SHORT COMMUNICATION

NAPLES, ITALY

The effect of extended culture of cumulus–oocyte complexes in follicular fluid during *in vitro* fertilisation cycles

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Purpose: To assess the clinical and biological effect of the preincubation of oocytes in follicular fluid prior to IVF and ICSI cycles.

Methods: A series of patients were treated by the preincubation of oocytes in the patients' follicular fluid for 3 h after oocyte retrieval followed by processing with standard protocols. Control oocytes were preincubated in normal IVF culture medium. Fluorescence techniques were used to examine oocyte mitochondrial membrane potential.

Results: Fertilisation, pregnancy, and implantation rates were all significantly improved after the preincubation of oocytes in follicular fluid. Further tests suggested that differences in pH between follicular fluid and artificial culture medium may be critical to these differences.

Conclusions: Preincubation of human oocytes in follicular fluid improves the results after IVF. This may be partly due to the use of a non-“physiological” pH in artificial culture media during *in vitro* fertilisation procedures.

KEY WORDS: Assisted reproduction; follicular fluid; human oocyte; IVF and ICSI; pH regulation.

INTRODUCTION

The development of protocols and culture systems for *in vitro* fertilisation has led to a progressive increase in the clinical success rate after IVF, which national statistics now report in the range of 20–30% (1–3). However, the use of artificial, defined media for the *in vitro* culture of human embryos is limited to the extent of scientific knowledge of the *in vivo* environment. In human reproduction, the difficulty of access to the *in vivo* embryo environment together with the changes in environment that occur as the human embryo progresses

along the fallopian tube towards the uterus, augment the difficulties in developing adequate *in vitro* substitutes.

The large amount of follicular fluid that is expelled with the oocyte at ovulation suggest that cumulus–oocyte complexes (COCs) will remain within an environment that is similar to follicular fluid for the first hours after natural ovulation. However, current protocols of assisted reproduction require the immediate removal of oocytes from follicular fluid after oocyte retrieval. This protocol may cause abrupt changes in the environment of the oocyte at a critical point in the reproductive cycle. One of the critical parameters of this environment could be the pH of fluids surrounding the oocyte. In fact, although the osmolarity and temperature of *in vitro* culture medium mimic the *in vivo* environment, the difficulty of measuring the *in vivo* pH of reproductive fluids means that questions still remain as to the correct pH of human embryo culture medium. Although a pH of 7.1–7.3 is commonly achieved with artificial media in culture conditions, measurements of the pH of the fluid contained within the human follicle (4–9), fallopian tube (10–12), and uterus (13–15) vary, suggesting that the “physiological” pH of culture media may not accurately reflect the *in vivo* environment of the human oocyte and embryo. The maintenance of correct cytoplasmic pH is vital for virtually all cell processes. Although human oocytes contain mechanisms to balance cytoplasmic pH against changes in the external environment (9,16–18), these may lose efficiency in the long term, causing a deviation in the cytoplasmic pH.

In this work, we have examined the clinical effect of the extended culture of freshly retrieved human oocytes in follicular fluid during cycles of *in vitro* fertilisation. We further examine the physiological pH and buffering capacity of human follicular fluid with respect to both human blood serum and commercially available IVF culture media. Because pH is critical to the maintenance of the H⁺ potential across the mitochondrial membrane and therefore the production of adenosine triphosphate (ATP) through aerobic respiration, we use a mitochondrial membrane potential (mitochondrial $\Delta\Psi$)-sensitive fluorescence probe to examine the effect of extended culture in non-“physiological” pH on the oocyte mitochondrial $\Delta\Psi$. Although many differences exist between defined artificial culture medium and follicular fluid, our results suggest that the pH difference between the two fluids is a critical factor.

MATERIALS AND METHODS

Patients

A prospective, multicenter, randomised controlled trial was initiated to test the clinical outcome of preincubation of human oocytes for 3 h in follicular fluid as opposed to preincubation in IVF culture medium. IRB approval was not sought due to the absence of such a board for private IVF centers; however, the guidelines of the Helsinki Declaration 1975 were adhered to and the research would qualify for 45CFR, 46.101 criteria. The trial followed CONSORT guidelines (19). Patients were attending IVF centers for IVF and ICSI procedures between 2000 and 2004. Patients agreed to the trial and signed an informed consent form. All centers used identical procedures both for patient preparation and in the laboratory, and these were coordinated by one member of the medical and biological team. Patients were prepared for IVF and ICSI using standard controlled ovarian hyperstimulation protocols including down-regulation of the pituitary gland with a GnRH agonist (Decapeptyl, Ipsen, Italy) followed by ovarian stimulation with exogenous FSH. Oocyte retrieval was performed 36 h after the administration of 10,000 IU β -hCG when two to three follicles of 18–20 mm diameter were observed by ultrasound examination, and blood 17 β -oestradiol levels reached 150–200 pg/mL/follicle over 18 mm. Oocytes were processed for standard IVF and ICSI protocols using commercial IVF medium (Medicult, Copenhagen, Denmark) pre-equilibrated to 37°C and 5% CO₂. The establishment of a pregnancy was considered as a positive β -hCG test 14 days after embryo transfer, followed by the observation of a gestational sac with foetal heart beats after ultrasound analysis, 8 weeks after the establishment of pregnancy.

A sample of 5 mL of clear, blood-free follicular fluid observed to have contained a COC was collected during oocyte retrieval, filtered and conserved at 37°C in a closed polypropylene tube (Falcon 2063, Becton Dickinson, NJ, USA). COCs retrieved during oocyte retrieval were placed into this sample and maintained at 37°C with the stopper closed for 3 h after the completion of the oocyte retrieval (39 h post-hCG). Control COCs were collected into equivalent tubes filled with commercial IVF medium (Medicult) and maintained in an atmosphere of 37°C and 5% CO₂ until 39 h post-hCG. Oocytes were then washed into standard commercial IVF medium (Medicult) and treated as for routine IVF and ICSI protocols.

The primary outcome measure of the trial was the clinical success rate (i.e., pregnancy and implantation rates). Secondary outcome measurements were the other biological parameters recorded during the trial (such as fertilisation rate and embryo quality scores). Embryo quality scores were calculated by previously described methods (20). All embryo transfers were performed on day 2 (43–44 h post-insemination). Three exclusion criteria were applied to the trial. In the first, patients requiring the use of spermatozoa derived from testicular aspiration or biopsy were excluded due to the lower fertilisation and implantation rates observed in these cycles. Secondly, patients diagnosed with hydrosalpinx, endometriosis, or polycystic ovarian syndrome were excluded from the study due to the possible influence of these factors on oogenesis or endometrial receptivity. Thirdly, patients undergoing their second or subsequent cycle were excluded from the trial. Patients undergoing the trial were treated in batches of at least 25. Patients in a single batch were selected for the trial and control groups by the assignment of numbers followed by the use of random number tables to allot to groups. Analysis of the results were performed blind, i.e., the statistician was unaware which of the groups was the test group.

pH Measurements

Measurements of pH were taken by the use of litmus paper (Sigma, Milan, Italy) and a Hanna instruments HI 9321 pH meter (Hanna Instruments, Rhode Island, USA). The calibration curve for the pH meter was made with pH 7.0 and 4.0 standards as references. The measurement of extracellular fluids was standardised to ensure reliability in the measurements. In this sense, the pH of clear, blood-free follicular fluid was measured immediately after examination for the presence of oocytes during oocyte retrieval. The total time lapse between fluid retrieval and pH measurement was less than a minute. Measurements on IVF culture media were made after equilibration in an atmosphere of 37°C and 5% CO₂. The pH of human blood serum was measured immediately after the removal of blood cells by centrifugation. Measurements of the change in pH over time were made by placing 10 mL of fluid in an open container in an atmosphere of 37°C in air. The buffer capacity of the above-mentioned fluids was tested by the measurement of pH after the addition of 0.1 M aliquots of either HCl or NaOH to fluids maintained in an atmosphere of 37°C and 5% CO₂.

Fluorescence Labeling and Confocal Microscopy

Fresh metaphase-II human oocytes were donated for research after informed consent and in cases where excess oocytes were available after IVF treatments without affecting the clinical potential of the IVF cycle. The potential-sensitive fluorescence dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Oregon, USA) was used to measure mitochondria $\Delta\Psi$ (21,22). The dye was dissolved to a stock concentration of 0.5 mM in a solubilising agent containing 10% Pluronic F-127 in dimethylsulphoxide and diluted into pre-equilibrated IVF medium (Medicult), using a vortex to aid the dissolution of the dye, as required. An Olympus Fluoview (Olympus, Segrate, Italy) confocal microscope, based on an Olympus IX-70 inverted microscope, was used for all experiments. A Kr/Ar laser was used to produce the excitation laser line at 488 nm and emission wavelengths were separated by a 530 nm dichroic mirror followed by analysis in a photo multiplier after further filtering through a 515–530 nm band pass filter (green emission) or a 585 nm long pass filter (red emission). Laser power and photo multiplier settings were kept constant for all experiments. Oocytes were positioned with the polar body in the plane of focus where present and a single scan through the center of the oocyte was used for the analysis. Oocytes were used only once before being discarded. Images were processed by the confocal software and Adobe Photoshop. The relative mitochondrial membrane potential was calculated by ratiometric analysis of the images obtained. Ratiometric measurements of individual oocytes and embryos was calculated by pixel-by-pixel division of the mitochondrial $\Delta\Psi$ -sensitive confocal channel red channel (J-aggregates) by the green emission channel (JC-1 monomers). The result is an indication of mitochondrial activity where dye distribution artefacts have been removed. These data were plotted as mean pixel intensity of the ratio calculated using confocal software to indicate mean mitochondrial $\Delta\Psi$ of the scanned region.

Statistics

All data were plotted as mean $\pm$ SD unless stated. All plots and statistical analysis was calculated using the Sigma Plot and Sigma Stat software packages (SPSS, Erkrath, Germany). Student's *t*-test was used to test for differences between groups. The Mann–

Whitney rank sum test was used where required to adjust the *t*-test for small populations of data. The *z*-test was used to determine the significance of proportions.

RESULTS

The Preincubation of Human Oocytes in Follicular Fluid Increases the Clinical IVF Success Rate

A total of 512 patients, matched for maternal age, BMI, and sperm quality, agreed to the trial and signed an informed consent form during 2000–2004. These patients were randomly selected into controls (group A) or experimental protocols (group B) according to random number tables. In total, 157 patients were selected for IVF protocols and 355 patients were treated with ICSI (Tables I and II). After 8 cycle cancellations for poor response to the ovarian stimulation protocol, 149 patients undergoing IVF had oocyte retrieval cycles (Table I). Three hundred and thirty-seven oocyte of the 355 patients attending for ICSI had oocyte retrieval (Table II). The numbers of oocytes retrieved was not statistically different between control and experimental procedures in either IVF or ICSI patients (Tables I and II). We examined fertilisation rates, embryo quality, and clinical success rates under these conditions. Group A IVF cycles were characterised by a fertilisation rate of 56% (474/846 COCs retrieved, Table I). Group B IVF cycles were characterised by a significantly higher fertilisation rate of 67% (483/720 COCs retrieved, Table I). ICSI cycles, in which the number of metaphase-II oocytes is precisely known, are a statistically more accurate test of the fertilisation rate in control and experimental protocols. In our trial, group A fertilisation rates after ICSI were 60% (805/1341 M-II oocytes, Table II). Group B fertilisation rates were again significantly higher (999/1297 M-II oocytes fertilised, 77%, Table II), confirming the data from IVF cycles. All centers in the trial noted the same differences between control and experimental groups. These data suggest that the pretreatment of COCs in human follicular fluid significantly increases the fertilisation rate after IVF or ICSI.

In addition to an increase in the fertilisation rate, clinical pregnancy and implantation rates were significantly higher in patients in which oocytes were preincubated in follicular fluid. Of a total of 83 oocyte retrieval cycles in group A patients attending for IVF, 31 achieved clinical pregnancy (37.8%,

Table I. Clinical Results After the Preincubation of Human Oocytes in Follicular Fluid During IVF Cycles

	Controls (group A)	Follicular fluid (group B)	Significance (<i>p</i> -value) ^a
Patients	83	66	N/A
Maternal age (years)	34.0 ± 3.1	33.2 ± 2.7	0.100
Oocyte retrievals	83	66	N/A
Number of oocytes (mean ± SD)	846 (10.1 ± 3.5)	720 (10.8 ± 4.4)	0.281
Number fertilised (%)	474 (56)	483 (67)	<0.001
Number of transfers	82	66	N/A
Number transferred (mean)	171 (2.2 ± 1.2)	144 (2.3 ± 1.0)	0.589
Embryo quality transferred embryos ^b	428 ± 122	468 ± 189	0.024
Top quality embryos (%)	19	21	0.763
Number of clinical pregnancies	31 (37.8)	37 (56.1)	0.04
Number of gestational sacs (implantation rate %)	34 (19.9)	40 (27.8)	0.13
Number of live births	32 (18.7)	39 (27.1)	N/A

Note. Data are presented as actual figures. Percentages are presented in parentheses where necessary. N/A: not applicable.

^a*p* < 0.05 is considered significant.

^bEmbryo quality according to ref. (20).

Table I). The pregnancy rate in group B IVF cycles was significantly higher than group A IVF cycles, 56.1% of cycles resulting in clinical pregnancy (37/66 cycles, Table I). Group B ICSI pregnancy rates were also significantly higher than group A ICSI cycles. Of a total of 177 ICSI cycles, 74 resulted in clinical pregnancies in group A (42%, Table II). Group B ICSI cycles were characterised by a clinical pregnancy rate of 56% (90/160 cycles, Table II). Implantation rates were also significantly higher in both group B IVF and ICSI cycles (Tables I and II). Although differences in embryo morphology were noted between control and trial groups (Tables I and II), no differences in the number of top quality embryos was noted at transfer (Tables I and II).

Measurement of pH of Extracellular Fluids During Cycles of IVF

At the moment of oocyte retrieval, oocytes are removed from a physiological environment (follicular fluid) and placed into an artificial ambient (culture medium). We tested whether these two environments coincided with respect to pH. The pH of follicular fluid was measured within 60 s of aspiration of oocytes during oocyte retrieval cycles, after the removal of COCs for use in assisted reproduction. We noted a value for pH of 7.65 ± 0.10 (mean ± SD, *n* = 242). Because the change of atmosphere during oocyte retrieval (i.e., transfer from a CO₂-containing atmosphere within the body to a CO₂-deficient atmosphere outside) could cause a rapid increase of pH in poorly buffered fluids, we measured the pH at

Table II. Clinical Results After the Preincubation of Human Oocytes in Follicular Fluid During ICSI Cycles

	Controls (group A)	Follicular fluid (group B)	Significance (<i>p</i> -value) ^a
Patients	177	160	N/A
Maternal age (years)	33.6 ± 3.3	34.0 ± 3.6	0.288
Oocyte retrievals	177	160	N/A
Number of oocytes (mean ± SD)	1412 (7.7 ± 4.3)	1336 (8.2 ± 3.2)	0.231
Number of mature oocytes injected	1341	1297	N/A
Number fertilised (%)	805 (60)	999 (77)	<0.001
Number of transfers	176	160	N/A
Number of embryos transferred (mean embryos/transfer)	442 (2.6 ± 0.6)	398 (2.4 ± 0.4)	<0.001
Embryo quality transferred embryos ^b	406 ± 144	476 ± 161	<0.001
Top quality embryos (%)	22	20	0.532
Number of pregnancies (pregnancy rate)	74 (42)	90 (56)	0.014
Number of gestational sacs (implantation rate %)	82 (18.6)	104 (26.1)	0.011
Number of live births	78 (17.6)	98 (24.6)	N/A

Note. Data are presented as actual figures. Percentages are presented in parentheses where necessary. N/A: not applicable.

^a*p* < 0.05 is considered significant.

^bEmbryo quality according to ref. (20).

Fig. 1. Change in pH of blood serum, follicular fluid, and IVF culture media after exposition to air. The graphs show the pH response of the fluids mentioned after the exposition to air. (a) The expansion of the trace to illustrate the first 5 min of exposure and (b) the entire trace of 120 min exposition. *Black circles* represent human follicular fluid, *white circles* IVF culture medium, and *triangles* human blood serum. In (a), *lines* represent regression lines, the *solid line* is the regression line for follicular fluid, *dashes* represent the regression line for serum, and *dots* the line for IVF culture media. A single experiment is shown out of a total of five experiments per fluid.

further 60-s intervals to test for a steady increase in it. pH readings in the subsequent intervals were equivalent to the initial measurement (Fig. 1). pH readings in follicular fluid changed slowly over the course of a further 60 min when exposed to air, demonstrating that CO_2 was expelled slowly and confirming that the initial reading was accurate (Fig. 1). The pH of fresh follicular fluid measured in the current experiments was significantly greater than the pH of commercial culture media equilibrated at 37°C and in an atmosphere of 5% CO_2 ($p < 0.001$, two-tailed t -test). In 242 tests, the pH of a wide range of batches of IVF medium was 7.2 ± 0.1 (mean $\pm$ SD, $n = 242$).

Fig. 2. pH buffering capacity of blood serum, follicular fluid, and IVF culture media. The graphs show the pH response of the three fluids mentioned to the addition of 0.1 M aliquots of HCl or NaOH. *Black circles* represent human follicular fluid, *white circles* IVF culture medium, and *triangles* human blood serum. A single experiment is shown out of a total of five experiments per fluid.

Human blood serum is known to have a pH of 7.4. As a control of our measurement technique, we tested simultaneously whether the pH of blood samples taken from patients undergoing oocyte retrieval matched that of follicular fluid. In our experiments, the pH of blood serum was 7.4 ± 0.05 (mean $\pm$ SD, $n = 238$). This was significantly lower than that of follicular fluid retrieved from the same patients. These data suggest that the pH of follicular fluid of human subjects at oocyte retrieval is significantly more alkaline than that of the blood serum from the same patients, and of *in vitro* culture medium.

We examined the pH buffering capacity of human follicular fluid, IVF culture medium, and human blood serum by measuring the pH obtained after the addition of 0.1 M aliquots of HCl or NaOH to the fluids. At the equilibrium pH established under these experimental conditions, the calculated buffer capacity of follicular fluid was 70.4 ± 3.3 M/pH unit/L (mean $\pm$ SD, $n = 5$, Fig. 2). This was slightly larger than the buffer capacity of human blood serum (55.6 ± 1.4 M/pH unit/L, mean $\pm$ SD, $n = 5$, $p = 0.03$, two-tailed t -test, Fig. 2). The calculated buffer capacity of *in vitro* culture medium measured was 29.8 ± 0.8 M/pH unit/L (mean $\pm$ SD, $n = 5$, Fig. 2), significantly lower than that of both follicular fluid and blood serum ($p < 0.001$, two-tailed t -test). Our data suggests that follicular fluid is a significantly stronger buffer than IVF culture medium at the equilibrium pH.

Table III. Extracellular pH and Mitochondrial Membrane Potential of Human Oocytes

	Mitochondrial membrane potential	Significance (<i>p</i> -value) ^a
7.0 (<i>n</i> = 18)	1.56 ± 0.1	<0.001
7.5 (<i>n</i> = 21)	1.88 ± 0.07	N/A
8.0 (<i>n</i> = 20)	1.62 ± 0.2	<0.001

Note. Data are presented as mean ± SD.

^aSignificance testing was performed by Students *t*-test and refers to the significance with respect to the measurements at pH 7.5.

Extracellular pH Affects the Mitochondrial $\Delta\Psi$ of Human Oocytes and Preimplantation Embryos

We examined the effect of extracellular pH on the mitochondrial $\Delta\Psi$ of 59 fresh human oocytes donated to research. Solutions of extracellular media were buffered to pH 7.0, 7.5, and 8.0. Oocytes were cultured for 24 h in these solutions prior to loading with the mitochondrial $\Delta\Psi$ sensitive dye JC-1 in order to offset any short-term changes in intracellular pH through the action of ion pumps (9,16–18). The two non-physiological pH-buffered media gave readings significantly lower than the mitochondrial $\Delta\Psi$ measured in physiological pH. At near physiological pH (7.5), oocytes were characterised by a relative mitochondrial $\Delta\Psi$ of 1.88 ± 0.07 (mean ± SD, *n* = 21, Table III). When oocytes were incubated in solutions buffered to pH 7.0, a relative fluorescence after JC-1 loading of 1.56 ± 0.01 (mean ± SD, *n* = 18, Table III) was measured, significantly lower than that of pH 7.5 (*p* < 0.001, Student's *t*-test). pH 8.0-exposed oocytes were characterised by a relative membrane potential of 1.62 ± 0.02 (mean ± SD, *n* = 20, Table III), again significantly different to that of pH 7.5 (*p* < 0.001, Student's *t*-test).

DISCUSSION

Current protocols for clinical assisted reproduction techniques (ART) involve the immediate removal of human COCs from follicular fluid at oocyte retrieval followed by the culture in artificial media until embryo transfer. However, although the technology of these media is continually improving, formulations may still not accurately reflect the *in vivo* environment because of a lack of understanding of human *in vivo* fertilisation and embryo physiology. In the present trial, we tested the clinical effect of extending the culture of COCs in follicular fluid on the success

rate of an ongoing IVF program in diverse centers. Our data shows that three critical parameters of clinical success, i.e., the fertilisation rate, pregnancy rate, and the implantation rate are significantly improved with this modified protocol.

How does the preincubation of COCs in follicular fluid significantly improve the clinical success rate after IVF and ICSI? Follicular fluid could act by increasing the fertilisation rate and therefore making more embryos available for transfer. In alternative, the preincubation of human oocytes in follicular fluid could cause an improvement at the level of the oocyte cytoplasm, increasing the implantation potential of the embryos. In practice, the preincubation of oocytes in follicular fluid appears to affect both steps through a positive effect at the level of the oocyte cytoplasm.

The fertilisation of human and mammalian oocytes depends on several factors: The ability of sperm to penetrate the cumulus and zona pellucida and come into contact with the oocyte membrane, the fusion of the sperm and the oocyte plasma membrane, the activation of the oocyte by the spermatozoa, and the decondensation of the sperm and oocyte partial genome followed by the formation of the unique genome of the zygote. Since the fertilisation rate was improved after both IVF and ICSI, we suggest that the latter phases were positively affected by the protocol. The increase in fertilisation however did not lead to a simple increase in the probability of obtaining top quality embryos for transfer. In Switzerland, and currently in Italy, embryo selection is not permitted, and therefore the increase in clinical success is not due to the formation of a higher percentage of "top quality" embryos that are selected for transfer. Here, we show that the percentage of top quality transferred embryos is not significantly greater between controls and experimental protocols. These data suggest therefore that the improvement in the clinical success rate is due to an improvement in oocyte and embryo quality more than a simple increase in the number of embryos available for transfer.

What could follicular fluid provide that augments oocyte quality? Many differences exist between follicular fluid and artificial culture medium, and therefore no simple answer to this question exists. Follicular fluid, as any external environment, has several properties that maintain the oocyte in an optimal state. Such properties include the provision of an environment of the correct osmolarity, pH, ion concentration, and temperature. Follicular fluid may

also act as a strong buffer of free radicals (25,26). Furthermore, follicular fluid contains many proteins and other factors that may be beneficial to human oocytes, such as transport proteins, intercellular signals, and hormones (23,24,27–32). Although some or all of the above factors could have positive effects on oocyte quality, we speculate in this work that one of the fundamental differences between follicular fluid and artificial culture media is the pH of the two fluids. Our present data demonstrates that follicular fluid is maintained and strongly buffered to pH 7.65 ± 0.10 (mean $\pm$ SD, $n = 242$), a significantly more alkaline pH than that of *in vitro* conditions. These results differ from some reports of the pH of human follicular fluid (4–8), but agree with reports in which follicular fluid is aspirated from human follicles *in situ* (6). We suggest that the excision of ovaries or use of CO₂ gas during laparoscopy causes the production of excess acid in the follicle and leads to artefactual pH measurements. The differences in pH between follicular fluid and *in vitro* culture medium could be significant for oocyte physiology because pH is a fundamental biological parameter affecting many aspects of cell physiology including enzymatic reactions and metabolism. In fact, aerobic metabolism in all eukaryotic cells is dependent on a gradient of pH (i.e., H⁺ ions) across the mitochondrial inner membrane (33,34). We used a fluorescence indicator of mitochondrial $\Delta\Psi$ to test whether the extracellular pH could influence this in human oocytes. We show that extended culture of oocytes in media with an extracellular pH outside the “physiological” level significantly decreases the mitochondrial $\Delta\Psi$. Our data suggests that, although human oocytes and embryos have pH compensatory mechanisms (9,16–18), in the long term the external pH has a direct effect on the mitochondria. From these data, we speculate that at least one of the fundamental differences between follicular fluid and artificial culture media is the pH that human oocytes are exposed to, and suggests that this affects human oocyte physiology at the level of the mitochondrial $\Delta\Psi$. We are currently performing a trial to test whether simple adjustment of the pH of *in vitro* culture medium can mimic these effects. Due to legal restrictions, it was not possible to test the effects of non-“physiological” extracellular pH on the mitochondrial $\Delta\Psi$ of developing human embryos. However, our previous data have suggested that extracellular pH does affect human embryo development *in vitro* (9), suggesting that in the long term, a non-“physiological” external pH does have

physiological consequences for developing human embryos.

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