomal errors which underpin the observed reduced oocyte quality in these patients it is therefore important that full karyotype evaluations are conducted to ensure accurate analysis.

This study was therefore conducted to measure multiple parameters of oocyte metabolism and mitochondrial activity in oocytes from patients with PCOS and to determine whether the syndrome was associated with numerical chromosomal errors.

**Materials and Methods**

Unless otherwise stated, all chemicals and reagents used were obtained from Sigma Aldrich Chemical Company (Poole, Dorset, UK).

**Patients, consent and ethical approval**

Patients attending the intracytoplasmic sperm injection (ICSI) programme at the Reproductive Medicine Unit at Leeds General Infirmary consented to the donation of their surplus immature oocytes for research under protocols approved by the local research ethics committee and the UK Human Fertilization and Embryology Authority. Control patients were
women seeking treatment for male factor or tubal infertility but who had normal ovarian morphology and endocrine profiles. Patients with an ultrasound appearance of multiple cysts on one or both ovaries were grouped as PCO, whereas patients who also fulfilled two of the three criteria defined in the 2003 Rotterdam consensus definition for PCOS (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004a, b), including polycystic ovaries (Balen et al., 2003), were categorized as PCOS. None of the patients with PCOS/PCO who were recruited to the study had metabolic abnormalities as confirmed by screening for impaired glucose tolerance, only those patients with normal glucose tolerance were included in the study. Furthermore none of the patients had metabolic syndrome.

**Ovarian stimulation**

Patients underwent a long protocol of controlled ovarian stimulation (Tang et al., 2006b). In summary, ovarian function was down-regulated by chronic administration of a gonadotrophin releasing hormone agonist (Nafarelin; Synarel, Pharmacia, Surrey, UK), delivered by nasal spray. When a minimum endometrial thickness (<3 mm) had been achieved, controlled ovarian stimulation commenced. This consisted of a daily subcutaneous injection of a recombinant FSH preparation (Puregon, Organon Laboratories, Cambridge, UK), for 10–13 days. Follicle development was tracked by ultrasound scan and when at least three follicles of 17 mm diameter or over were present in the ovaries, 5000–10 000 IU human chorionic gonadotrophin (hCG) (Profasi Pharmaceuticals, Serono, Middlesex, UK) was administered. Some PCOS patients were treated with mefinor, receiving 850 mg twice per day beginning at the start of pituitary down-regulation and continuing until egg collection (~4 weeks).

**Oocyte collection**

Oocytes were harvested transvaginally by ultrasound-guided oocyte pick-up ~36–38 h post-hCG. Prior to ICSI, oocytes were stripped of their cumulus cells by brief exposure to 80 IU/ml Hyaluronidase (Synvitro Hydase, Medicut, Surrey, UK) at 37°C. Only oocytes that were immature, i.e. had a visible germinal vesicle (GV) or had undergone GV breakdown (GVBD) but with no visible first PB, at the time of ICSI were donated for research.

**In vitro maturation**

Immature oocytes were transferred to the research laboratory in oocyte flushing medium (Medicut, Surrey, UK) at 37°C on the day of oocyte recovery. Oocytes were cultured individually under washed, equilibrated paraffin oil for 18 h in 2 μl micro-droplets of in vitro maturation (IVM) medium (Wynn et al., 1998). The IVM medium contained recombinant human FSH (0.01 IU/ml), recombinant human hCG (0.1 IU/ml), insulin (10 ng/ml) and a long-acting synthetic analogue of insulin-like growth factor I (100 ng/ml) in addition to 0.2 mM glucose and 0.4 mM sodium pyruvate in bicarbonate-buffered alpha-minimum essential medium at 37°C and 5% CO₂ in air. Oocyte nuclear maturity was assessed visually at the start and end of culture. Spent drops of IVM media were stored in their culture dishes under oil at -80°C for future metabolism assays. A subset of oocytes that had failed to mature to metaphase II (MII) after 16–18 h were cultured for a further 5–6 h in 1 μl of maturation medium and maturation rate and metabolism were subsequently re-assessed.

**Metabolism assays**

The glucose, pyruvate and l-lactate content of spent media were assayed using a non-invasive, ultra-microfluorometric method (Leese and Barton, 1984). Assays were performed on an epifluorescence Zeiss Axioplan microscope with photon multiplier tube and photometer attachments (Photon Technology International, Ford, W. Sussex, UK). Data were processed using Felix 32 software (Photon Technology International, Ford, W. Sussex, UK).

**Oocyte fluorescence assays**

All assays of oocyte fluorescence were performed on the photometry system previously described, using a 40× objective lens and with a photo-meter aperture set to 130 × 130 μm.

**Assay of intracellular nicotinamide adenine dinucleotide phosphate content analysed by oocyte autofluorescence**

Oocyte nicotinamide adenine dinucleotide phosphate [reduced NAD(P)H] content reflects intracellular REDOX potential and can be investigated by measuring oocyte autofluorescence (Dumollard et al., 2007; Johnson et al., 2007). Solutions of 1 mM NADH and 1 mM NADPH were subjected to an excitation scan to determine optimum excitation wavelength. After this calibration, a subset of oocytes was subjected to 10 ms of excitation and oocyte autofluorescence intensity was quantified.

**Oocyte mitochondria activity assessment**

Prior to assessment, oocyte background fluorescence readings were obtained. Oocytes were incubated in a 1 μg/ml solution of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen, Paisley, UK) in Hank’s buffered saline solution (HBSS) containing 0.01% v/v dimethylsulfoxide for 30 min at 37°C (Van Blerkom et al., 2003). This compound differentially fluoresces according to inner mitochondria membrane potential, with orange-fluorescing J-aggregates forming in the presence of polarized, active mitochondria and green-fluorescing monomers co-existing with inactive mitochondria. Oocytes were washed three times in fresh HBSS before being exposed to UV irradiation (488 ± 2 nm) for 10 ms. Background values for orange and green fluorescence were subtracted and the ratio of active:inactive mitochondria was produced by co-quantifying intensities of orange (active mitochondria) and green (inactive mitochondria) fluorescence.

**Oocyte chromosome preparation**

Mill chromosome preparations were obtained using the methodology previously detailed by Clyde et al. (2001, 2003). Briefly, oocytes with a visible first PB were incubated in a hypotonic solution of 1% w/v sodium citrate for 18–20 min at 30°C. Once sufficient swelling had been achieved, oocytes were transferred to fresh, ice-cold Carnoy’s fixative (3:1 methanol:acetic acid) for ~3 s. A finely pulled glass pipette was then used to drop the oocyte from a height of ~6 cm onto an ice-cold, positively charged slide (VWR UK Ltd., Lutterworth, UK). The oocyte spread was allowed to dry in air at 30°C containing 50% humidity and the location of the oocyte chromosomes was visualized under phase contrast microscopy and marked with a diamond-tipped slide marker (Zeiss, Welwyn Garden City, UK). The chromosome spread was allowed to age at room temperature overnight before storage at 4°C until karyotype analysis was performed.

**Oocyte karyotyping**

Prior to karyotyping, oocyte spreads were stained with 60 ng/ml 4′,6-diamidino-2-phenylindole in Vectorshield mounting medium (Vectorlabs, Burlingame, CA, USA) and examined on a Zeiss Axiosvert epifluorescence microscope in order to get an accurate chromosome count. Spreads were then soaked for 30 min in 2 × sodium chloride/sodium citrate (SSC) solution and washed in a further two changes of 2 × SSC followed by 30 min each in 70, 90 and 100% ethanol. Karyotyping was done using mFISH.
WCP (Spectravysion, Abbott Molecular Inc., Maidenhead, UK) in which chromosomes are labelled with a unique combination of six different fluorophores. The manufacturer’s protocol for mFISH was followed with the following modifications: prior to pepsin treatment, oocyte spreads were exposed to 1 M sodium thiocyanate at 80°C for 0, 4 or 8 min, depending on chromosome morphology. A charged coupled device was used for capturing images which were subsequently analysed using Genus software (Applied Imaging International, Genetix Ltd., New Milton, UK) to detect numerical chromosome abnormalities occurring in meiosis I, namely chromosome non-disjunction or premature PSSC.

**Statistical analyses**

Data were checked for normal distribution using the Anderson–Darling test. Where appropriate for parametric data, student’s t-test or one-way analysis of variance (ANOVA) followed by Fisher’s post hoc test were used to specify the contrast among means. The Mann–Whitney U-test was used to test for statistical significance between proportions. Significance was assumed if \( P \leq 0.05 \). Minitab software was used for all statistical analyses. Data from PCOS patients were also analysed on the basis of prior treatment with metformin.

**Results**

**In vitro maturation**

One hundred and twenty-five oocytes were donated from 46 control patients (mean age 34.6 ± 0.5 years, range 22–41), 40 oocytes were donated from 14 PCOS patients (mean age 31.7 ± 1.3 years, range 24–40) and 31 oocytes were donated from 14 PCO patients (mean age 33.9 ± 1.0 years). There were no significant differences in patient age between groups and no effects of patient aetiology were recorded for the IVM potential of oocytes from the different sources. For control, PCOS and PCO oocytes that were at the GV stage at the start of culture, 15–42% produced a PB after only 18 h of IVM (control \( n = 65 \), PCO \( n = 13 \), PCOS \( n = 14 \)). For oocytes that were at the GVBD stage, 62–83% produced a PB after 18 h of IVM (control \( n = 59 \), PCO \( n = 18 \), PCOS \( n = 26 \)). A subset of oocytes (\( n = 7 \)) which failed to progress from the GV stage after 18 h of IVM was cultured for a further 5–6 h. None of these oocytes progressed to MII after extended culture. Of the oocytes that were GVBD after 18 h of culture, 72% (\( n = 31 \)) went on to MII and extruded a first PB after a further 6 h of culture. Overall, 52% of GV and 68% of GVBD oocytes progressed to MII over 24 h, 14% degenerated. For the oocytes from PCOS patients, there was no significant effect of prior patient treatment with metformin on oocyte maturation potential (66.6% MII with metformin versus 54.5% without metformin).

**Karyotype**

There were no significant differences in frequency of oocytes with numerical chromosome abnormalities (mean 44.2%) observed in 57 control, 17 PCOS and 21 PCO MII oocytes (\( P \geq 0.05 \)), however, the frequency of different types of chromosome abnormality did differ significantly between aetiologies. A representative example of mFISH is shown in Fig. 1. In control oocytes, whole chromosome losses or gains accounted for 61% of numerical abnormalities, whereas predivision (balanced and unbalanced) accounted for 39% (Fig. 2A). In PCO and PCOS oocytes, the proportions for non-disjunction and predivision events were significantly different to controls, being 36 and 64%, respectively, for non-disjunction and predivision in PCO oocytes and 41 and 59%, respectively, in PCOS oocytes (Fig. 2A). When chromosome predivision was further analysed, no significant differences in predivision type were observed.

**Figure 1** Karyotyping of oocytes by mFISH. (A) Oocyte chromosomes labelled with DAPI, (B) composite of the six fluorophores used for combinatorial labelling, (C) pseudo-coloured image to facilitate chromosome classification and (D) karyotype. An extra chromatid 15 is present (arrowhead), indicating unbalanced predivision in this oocyte.
between different aetiologies (Fig. 2B) and the occurrences of the different predivision abnormalities were similar between the different chromosome groups (Fig. 2C). Retention of one chromatid was the most frequently observed abnormality, occurring in 36–43% of oocytes. Balanced predivision accounted for only 10–36% of predivision events. Group C chromosomes were most likely to be involved in non-disjunction (Fig. 2C), but when the data were weighted to account for the differing numbers of chromosomes in each group (for example, Group C contains eight chromosomes; 6–12 and X, while Group G is comprised of only two chromosomes; 21 and 22), Group F and G chromosomes were most at risk of non-disjunction (Fig. 2C). Conversely, Group G chromosomes were at most risk of predivision.

There were no significant differences ($P = 0.786$) in the incidence of chromosome abnormalities in oocytes maturing to MII in the first 18 h of culture and those reaching MII during the second culture period. There were no significant differences in the types of chromosome abnormality detected in oocytes after both culture periods. A small subset of oocytes ($n = 7$) progressed to MII prior to overnight culture. These gametes were fixed immediately for chromosome analysis, and showed no significant difference in incidence of chromosome abnormality compared with oocytes cultured overnight ($P = 0.878$).

**Metabolism**

Within each patient group, there were no significant differences in metabolism between GV and GVBD oocytes which matured to MII, so data were pooled (Fig. 3). The PCOS aetiology was associated with increased glucose consumption by both GV and GVBD oocytes maturing to MII, although there was no significant difference according to whether or not the PCOS patients had previously been prescribed metformin (Fig. 3). Pyruvate consumption by PCO oocytes in vitro was not significantly different to controls (Fig. 3). In marked contrast, pyruvate consumption by oocytes from PCOS ($+/−$ metformin) was significantly different to controls. The PCOS aetiology was associated with elevated oocyte pyruvate consumption, and prior exposure to metformin in vivo significantly reduced oocyte uptake of this metabolite in vitro. There were no significant differences in oocyte lactate output, although the difference between PCOS groups ($+/−$ metformin) approached significance ($P = 0.06$; Fig. 3).

There were no other major differences in oocyte metabolism profiles relating to specific metabolite or stage of meiosis. For oocytes maturing to MII, oocytes from PCOS patients who had prior treatment with metformin were divided according to whether or not they had been exposed to metformin in vivo. Glucose consumption by oocytes from PCOS patients who had prior treatment with metformin was $2.3 ± 1.3 \text{ pmol/h}$, whereas oocytes from PCOS patients not exposed to metformin in vivo had a lower rate of glucose consumption at $1.8 ± 0.4 \text{ pmol/h}$, although this difference was not significant. Both groups were significantly different to glucose consumption by control oocytes (Fig. 3). Oocyte metabolism was compared between oocytes which progressed to MII over 18 h compared with 24 h. The data show that oocytes which matured from GVBD to MII during the second culture had a significantly higher rate of pyruvate consumption ($16.4 ± 1.2 \text{ pmol/oocyte/h}$, $n = 39$) compared with oocytes that had matured during the initial culture ($12.2 ± 0.8 \text{ pmol/oocyte/h}$, $n = 64$; $P = 0.004$). Irrespective of oocyte aetiology, pyruvate consumption by individual oocytes was significantly associated with lactate production ($P < 0.0001$; Pearson coefficient $= 0.377$). Control MII oocytes with an abnormal karyotype had a significantly higher pyruvate consumption.
(19.9 ± 2.1 pmol/h, n = 19) compared with chromosomally normal oocytes (13.4 ± 1.9 pmol/h, n = 22; P = 0.028).

**NAD(P)H content**

At the end of culture, there were no significant differences in NAD(P)H content between GVBD and MII oocytes. Oocytes from control patients had significantly lower levels of intracellular NAD(P)H compared with oocytes from PCO or PCOS patients (Table I). Irrespective of aetiology or karyotype, oocyte NAD(P)H content was significantly associated with oocyte pyruvate consumption (P = 0.001, Pearson coefficient = 0.431), lactate production (P = 0.002; Pearson coefficient = 0.413), lactate:pyruvate ratio (P = 0.016; Pearson coefficient = −0.32) and the difference between pyruvate consumption and lactate production (P = 0.009; Pearson coefficient = 0.321). Oocyte NAD(P)H content was also 1.35-fold higher in chromosomally abnormal oocytes (P = 0.013). Within the PCOS category, there was no significant difference in intracellular NAD(P)H between metformin-exposed and non-exposed oocytes, therefore Table I shows the pooled data.

### Table I  Level of intracellular NAD(P)H in oocytes from control, PCO and PCOS patients.

<table>
<thead>
<tr>
<th>Patient aetiology</th>
<th>No. of oocytes</th>
<th>Intracellular NAD(P)H (arbitrary units)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>45</td>
<td>40.067 ± 2234&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCO</td>
<td>20</td>
<td>53.822 ± 2157&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCOS</td>
<td>21</td>
<td>49.671 ± 1696&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SEM of the number of oocytes shown. Different superscripts indicate significant differences (P < 0.001).

**Mitochondria**

Patient aetiology, karyotype and glucose, pyruvate and lactate metabolism were not associated with oocyte mitochondria activity (P > 0.1), nor was there any significant difference in mitochondrial activity between GVBD and MII oocytes. However, mitochondrial activity was found to be significantly associated with control oocyte NAD(P)H content (P < 0.001; Pearson coefficient = −0.676). Similar trends were seen in PCOS and PCO oocytes but these were not significant. There was no significant difference in the mitochondrial activity of oocytes from PCOS patients who had been prescribed metformin prior to oocyte recovery and those that had not been treated with metformin.

### Discussion

This report is the first to demonstrate an association between the metabolic profile of maturing oocytes from control patients and patients with PCO and PCOS in relation to chromosome normality, oocyte NAD(P)H content and mitochondrial activity. The IVM potential of surplus GV and GVBD oocytes was not significantly different between control, PCOS and PCO groups. All patients received hCG ~36 h prior to egg collection, a procedure that has been shown to improve the outcome in PCOS patients undergoing IVM.
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treatment cycles (Chian et al., 2000). It is important to note that all of the oocytes donated for research in the study had failed to respond fully to hCG in vivo, probably due to their harvest from less advanced follicles in the ovulatory cohort which lack sufficient granulosa cell LH receptors to respond to the hCG (Grondahl, 2008). These oocytes are likely to have some degree of cytoplasmic immaturity which has underpinned their delayed maturation. Despite this, the majority of the oocytes harvested from smaller follicles have been shown to retain the capacity to progress to MII when exposed to the appropriate signals in vitro and in some cases oocytes from this source have been successfully fertilized in vitro (Hassan et al., 2001), albeit with a significantly reduced developmental competence. Furthermore, a limited number of reports have suggested that given time and suitable conditions immature oocytes from this origin have produced a pregnancy and live birth (Barnes et al., 1995). The underlying cause of the reduced developmental potential and fertility of these immature oocytes may be due to asynchrony between the follicle environment and oocyte maturation or to extended culture which is manifest by increased chromosome errors (Clyde et al., 2003) in this population relative to oocytes matured wholly in vivo or in vitro (Smitz et al., 2007). Encouragingly, both the IVM rates recorded in the current study and the aneuploidy rates are consistent to those for oocytes from hCG-primed PCOS patients (Yang et al., 2005) as reported in our earlier work (Clyde et al., 2003), respectively. The IVM of immature oocytes from ICSI cycles therefore represents a valuable experimental model system which can be used in tightly controlled studies to investigate the impact of patient aetiology on many aspects of oocyte biology in vitro including metabolism and cytogenetics. Furthermore, the rationale of the present study permitted direct comparison of these parameters in oocytes from PCOS patients with those from PCO morphology compared with normal controls. The question of whether PCO is pathological or a normal variant of ovarian morphology is a subject of considerable debate. By segregation of the three different aetiologies, this study has for the first time permitted the direct evaluation of the impact of full blown PCOS on oocyte metabolism and karyosomal health while allowing the investigation of the normality of PCO oocytes compared with controls.

During development, the oocyte is dependent on its host follicular environment to provide an appropriate milieu of nutrients, proteins and energy supplies at suitable concentrations. Granulosa cells metabolize glucose, metabolites of which are then secreted into follicular fluid (Hillier et al., 1985; Leese and Barton, 1985; Harris et al., 2005) or passed directly to the oocyte via gap junctions (Downs, 1995; Eppig et al., 2005). In patients with insulin resistance, there is a question of whether the perturbed insulin and glucose metabolism reported for granulosa cells (Rice et al., 2005) significantly affects nutritional availability to the developing oocyte, with consequent alteration in the oocyte’s own metabolism profile. Follicular fluid glucose concentration is in the range of 3–3.4 mM (Gull et al., 1999; Leese and Lenton, 1990), but we have been unable to find data specific for PCOS follicles. Although the effects of oocyte exposure to an altered follicular metabolism during growth and maturation upon developmental competence are unclear, it has previously been shown that the metabolic and maturation potential of human oocytes vary with the nutrient composition of culture media (Roberts et al., 2002). Similar observations have been made in human embryos, in which sub-optimal nutrient levels induce metabolic transformation (Leese, 1995) with subsequent reduced developmental potential (Lane and Gardner, 1998).

In the current work, altered oocyte metabolism was demonstrated in maturing oocytes from PCOS patients. Glucose was consumed in significantly greater amounts by oocytes derived from patients with PCOS, but this alteration was not solely caused by increased sensitivity to insulin as defined by prior exposure to metformin in vivo. Hyperglycaemia is often associated with the generation of reactive oxygen species (ROS). In some cell types, metformin reduces the effect of ROS, in part through the suppression of NADPH oxidase (Bellin et al., 2006). This reduces NADPH metabolism and would potentially raise intracellular availability, which provides a possible explanation for the present observation that NAD(P)H was higher in PCOS oocytes.

There is little doubt that PCOS is associated with perturbed energy metabolism in many somatic tissues, including the ovary (Rice et al., 2005), which will contribute to an altered endocrine and nutritional environment within the follicle. In this study, we have confirmed that glucose is consumed at low rates by human oocytes. It is often thought that mammalian oocytes do not consume glucose, indeed, mouse oocytes can survive oogenesis without an intact glycolytic pathway (Kelly and West, 2002). In species such as mouse, mature oocyte glucose consumption is barely detectable (Downs and Utecht, 1999), but in other species with larger oocytes, including pigs (Krisher et al., 2007), cows (Krisher and Bavister, 1999), cats (Krisher and Bavister, 1999) and humans (Hardy et al., 1989), low rates of glucose consumption have been demonstrated. In human oocytes, GLUT-1 (SLC2A1) expression has been shown (Dan-Goor et al., 1997) but GLUT-2, 3 and 4 are not expressed (Dan-Goor et al., 1997). In contrast, in monkey oocytes, GLUT-4 and 6 appear to be the predominant glucose transporters (Zheng et al., 2007). Glycolytic pathway components are expressed in mammalian (Chi et al., 1988; Zheng et al., 2007) and human (Chi et al., 1988; Tsutsumi et al., 1990; el Mouatassim et al., 1999) oocytes. Pentose phosphate pathway (PPP) components are also expressed in mammalian (Mangia and Epstein, 1975; Tsutsumi et al., 1992; Ferrandi et al., 1993; Zheng et al., 2007) and human (Chi et al., 1988) oocytes. Insulin signalling pathways are also active (Acevedo et al., 2007; Zheng et al., 2007). In human oocytes, glucose metabolism is very low in comparison to pyruvate and it is not thought to be a major energy substrate, however, it is possibly metabolized via the PPP to produce NADPH, ribose-5-phosphate and purine precursors.

The metabolism of pyruvate, the key energy substrate used by human oocytes (Hardy et al., 1989), was greater by PCOS oocytes. It was previously thought that oocyte pyruvate utilization was not significantly affected by PCOS (Franks et al., 2002), however, when the current PCOS oocyte data were pooled the results were not significantly different to controls, and it was only when metformin pretreatment was accounted for that differences in pyruvate metabolism became apparent. Prior exposure to metformin in vivo also significantly attenuates pyruvate uptake. One of the mechanisms of action of metformin is activation of the AMP-activated protein kinase (AMPK) cascade (Hardie, 2008) through accumulation in mitochondria (Owen et al., 2000) where it inhibits Complex I of the respiratory chain (Brunmair et al., 2004). This stimulates glycolysis (Katzung, 2006; Hardie, 2008) and attenuates Krebs cycle activity. The half-life of metformin in vivo is ~6 h due to renal clearance but in culture it could exist in cells for longer, due to prior mitochondrial accumulation.
and the absence of clearing processes. This provides a plausible explanation for reduced pyruvate consumption observed in the present studies by oocytes which had been exposed to metformin in vivo. There was a range of oocyte pyruvate consumption rates in the current study but these were not reflected by differences in mitochondrial activity in the same cells. Increased pyruvate consumption tended to be associated with increased lactate production, a phenomenon that has previously been postulated to be related to adjusting cellular pH (Butcher et al., 1998). In human embryos, the majority of pyruvate consumed is not accounted for by oxidation (Butcher et al., 1998) and a large proportion of pyruvate appears to be exported as lactate (Butcher et al., 1998), which echoes the current results in human oocytes.

Many tissues in women with PCOS or insulin resistance demonstrate reduced glucose consumption; however, interestingly, we have observed an increased capacity for glucose consumption by oocytes from patients with PCOS. The cause of this surprising observation is unclear. It is unlikely that oocytes use glucose for significant energy production, as pyruvate is consumed in far greater quantities; however, it is possible that oocyte glucose consumption is related to production of NADPH or purine precursors via the PPP.

In support of this idea in the present study, we have shown that NAD(P)H is higher in oocytes from PCOS patients compared with controls.

Mitochondrial activity was measured in a subset of oocytes. There was no significant association with patient aetiology, stage of oocyte maturation or metabolism of glucose, lactate or pyruvate. Mitochondrial activity, however, was significantly correlated with oocyte NAD(P)H content, with oocytes with a greater proportion of active mitochondria tending to have higher NAD(P)H content. There was a similar pattern in PCOS and PCO oocytes but the trend was not significant. The observed NAD(P)H fluorescence can come from two major sources: (i) the cytoplasm, where PPP activity produces NADPH and glycolysis produces a small amount of NADH; or (ii) the mitochondria, where the Krebs cycle activity generates NADH and it is probable that the association of mitochondrial activity with NAD(P)H content is due to increased mitochondrial NADH generation.

Numerical chromosome errors are common in human oocytes (Clyde et al., 2003; Smits et al., 2007) and are an underlying cause of aneuploidy in human embryos (Dailey et al., 1996). In the current study, numerical chromosome abnormality occurred in ~44% of oocytes. This is similar to abnormality rates reported by other workers using either unfertilized or meiotically delayed oocytes. This is similar to abnormality rates reported by other workers using either unfertilized or meiotically delayed oocytes which subsequently underwent IVM (Clyde et al., 2003; Gutierrez-Mateo et al., 2004; Fragogli et al., 2006). The observed result is higher than the chromosomal error rate of ~20% reported for fresh oocytes which were analysed immediately after oocyte harvest (Sandalinatas et al., 2002; Smits et al., 2007) and fresh oocytes from unstimulated ovaries (Volarcik et al., 1998). It is well-known that the smaller chromosomes in Groups F and G are most susceptible to involvement in meiotic errors (Fragoli et al., 2006). This agrees with the current oocyte data.

Advancing maternal age is a major predisposing factor for numerical chromosome abnormalities in oocytes (Dailey et al., 1996; Sandalinatas et al., 2002; Pellestor et al., 2005) which has been implicated in declining functionality of oocyte mitochondria (Wilding et al., 2001). Oocyte meiosis is an energy-dependent process and age-related mitochondrial insufficiency has been proposed as a causative factor in aneuploidy induction (Schon et al., 2000). In this study, we found no significant effect of PCOS or PCO pathologies upon overall incidence of oocyte chromosome abnormalities. This incidence of chromosome errors agrees with previous observations that PCOS oocytes have similar numbers of chromosomes to non-PCOS oocytes (Sengoku et al., 1997). This suggests that energy production in PCOS and PCO oocytes is not significantly compromised and there is sufficient ATP to support energy-demanding processes such as meiosis. This idea is backed up by the observation that oocyte mitochondrial activity was not significantly different within the three different aetiologies studied.

Although overall incidence of chromosome abnormalities was not significantly different to Controls, in PCOS and PCO oocytes there was an increased frequency of predivision compared with non-disjunction events. Non-disjunction and predivision are the two major mechanisms inducing numerical chromosome abnormality in human oocytes (Dailey et al., 1996), accounting for ~50% of abnormalities observed (Rosenbusch et al., 2001). These proportions do vary, however, between studies (Rosenbusch, 2006) and can be influenced by maternal age and time in culture (Dailey et al., 1996). Predivision occurs when the two chromatids split prior to the separation of chromosome homologs during meiosis I. This phenomenon can be caused by lack of centromeric cohesion (Viallard et al., 2006) and inappropriate expression of genes including shugoshin 1 (Yin et al., 2008) and separase (Zhang et al., 2008). Maternal ageing is associated with down-regulation of genes involved in the oocyte spindle checkpoint (Steuerwald et al., 2001), however, in the current study patient age could not account for the observed differences in predivision and non-disjunction frequency in PCO/PCOS oocytes. For predivision to occur, separation of sister chromatids must occur prior to whole chromosome separation. The current data suggest that PCO and PCOS may predispose oocytes to predivision, indeed, aberrant expression of many genes regulating meiosis has been observed in oocytes from PCOS patients (Wood et al., 2007). However, overall incidence of numerical chromosome abnormalities was not significantly higher in MII oocytes from PCO or PCOS MII patients, indicating that chromosomal abnormalities are not the major cause of the lower pregnancy outcomes associated with PCOS. It is unclear how the altered transcriptome in MII oocytes (Wood et al., 2007) affects completion of meiosis II and subsequent mitotic divisions in the embryo, however, there is evidence to suggest that there is little difference in incidence of aneuploidy in embryos from Control and PCOS patients (Weghofer et al., 2007).

Ovarian stimulation is associated with reduced egg quality in mice and humans (Akaebosu et al., 1998; Van der Auwera and D’Hooghe, 2001), possibly due to altered tract milieu. Excess vascular endothelial growth factor is often associated with ovarian stimulation (Gomez et al., 2002; Wang et al., 2002), particularly in PCOS (Agrawal et al., 2002), and this can cause increased oviduct permeability, resulting in increased plasma transudation and thus altering the oviduct fluid composition; incubation fluid composition is well-known to influence oocyte and embryo quality (Gardner and Leese, 1990; Roberts et al., 2002; Lane and Gardner, 2005). Furthermore, excess androgens appear to contribute, at least partially, to altered gene expression in PCOS oocytes (Wood et al., 2007).
In human embryos, metabolic profiling has the capacity to predict developmental outcomes (Houghton et al., 2002; Brison et al., 2004). The factors contributing to altered metabolism by good and poor quality embryos are not understood. Pyruvate, which is the predominant energy metabolite in human oocytes and early preimplantation embryos, was consumed at significantly higher rates by oocytes harbouring chromosome abnormalities. Pyruvate consumption has previously been shown to be higher by human embryos that fail to implant (Conaghan et al., 1993) and the current data provide a plausible explanation for why this may occur.

In conclusion, we have shown that the metabolic profile of oocytes is associated with chromosome abnormality. The data demonstrate that oocyte metabolism is compromised during assisted conception cycles in patients with PCOS compared with healthy controls and this metabolic disturbance can be detected both within and between individual oocytes. Furthermore, whereas oocyte metabolism during IVM can be moderated by prior in vivo exposure to insulin-sensitising agents such as metformin, oocytes from PCOS patients are not likely to harbour an increased number of numerical chromosome abnormalities compared with controls. Oocytes from PCO patients had intermediate metabolism profiles compared with controls and PCOS.

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References


