Results and Discussion

Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome with Sperm DNA Fragmentation in Art Cycles


APPENDICES

APPENDIX I

To determine the quantitative assay of PROLACTIN (PRL)

**ASSAY PRINCIPLE**

The prolactin assay is a two step immunoassay to determine the presence of prolactin using chemiluminescence micro particle immunoassay.

- Sample + anti-prolactin (mouse, monoclonal) coated paramagnetic micro particles are combined.

- Prolactin present in the sample binds to the anti-prolactin (mouse, monoclonal) coated micro particles after washing → anti-prolactin (mouse, monoclonal) acridium labeled conjugate is added.
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• pre trigger and trigger solutions are added → mixing → the resulting chemiluminescent reaction is measured as relative light units detected by the optical system.

REQUIREMENTS

REAGENT:

• MICROPARTICLES: 1 or 4 bottles (6.6/27.0ml) anti –Prolactin (mouse, monoclonal) coated micro particles in TRIS buffer with protein (murine and bovine) stabilizers. Preservative: antimicrobial agents

• CONJUGATE: 1 or 4 bottles (5.9/26.3 ml) anti –Prolactin (mouse, monoclonal) acridium labeled conjugate in phosphate buffer with protein (piscine and bovine) stabilizers. Minimum concentration: 0.05µg/ml Preservative: antimicrobial agents.

• PRE-TRIGGER SOLUTION: pre-trigger solution containing 1.32% (w/v) hydrogen peroxide.

• TRIGGER SOLUTION: trigger solution containing 0.35N sodium hydroxide.

• WASH BUFFER: wash buffer containing phosphate buffer saline solution

• Preservative: antimicrobial agents.

PROCEDURE

• Turn on the instrument

• check the wash buffer levels & cuvettes & trigger, pre-trigger and empty the waste container

• Do initialization the instrument.
• Do routine maintenance if any
• Check all the reagents are loaded and ready to use
• process the QC’s samples
• if the QC’s values are with in the acceptable range
• start processing the samples
• load the samples and press the start key
• make sure the instrument is connected to LIS
• After starting the instruments will read to barcode and the assays are performed
• The results are transferred to LIS if the results are low (or) high the samples are repeated

APPENDIX II

FOLLICLE STIMULATING HORMONE (FSH):

FSH is an automated quantitative test for use on the VIDAS instruments for the determination of human follicle stimulating hormone in human serum or plasma (lithium heparinate), using the ELFA technique (Enzyme Linked Fluorescent Assay).

PRINCIPLE

The assay principle combines an enzyme immuno assay sandwich method with the final fluorescent detection (ELFA). The sample /conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a “sandwich”.

Assessment of Oocyte Quality with AMH, Inhibin B in Serum and Follicular Fluid and Predicting Pregnancy outcome

Sperm DNA Fragmentation in ART Cycles
PROCEDURE:

- Only remove the required reagents from the refrigerator and allow them to come to room temperature for 30 minutes before use.

- Use one FSH strip and one FSH SPR for each sample control or calibrator to be tested make sure the storage pouch has been resealed after the required SPRs have been removed.

- Type or select “FSH” on the Instrument to enter test code. The calibrator must be identified by “S1” and tested in triplicate. If the control needs to be tested, it should be identified by C1.

- Mix the calibrator, control and samples with a vortex type mixer in order to improve result reproducibility.

- Pipette 200µl of sample calibrator or control into the sample well

- Insert the SPRs and reagent strips into the instrument in the position indicated on the screen. Check to make sure the color labels with the assay on the SPRs and the reagent strips match.

- Initiate the assay as directed in the operator’s manual. All the assay steps are performed automatically by the instrument. The assay will be completed within approximately 40 minutes.

- After the assay is completed, remove the SPRs and the strips from the instruments.

- Dispose of the used SPRS and strips into an appropriate recipient.

APPENDIX III

LH (LUTEINIZING HORMONE)
LH is an automated quantitative test for use on the VIDAS instruments for the determination of human luteinizing hormone in human serum or plasma (lithium heparinate), using the ELFA technique (Enzyme Linked Fluorescent Assay).

**PRINCIPLE:**

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection (ELFA). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to the antibodies coated on the SPR and to the conjugate form of a “sandwich”. Unbound components are eliminated during the washing steps. The fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample.

At the end of the assay, results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

**PROCEDURE**

- only remove the required reagent from the refrigerator and allow them to come to room temperature for at least 30 minutes
- Use only LH strip and then one Lhs pr for each sample, control or calibrator to be tested. Make sure that the storage pouch has been resealed after the required SPRs have been removed
- type or select “LH” to enter the test code. The calibrator must be identified by “S1”, and tested in duplicate. If the control needs to be tested, it should be identified by C1
- If necessary, clarify samples by centrifugation.
• mix the calibrator, control and samples using a vortex type mixer

• Pipette 200 µL of sample, calibrator or control in to sample well.

• Insert the SPRs and strip into the instrument. check to make sure the color labels with the assay code on the SPRs and the reagent strip match

• Initiate the assay as directed in operators manual. All the assays strips are performed automatically by the instrument. The assay will be completed with in approximately 40 minutes

• After the assay is completed, remove the SPRs and strips from the instruments

• Dispose of used SPRs and reagent strips in an appropriate recipients

APPENDIX IV

ESTRADIOL II

Estradiol II is an automated quantitative test for use on the VIDAS instruments for the quantitative measurement of total 17β –estradiol in human serum or plasma (lithium heparinate) using the ELFA technique (Enzyme linked fluorescent assay) the Vidas E2 II to assay is intended for use as an aid in the diagnosis and treatment of various hormonal sexual disorders and in assessing placental function in complicated pregnancy.

PRINCIPLE
The assay principle combines a competition method with a final fluorescent detection (ELFA). The estradiol present in the serum and estradiol derivative in the conjugate compete for the anti-estradiol specific antibody sites coated to the inner surface of SPR. During the final detection step, the substrate (4methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolyzes of this substrate into a fluorescent product (4methyl-umbelliferone) the fluorescence of which is measured at 450nm.

PROCEDURE:

• Only remove the required reagents from the refrigerator and allow them to come to room temperature for 30 minutes before use

• Use one E2 II strip and one E2II SPR for each sample control or calibrator to be tested make sure the storage pouch has been resealed after the required SPRs have been removed

• Type or select “E2II” on the Instrument to enter test code. The calibrator must be identified by “S1” and tested in triplicate. If the control needs to be tested, it should be identified by C1.

• Mix the calibrator, control and samples with a vortex type mixer in order to improve result reproducibility.

• Pipette 200µl of sample E2II calibrator or control into the sample well

• Insert the SPRs and reagent strips into the instrument in the position indicated on the screen. Check to make sure the color labels with the assay on the SPRs and the reagent strips match.

• Initiate the assay as directed in the operators manual. All the assay steps are performed automatically by the instrument. The assay will be completed within approximately 60 minutes.
• After the assay is completed, remove the SPRs and the strips from the instruments.

• Dispose of the used SPRS and strips in to an appropriate recipient.

APPENDIX V
PROGESTERONE

Progesterone is an automated quantitative test for use on the VIDAS instruments for the quantitative measurement of progesterone in human serum or plasma (lithium heparinate or EDTA) using the ELFA technique (enzyme linked fluorescent)

PRINCIPLE:

The assay principle combines a enzyme immuno assay competition method with a final fluorescent detection (ELFA). After sample dilution, the progesterone in the sample binds with the specific monoclonal antibody coating the interior of the SPR. Unbound components eliminated during the washing stages. The conjucate is then cycled in to the SPRs. the remaining free antibody sites are saturated by the conjucate, which is an alkaline phosphatase labeled progesterone derivative .Unbound conjucate is removed by washing. Fluorescent product (4-methyl umbelliferone),the fluorescent of which is measured at 450nm.the intensity of the fluorescent signal is inversely proportional to the concentration of progesterone present in the sample.

PROCEDURE:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for 30 minutes before use
2. Use one PRG strip and one PRG SPR for each sample control or calibrator to be Tested make sure the storage pouch has be resealed after the required SPRs have been removed.

3. Type or select “PRG” on the Instrument to enter test code. The calibrator must be identified by “S1” and tested in triplicate. If the control needs to be tested, it should be identified by C1.

4. Mix the calibrator, control and samples with a vortex type mixer in order to improve result reproducibility.

5. Pipette 200µl of sample calibrator or control into the sample well.

6. Insert the SPRs and reagent strips into the instrument in the position indicated on the screen. Check to make sure the color labels with the assay on the SPRs and the reagent strips match.

7. Initiate the assay as directed in the operators manual. All the assay steps are performed automatically by the instrument. The assay will be completed within approximately 45 minutes.

8. After the assay is completed, remove the SPRs and the strips from the instruments.

9. Dispose of the used SPRS and strips in to an appropriate recipient.

APPENDIX VI

MIS/AMH ELISA

The active mullerian inhibiting substance/anti-mullerian hormone (MIS/AMH) enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of MIS/AMH in serum.

PRINCIPLE

The active MIS/AMH ELISA is an enzymatically two-site immunoassay. In the assay, standards, controls and samples are incubated in microtitration
wells which have been coated with anti-MIS/AMH antibody. After incubation and washing anti-MIS/AMH detection antibody labeled with biotin is added to each well. After a second incubation and washing step, HRP is added to the wells. After a third incubation and washing step, TMB is added to the wells. Lastly acidic stopping solution is added. The degree of enzyme turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm and between 600 and 630 nm.

MATERIALS

1. Anti-MIS/AMH antibody COATED MICROTRITATON STRIPS
2. MIS/AMH standard A / sample diluent:
3. MIS/AMH standard B –G
4. MIS/AMH controls
5. MIS/AMH antibody –biotin conjugate
6. streptavidin- enzyme conjugate
7. MIS/AMH assay buffer
8. TMB chromogen solution
9. wash concentrate-1
10. stopping solution A

SAMPLE PREPARATION

- Serum is the recommended sample
- Within two hours after centrifugation transfer at least 500 µl of cell free sample to a storage tube. Tightly stopper the tube immediately
- Store samples tightly stoppered at 2 to 8° C
- If the assay will not be completed within 24 hours, or for shipment of samples, freeze at -20° C or colder.

PROCEDURE
• mark the micro titration trips to be used
• pipette 20 µl of the standards, controls and samples to the appropriate wells
• add 100 µl of MIS/AMH buffer to each well using a precision pipette
• incubate the wells, shacking at 500-700 rpm on an orbital micro plate shaker for one hour at room temperature
• aspirate and wash each well five times for 30 seconds with wash solution
• add 100 µl of antibody-biotin conjugate solution to each well using a precision pipette
• incubate the well, shacking at 500-700 rpm on an orbital micro plate shaker for one hour at room temperature
• aspirate and wash each well five times for 30 seconds with wash solution
• add 100 µl of streptavidin-enzyme conjugate solution to each well using a precision pipette
• incubate the wells, shacking at 500-700 rpm on an orbital micro plate shaker for 30 mins at room temperature
• aspirate and wash each well five times for 30 seconds with wash solution
• add 100 µl of TMB chromogen to each well using a precision pipette
• incubate the wells, shacking at 500-700 rpm on an orbital micro plate shaker for 10-15 mins at room temperature
• add 100 µl of stopping solution to each well with in 30 mins using a precision pipette
• read the absorbance of the solution in the wells within 30 mins using a microplate reader set to 450 nm

APPENDIX VII
ACTIVE INHIBIN-B ELISA

Inhibin-B ELISA used to quantitative measurement of the dimeric Inhibin-b in human serum. This assay is intended for in vitro diagnostic use.

PRINCIPLE

Inhibin-B ELISA is an enzymatically amplified two-site two-step sandwich-type immunoassay. In this assay standard, controls, and unknown serum samples are incubated in micro titration well, which have been coated with anti-Inhibin βb subunit antibody. After incubation and washing the wells are incubated with biotinylated anti-Inhibin α- subunit detection antibody and the immunoreactions monitored by subsequent addition of streptavidin labeled with HRP. After a third incubation and washing step the wells are incubated with the substrate TMB. Then the acidic stopping solution is added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement of 450 and 620 nm.

MATERIALS
1. Anti- Inhibin B coated micro titration strips
2. Inhibin –B standard A/ sample diluent
3. Inhibin - B standards
4. Inhibin –B controls
5. Inhibin –B sample buffer A
6. Inhibin –B sample buffer B
7. Inhibin –B antibody- biotin conjugate
SPECIMEN COLLECTION AND PREPARATION

Serum should be used and the usual precaution for venipuncture should be observed. The samples may be stored at 2-8° C for up to 24 hours or frozen at -20° C or lower for up to 30 days. Avoid repeated freezing and thawing of samples. Do not use hemolyzed or lipemic samples. Frozen samples should be thawed and mixed thoroughly by gentle swirling or inversion prior to use.

ASSAY PROCEDURE

- mark the micro titration trips to be used
- pipette 50 µl of the standards, controls and samples to the appropriate wells
- Add 25 µl of Inhibin –B sample buffer A to each well using a semi-automatic dispenser
- Add 25 µl of Inhibin –B sample buffer B to each well using a semi-automatic dispenser
- cover and incubate the wells, shaking at 300-400 rpm on an orbital micro plate, over night at room temperature
- Aspirate and wash each well 3 times with deionized water using an automatic micro plate washer. Blot dry by inverting plate on absorbent material.
- Add 50 µl of Inhibin –B-antibody biotin conjugate using a semi-automatic dispenser
- cover and incubate the wells on an orbital micro plate shaker set at 300-400 rpm for 1.5 hrs at room temperature

- Aspirate and wash each well 6 times with deionized water using an automatic micro plate washer. Blot dry by inverting plate on absorbent material.

- add 50 µl streptavidin-enzyme conjugate to each well using a semi-automatic dispenser

- Aspirate and wash each well 3 times with deionized water using an automatic micro plate washer. Blot dry by inverting plate on absorbent material.

- Add 100 µl of MIS/AMH buffer to each well using a precision pipette

- Incubate the wells, shaking at 500-700 rpm on an orbital micro plate shaker for one hour at room temperature

- Aspirate and wash each well five times for 30 seconds with wash solution

- Add 100 µl of antibody-biotin conjugate solution to each well using a precision pipette

- Incubate the well, shaking at 500-700 rpm on an orbital micro plate shaker for one hour at room temperature

- Aspirate and wash each well five times for 30 seconds with wash solution

- Add 100 µl of streptavidin-enzyme conjugate solution to each well using a precision pipette

- Incubate the wells, shaking at 500-700 rpm on an orbital micro plate shaker for 30 mins at room temperature

- Aspirate and wash each well five times for 30 seconds with wash solution
• add 100 µl of TMB chromogen to each well using a precision pipette
• Incubate the wells, shacking at 500-700 rpm on an orbital micro plate shaker for 10-15 mins at room temperature
• add 100 µl of stopping solution to each well within 30 mins using a precision pipette
• Read the absorbance of the solution in the wells within 30 mins using a microplate reader set to 450 nm

APPENDIX VIII
(Semen Analysis AND SPERM MORPHOLOGY (WHO manual 1999))

The semen analysis evaluates certain parameters of an ejaculated specimen. These parameters include:

MACROSCOPIC EXAMINATION:
- Appearance
- Odour
- Liquefaction
- Viscosity
- Ph
- Volume

MICROSCOPIC EXAMINATION:
- Sperm concentration
- Sperm motility
- Sperm vitality
- Sperm morphology
- Sperm agglutination or aggregation
Presence of debris and cellular elements

Sample Collection

The sample should be produced by masturbation into a sterile, non-toxic jar and preferentially be in the laboratory within half an hour of collection. It is important that the laboratory provide private rooms for specimen collection. A period of three days ejaculatory abstinence is compulsory but this period may be extended to 2 to 5 days in certain circumstances.

Semen Analysis Flow Chart

The flow chart below outlines the schematic analysis of a semen sample:

1. Place the sample details (i.e. name, date of birth, days of abstinence, completeness of the specimen, time of production, etc.) in the laboratory register.
2. The sample should be placed in a 37°C incubator for 10 to 20 minutes or until the sample has liquefied.
3. The sample is then examined for appearance, odour and completeness of liquefaction.
4. The volume and viscosity is then measured.
5. Aliquots are then withdrawn for microbiological, cellular / debris, sperm agglutination / aggregation and motility analysis.
6. Slides are prepared for eosin-nigrosin and morphology stain assessment.
7. A concentration vial is made for sperm concentration evaluation.
8. A pH test is performed.
9. The sample is prepared for a trial wash and an Immunobead test.
10. A peroxidase stain may be set up for leucocyte determination.
11. Re-assess sperm motility after 3 hours.
12. Estimate the sperm concentration. □

13. Read the stain slides for morphology and vitality estimation.

Performing a Semen Analysis

After 10 to 20 minutes remove the specimen container from the incubator and mix the sample thoroughly.

Check that all the preliminary details such as: name, days of abstinence, patient’s date of birth, address, referring doctor, method of production and completeness of the sample are recorded on the semen analysis proforma sheet. Check that the sample is fully liquefied. Return any un-liquefied samples to the incubator for another 10 minutes before re-analysis.

Appearance:

Colour - is it whitish grey, translucent, pinkish, or yellow? Does it have mucus streaks present (gelatinous looking globules)? Normal semen has a homogenous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low, red brown when red blood cells are present or yellow in a patient with jaundice or taking some vitamins

Odour:

- Normal
- Strong
- Putrescent
- Presence of a urine smell

Volume and viscosity:

Measure the volume of the semen using a graduated pipette. At the same time, measure the viscosity of the sample by noting the way it moves up and down the pipette. Note that a sterile pipette should be used if the sample is to be used for
insemination or is to be sent for microbiological analysis. The viscosity can be graded as follows:

- Water or reduced
- Normal
- Moderately increased.
- Greatly increased.

**pH:**

This test is usually determined via the use of a pH test strip range 6.5 to 10.00. The normal semen pH is around 7.2 to 8.2. Note that the pH changes with time after ejaculation and hence should be performed within half an hour of specimen collection. The pH may be out of range with certain inflammatory disorders of the prostate and the seminal vesicles. Take care not to plunge a non-sterile test strip into a semen sample that is to be used for insemination.

**Sperm clumping**

A wet mount preparation is made by placing 10 µl of the well-mixed semen on a clean microscope slide. Cover the micro-droplet with a cover slip (e.g. 2.2 cm x 2.2 cm) so that the resulting depth of the semen is approximately 20 µm. Wait for approximately 30 seconds to allow the specimen to settle. Using phase contrast optics, assess 10 random fields of view for sperm agglutination or aggregation. Sperm aggregation is noted when sperm are stuck together in between other cellular elements. Sperm agglutination is noted only when sperm are seen stuck together without involvement of cellular elements. This phenomenon is immunological where as aggregation is a common phenomenon that occurs to dead sperm. The percentages of sperm that are stuck together are evaluated and any specific sight of connection noted down on the proforma sheet.
Other cellular elements

It is common to see some round cells in a semen sample and they may be immature germinal cells, or even residual cytoplasm stemming from seminiferous epithelial. It is vital that you distinguish them from leucocytes. Perform a differential leukocyte stain if there is more than 1 round cell per x40 field of view.

Erythrocytes should not be present in the semen but should be noted when present in large numbers. These are easy to distinguish as they have a unique circle in the center of their cytoplasm.

Epithelial cells look like large, flat bodies and their presence is usually not pathological. An abundance of epithelial cells usually indicate that the sample was collected via coitus interruptus.

The presence of slight debris is typical and not abnormal. One should take care not to misinterpret debris that is moving due to Brownian motion as motile bacteria.

SPERM MOTILITY

A quantitative sperm motility analysis must be made soon after and the time post ejaculation noted on the proforma sheet. The analysis should be completed before the semen cools down or starts to dry out. It is determined by counting the number of both motile and immotile spermatozoa in several fields of view. Take 10 µl of liquefied semen and place it on a microscope slide. Lower a microscope slide onto the microdroplet and focus on the spermatozoa. Count at least 200 spermatozoa under x40 magnification and score them as either motile (showing some tail movement) or immotile. Express the number of motile sperm as a percentage by using the following formula:
% Motile = \frac{\text{No. Of motile sperm seen}}{\text{Total number of sperm counted (both motile and non-motile)}}

The motility count must be repeated on a second micro droplet of semen prepared in the same manner. There should be less than 10% discrepancy between the two results or the test should be repeated.

Count at least 200 spermatozoa and calculate the percentage of motile sperm, keeping away from the areas nearest to the edge of the slide. Count the number of rapid (WHO class a) and slow / sluggish motile sperm (WHO class b) in each field first and then tally the number of non-progressive but motile sperm (WHO class c). Express the percentage of spermatozoa in each of the following categories:

- % Rapid progressive
- % Slow progressive
- % Non-progressive
- % Immotile

A normal semen sample should contain at least 50% progressively motile (Class a+b according to WHO). Record all irregular patterns of sperm movements such as shaking or circular trajectories as well as the percentage of sperm exhibiting the irregular movement.

Qualitative sperm motility is subjectively assessed by grading the forward progression of the sperm exhibited by the largest proportion of the motile sperm.

- Non progressive motile only
- Poor, non directed progression
• Moderate progression
• Good progression
• Excellent progression

SPERM VITALITY

Sperm vitality assessment is performed via an eosin and nigrosin stain. Sperm that are immotile are not always dead. With the advent of ICSI, it is now possible to treat patients who have 100% immotile sperm but have some live sperm in their ejaculate. The presence of live / vital sperm in a sample that has 100% immotile sperm may indicate a defect in the sperm flagellum. The stain works on the principle that dead sperm have damaged plasma membranes. The damaged plasma membrane allows the red color of the eosin whilst the nigrosin component acts like a counter stain to allow easier visualization of the unstained, live sperm. The eosin-nigrosin stain is made by:

1. Dissolving 0.67 g eosin Y in 100 ml of tap water. Mix and heat gently.
2. Add 10.0 g nigrosin to this and bring to boil.
3. Allow to cool and then filter.
4. Store the final stain in a stoppered glass bottle at 4°C (but use at ambient temperature).
5. It is good to quality control each batch of stain by comparing the vitality results of two slides made from one sample. Stain one slide in the old batch and the one in the new and check for correlation between the two results.

Procedure:

Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome

Sperm DNA Fragmentation in Art Cycles
1. Add 10 µl of well-mixed semen to 10 µl of the eosin-nigrosin stain on a microscope slide. Mix well with the end of the yellow tip. Smear the slide between another and allow it to air dry. Do not allow the smear to get too thick or the visualization of the sperm will be hard due to the dark background. Cover the slide with a permanent mountant and cover slip. Allow to air dry.

2. Examine at least 100 spermatozoa under x1000 magnification and count the number of white-unstained (live) and pink-red (dead) sperm. Note the percentage of vital or live sperm.

**Sperm Concentration**

A fixed volume of 10 ul of semen is taken in a makler chamber. There are 100 squares in a the chamber Count 10 squares and that will give you the sperm concentration / ml. (62 sperms in 10 squares means sperm concentration = 62M/ml)

The sperm concentration per ejaculate is found by multiplying the total sperm count per ml by the volume of the ejaculate (ml). If the sperm count is less than 20 in 10 squares count 100 squares and dive it by 10 which will give the sperm count/ml(Eg: 135 sperms in 100 squares then dive by 10 which is 13.5m/ml)

**Sperm Morphology**

A normal mature spermatozoon will have an oval shaped head (4.0 to 5.5 µm long and 2.5 to 3.5 µm wide) with a distinct outline. The head can be divided into a pale, anterior area called the acrosome and a darker posterior region containing the chromatin. The length / width ratio should be 1:5 and 1:75 respectively. The normal sperm would not possess any neck, mid-piece, tail defects nor have any Cytoplasmic droplets greater than the size of a normal head.
The sperm tail is located at the base of the head and is usually about 50 µm in length. The tail should be present singly and should not be coiled, kinked nor folded back on itself. The mid-piece region is about 7 to 8 µm long and should not appear swollen. A swollen mid-piece usually indicates a defect or remnants of the Cytoplasmic droplet which would indicate sperm immaturity. (In this case, the Cytoplasmic droplet would stain green in the Papanicolaou stain.)

The Papanicolaou stain distinguishes between basophilic and acidophilic cellular components. It is the preferred staining method recommended by the World Health Organization (WHO) for morphology assessment.

**Method for the Papanicolaou Stain**

1. Place two 10-µl droplets of well-mixed semen on to a named / dated microscope slide approximately two centimeters apart. Smear the slide by placing another slide over the droplets and slowly pulling the slides apart.

2. Allow the slides to dry overnight.

3. Fix one slide and allow air-drying. Leave the other slide as a back up just in case there is a problem with the stain.

4. Fill the Coplin jars with the following and place the slides into each jar face-out.

   NB: Each dip is equivalent to approximately 1 second.

   - Ethanol 80% 10 dips
   - Ethanol 70% 10 dips
   - Ethanol 50% 10 dips
   - Distilled water 10 dips
   - Harris Haematoxylin 3 minutes exactly
<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Running tap water 2 dips</td>
</tr>
<tr>
<td>2.</td>
<td>Acid ethanol 2 dips</td>
</tr>
<tr>
<td>3.</td>
<td>Running tap water 2 to 3 minutes</td>
</tr>
<tr>
<td>4.</td>
<td>Scott’s solution 4 minutes</td>
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<tr>
<td>5.</td>
<td>Distilled water 1 dip</td>
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<tr>
<td>6.</td>
<td>Ethanol 50% 1 dip</td>
</tr>
<tr>
<td>7.</td>
<td>Ethanol 70% 10 dips</td>
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<tr>
<td>8.</td>
<td>Ethanol 80% 10 dips</td>
</tr>
<tr>
<td>9.</td>
<td>Ethanol 95% 10 dips</td>
</tr>
<tr>
<td>10.</td>
<td>Orange G 6 2 minutes</td>
</tr>
<tr>
<td>11.</td>
<td>Ethanol 95% 10 dips</td>
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<tr>
<td>12.</td>
<td>Ethanol 95% 10 dips</td>
</tr>
<tr>
<td>13.</td>
<td>Ethanol 95% EA-50 5 minutes</td>
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<tr>
<td>14.</td>
<td>Ethanol 95% 5 dips</td>
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<tr>
<td>15.</td>
<td>Ethanol 95% 5 dips</td>
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<tr>
<td>16.</td>
<td>Ethanol 95% 5 dips</td>
</tr>
<tr>
<td>17.</td>
<td>Ethanol 99.5% 2 minutes</td>
</tr>
<tr>
<td>18.</td>
<td>Xylene 1 minute</td>
</tr>
<tr>
<td>19.</td>
<td>Xylene 1 minute</td>
</tr>
<tr>
<td>20.</td>
<td>Xylene 1 minute</td>
</tr>
</tbody>
</table>

5. Mount immediately with DPX or equivalent mountant. Leave to dry overnight or until slides are ready to be scored.

6. Count at least 100 spermatozoa under x1000 magnification under oil immersion and score each according to the guidelines noted below.
The following four categories should be scored:

I Head shape and size defects: This includes spermatozoa that have small, large tapering, pyriform, amorphous, double, 20% or more vacuolation of the head or a combination of these.

II Neck and midpiece defects: These defects include an absent tail (free head), non-inserted, bent (the tail forms an angle of about 90° to the long axis of the head). Mid-pieces that are abnormal present themselves as either distended, irregular shape, bent, or abnormally thin (absence of the mitochondrial sheath) or a combination of these.

III Tail defects: These defects include: short, multiple, hairpin, broken, irregular width, coiled and with terminal droplets or a combination of these.

IV Cytoplasmic droplets: Sperm, which possess a Cytoplasmic droplet greater than the size of a normal sperm head, is considered abnormal. The droplet is usually located at the neck / mid-piece junction however may be seen further down the tail in immature sperm.

Any sperm with one or more defect is scored as abnormal. Each defect is scored as its incidence per 100 spermatozoa. Loose or free heads are counted as abnormal under the neck / midpiece category. Note that pinheads are not counted in the head defect category, as they are not considered as spermatozoa as they do not contain chromatin or a head structure anterior to the basal plate. Their incidence however should be noted separately.

A large incidence of immature sperm may indicate frequent ejaculation or a short period of abstinence. Coiling of the sperm tail may indicate sperm senescence or hypotonic stress as seen in the HOS test.
Perfectly rounded heads indicate “Globozoospermia” and result from the absence of the acrosomal region. Such patients can only be treated with ICSI and the injection of calcium into the oocyte at the same time of sperm insertion.

The normal range of abnormal sperm in fertile men is 30%. This value may be extended to 50% in clinical patients.

The Teratozoospermia Index (TZI) is a good predictor of fertility. It is calculated as the mean number of defects per abnormal spermatozoa. It is achieved by scoring sperm abnormalities using multiple entry morphology scoring. The TZI will range around 0 to 3. A score over 1.6 is considered abnormal and ICSI should be considered, especially in cases where the value exceeds 2.0.

Diagram 1 - Normal and abnormal Forms of Human Spermatozoa
Diagram No:1

1-3 = Variations of normal spermatozoa: 4 = A normal spermatozoa with a low incidence of vacuoles;  
5 = A normal sperm head in side view; 6 = Small head or microcephalic; 7 = Large head or macrocephalic;  
8 = Tapering forms; 9+10 = Pyriforms; 11 Constricted; 12 +13 = Reduced acrosomal region;  
14 = Dense staining or amorphous; 15 = Vacuolated; 16 = Cytoplasmic droplets; 17 = Asymmetrical tail insertion;  
18 = Double or duplicate tail; 19 = Abnormal mid-piece; 20 = Thin mid-piece due to the absence of mitochondria;  
21 = Non-inserted tail (neck defect); 22 = Coiled tail; 23 = Short tailed form; 24 = Terminal droplet on the tail;  
25 = Hairpin tail; 26 = Broken tail; 27 = Conjoined forms.
APPENDIX IX

Sperm preparation for ICSI using density gradient method

Density gradients such as gradient (Sure life) fractionate sub-populations of spermatozoa according to each sperm’s density. It is helpful in removing bacteria from the original samples but care must be taken to wash the gradient (Sure life) out of the final sperm suspension.

a) Prepare two sperm gradients and allow to come to ambient temperature.

b) Dispense 1.5 ml of 90% sperm into the bottom of two test tubes. Gently layer 1.5 ml of 45% sperm solution over the 90% layer.

c) Divide the semen sample into two equal volumes and layer over the 45% sperm layer. Keep at least 500 µl for semen analysis.

d) Centrifuge the two tubes for 20 minutes at 1800 RPM.

e) A pellet should have formed at the bottom of the conical tube. This may be small or invisible if the semen concentration is low, as seen for most ICSI cases. Remove the supernatant and collect it into a sterile specimen jar.

f) Change to a clean pipette and move the pellet into a clean conical test tube.

g) The pellet is then washed as in steps b) to f) of the wash and rise procedure. After the final spin, remove the supernatant and discard.

h) The pellet may be resuspended in 0.5 to 1.0 ml of culture medium and the sperm concentration, motility and sperm progression analysed. Note that the pellet may be risen for 30 to 60 minutes in media if it contains many immotile sperm or red blood cells.
APPENDIX X


Protocol

20 µL of washed sperm suspension for each patient was smeared on a precleaned glass slide. The smeared slides were air dried and later fixed in Carnoy's solution (1 part glacial acetic acid:3 parts methanol) for 2 hours. After fixation, slides were air dried and stained with freshly prepared 0.19 mg/mL AO stain (Polysciences, Warrington, Pa) for 5 minutes in the dark as follows: 10 mL of 1% AO in distilled water added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.2 M Na$_2$HPO$_4$0.7H$_2$O and pH adjusted to 2.5. After staining, slides were washed with distilled water, covered with glass cover slips, and immediately evaluated using a Nikon (Eclipse E400) fluorescence microscope at the excitation wavelength of 450-490 nm. A total of 500 sperm cells were evaluated on each slide by the same examiner with no more than 40 seconds duration of observation per field. Spermatozoa displaying green fluorescence were scored as having normal DNA content, whereas sperm displaying a spectrum of yellow-orange to red fluorescence were considered to have damaged DNA.

APPENDIX XI

IDENTIFYING OOCYTES IN FOLLICULAR FLUID

Collect the following items: dissecting microscope; pipette holder; 2 sterile borosilicate glass pipettes with teats; heated test tube block; 2 insulin syringes with needle; a 10 ml tube containing flushing media with hepes, sterile gauze or tissues; 5 to 10 large falcon petridishes; and 10 to 20 sterile, individually wrapped 10 ml Falcon test tubes. Turn on the microscope and the heated stage on if present. Place two petridish to warm on the heated stage. Attach the teats to the pipettes and place the end of the pipette into two large
Falcon tubes as pipette stands. Remove the outer packaging of the insulin needles and place them on the microscope stage so that the needles hang off the end of the stage and are maintained sterile.

Place the heated test tube block in a convenient place so that the test tubes are easily retrieved. Perform a routine identification check of the patient’s name, their husband/partner’s name and cross check the names with those on the proformas sheet and 4 well dishes. This should be done before the patient is sedated and in front of another co-worker. Allow the patient to state their own name rather than you asking them if this is their name. Check that all the consent forms have been signed and witnessed. Adjust the lights down and lock the theatre door for patient privacy. Explain to the patient the sequence of events that occur in IVF from the oocyte pick up to embryo transfer and cryopreservation of spare embryos.

Make sure the “Wash” and the “IM” dishes are ready for use which contains the fertilization medium. Check the name and identification numbers on the two dishes.

The embryologist then removes the cap off the Falcon tube and decants the fluid into a sterile, large Falcon petridish. Note that the embryologist must wear non-powdered gloves, mask and a gown when dealing with the fluid. Note the start time of the pick up on the proformas sheet. The dish is then placed onto a dissecting microscope stage and cumulus masses and granulose cells are aspirated into a washed, sterile glass pipette. The remaining fluid is emptied into the lid of the petridish and moved to a side on a heated stage. The dish surface should be kept wet with some remnants of follicular fluid. Gently dispense the follicular fluid along with the cumulus masses onto the bottom of the wet petridish, moving the pipette away form each spread mass. Each mass will
spread out over the dish surface enabling the microscopic visualization of its cellular structure.

Carefully look at each cumulus mass and identify any oocytes. Note that the masses that look like clear, mucoid structures usually contain oocytes however, immature oocytes may present themselves within tight, granulose-like cells. Over mature oocytes may be seen free of any cumulus cells and hence the follicular fluid should be examined for “nude” oocytes. The oocytes may be scored for maturity at this stage, but be quick, as you do not want the oocytes to dry out. After locating the oocytes, fill your pipette with two inches of culture medium.

Dispense medium over the oocyte and then aspirate the oocytes into the pipette in a minimal amount of medium.

Deposit the oocyte-cumulus masses into the first well of the “Wash” dish. Move the oocyte around the well by aspirating it up and down the pipette shank. This will commence the washing process removing blood and follicular fluid from oocytes. Empty your pipette of medium and fill it with about one inch of medium from the second well. Pick-up the oocytes from the first well using this pipette and dispense into the second well. Repeat the washing procedure by aspirating the oocytes up and down the pipette. Change to a clean pipette with teat and fill it with an inch of culture medium. Suck up the oocytes into the new pipette and place the oocytes into well three. Try to transfer as little blood into this well as possible. Wash the oocytes again and finally move them into well 4. Remove the entire medium out of the second pipette. Fill the second pipette with about an inch of medium from the “IM” dish and aspirate the oocytes in the fourth well. Place the oocyte into one of the wells of the “IM” dish, noting their well position on the oocyte pick-up proformas sheet. Place up to five oocytes per well as over crowding will make the subsequent fertilization check a nightmare.
Double-check the lid of the petridish containing the spent follicular fluid. Make sure you have not left oocytes behind, especially on the edge of the dish. Make sure to swirl the fluid so that the oocytes are not hiding under any bubbles present. Follicular fluid that contains too much blood may be halved into two dishes so that it is easier to see through. You may turn up the lighting on the microscope if the fluid is extremely bloody. Occasionally you will find clots forming in the fluid. All clots should be grossly dissected into at least eight pieces and any visible cumulus should be cut away and spread onto a wet petridish. Cut as much of the clot off without interfering with the oocyte and wash it as you would if it was blood free. Discard the checked follicular fluid into a contamination bag. Repeat for the next Falcon tube containing follicular fluid. Note that it is wise to keep oocytes that are immature in a separate well so that they may be inseminated 2 to 4 hours after the mature oocytes. Oocytes that have blood or those that have been collected from endometriosis cysts should be cultured in separate wells so that they do not contaminate the others.

After all the tubes of follicular fluid have been screened for oocytes, note down the time of egg pick up completion on the proformas sheet. Place the dish containing the washed oocytes into the incubator. Check the “Wash” dish for oocytes prior to throwing the dish away. Tally the total numbers of oocytes found and inform the patient. Speak to the patient about the oocyte quality/maturity and inform their partner of a time to collect their specimen. Make sure the patient is aware of what the laboratory will be doing to their gametes especially if they are having ICSI or other forms of manipulation. Make a note of what time the male partner will be collecting his sample and indicate the approximate time for oocyte insemination according to the maturity of the oocytes.

APPENDIX XII
PREPARING ICSI DISH

The following information must be placed on the top and the bottom of the dish:

I. The patient’s name.
II. Laboratory reference numbers.

When writing on the bottom of the dish, always write on the edge as not to obscure the injection. Always take time to check the name on the dish and correlate it to the name on the sperm sample before loading the sperm.

1. Write the patient’s name and laboratory reference number on the outer edge of the bottom of the dish.

2. Draw a circle of 8 mm diameter in the centre of the outer surface of the bottom of the dish. This circle will contain the droplet of sperm and PVP (do not fill it yet). As you get more skilled, you will not have to draw this circle in the centre of your dish.

3. You can now label the eight droplets around the circle of sperm / PVP. Begin by labelling a number 1 at the 12 o’clock position, a number 5 at the 6 o’clock position, a number 3 at the 3 o’clock position and a number 7 at the 9 o’clock position. The remaining numbers are labelled in between the existing numbers. NB: Some ICSI set-ups use a hole in the heated stage of only 2 cm in diameter at 37°C. Any droplets that lay too close to the heated stage device will become too hot as only the centre of the 2 cm hole is at 37°C and it progressively becomes hotter towards the edges. Take care to make all 8 outer droplets within a compact circle, which fits neatly with the heated ring area.
Another problem may occur when the droplets are spread too far apart. This will result in the outer droplets lying over the actual microscope stage and hence the oocytes will not be visible and the dish will have to be moved in order to inject that oocyte.

4. You are now ready to place 5 µl medium droplets onto the inner surface of the dish. You may want to use non-powdered gloves for this or alternatively, wash your hands well. Begin by dispensing a 5-µl droplet into the middle droplet. Repeat by placing a droplet adjacent to each number. Take care to ensure that each droplet is equidistant from the other and placed so that they correspond to one of the eight numbers.

5. Remove the central droplet and replace it with 2.5 µl of PVP and then 2.5 µl of washed, diluted sperm. (Note that the sperm concentration should be diluted so that there are 1 to 5 sperm seen per X10 FOV. Over-concentrated samples will make sperm immobilisation and isolation very difficult.)

6. Cover the droplets with 3 to 4 ml of culture oil. (NB: Use carbon dioxide equilibrated oil if using a bicarbonate buffered medium.) Place these dishes in an incubator until needed. Note that dishes made with Hepes buffered medium should be placed in incubators that have no carbon dioxide.

7. Repeat, so that two ICSI dishes are made with microdroplets.

8. A dish is also made to house injected oocytes. Medium containing glucose is not necessary at this stage, as the fertilisation process has been completed. Cleavage medium is used to make an “After ICSI” dish. 1 ml of cleavage medium is inserted into well 1 of a 4 well Nunc dish. From this
well, 10 µl is dispensed into wells 2, 3 and 4 and quickly covered with oil (ovoil). This dish is equilibrated with CO₂ overnight prior to use.

9. A 5 ml Falcon tube containing 2 mls of oocyte wash buffer is also made. This medium will be used to flood the pipettes during pipette setting up phase. It should be tight capped and stored in the refrigerator. The medium is warmed in a heated block (no CO₂) for two hours prior to use. We name this tube “ICSI pipette”

10. A 5ml Falcon tube containing 4 ml of oocyte wash buffer is made for stripping the oocytes in hyaluronidase. It has to be stored in the refrigerator and pre-warmed prior to use as in point 9 above. We name this tube “HYAL”

11. As usual, dishes are made for oocyte recovery, oocyte washing and sperm media aliquoted for semen preparation.

APPENDIX - XIII

ADDING SPERM AND PVP IN ICSI DISH

Prepare the ICSI dishes for sperm injection as below:

1. Take out the PVP from the freezer and allow it to come to room temperature.

2. Take out the diluted sperm sample.

3. Take out the 2 pre-equilibrated ICSI dishes and place them on a 37° C heated stage.
4. Check whether the name on the prepared diluted semen sample matches with the name on the ICSI dishes.

5. Remove the 5 µl central droplet with a sterile yellow tip.

6. Aspirate 2.5 µl of PVP into a new yellow tip (NB: PVP is highly viscous, so slowly aspirate this solution. Take care not to contaminate the PVP with sperm as it is used for several patients.)

7. Dispense the 2.5 µl of PVP into the central droplet. (Make sure it does not float into any drops used to house oocytes as it will damage the oocyte).

8. Aspirate 2.5 µl of diluted sperm sample into a sterile yellow tip. Dispense directly into the central droplet (now containing PVP).

9. Check the dish on the ICSI system to make sure there is a good concentration of sperm. Note, with incubation, more motile sperm will appear at the bottom of the dish.

10. Return the dish to a CO\textsubscript{2} incubator (bicarbonate buffered medium) or a heated stage (HEPES buffered medium) for 20 minutes prior to use.

APPENDIX XIV

IMMOBILIZING THE SPERM FOR ICSI

Make sure the pipette is set up correctly as outlined earlier. It is imperative that the pipette is so angled that it is either parallel or slightly (10°) pointing down towards the stage of the microscope. Please note the diagram below.
Note that if you position the pipette more than the recommended 10° you will find that only the some of the pipette shank will be in focus and hence you will have problems observing the sperm during the breakage of the oolemma. If you have over-positioned the pipette, take the time to lift it up to the correct position so that your subsequent oocyte injection will be easier.

Once the pipettes are in their optimal position, check that that microscope is adjusted for your eye width (diopter adjustment on the eye pieces) and that you have checked that each eyepiece focus is optimal for your own eyesight.

Sperm will be in all the layers of the 5-µl droplet you have made. You can only immobilise the sperm that are swimming at the bottom of the dish, as you will require the bottom of the dish to crush the sperm tails after clubbing with the injection pipette. In order to find out if you are at the bottom of the dish, focus on the edge of the droplet. When the edge of the droplet comes into a sharp focus, you know you have found the bottom of the dish. The sperm at
the bottom of the dish should only swim in two dimension (X+Y) and should not seem as if they are going in and out of focus (swimming in the Z direction).

If you have centered your pipettes in the middle of the droplet, they should come down in the same position when using the coarse control motorised manipulators. You can opt to bring the injection pipette down towards the bottom of the dish using the coarse control manipulators or the Z-axis control knob (see diagram of the hydraulic joy stick). It takes a lot of skill to start using the coarse manipulators as you may break the fragile injection pipette by crashing it onto the bottom of the dish. So learn to use the Z control knob to begin with and later use them in combination as your skill level increases. Whichever method you choose, always look down the microscope whilst bringing the pipettes down into the droplets. This will prevent the phenomenon of pipette dragging as described earlier. You will know that the pipette is at the bottom of the dish when it comes into the same focus as the sperm or the edge of the drop.

Take some time to move the pipette left and right using the X and Y axis control knobs or joy stick. Note how they respond to the movement of the control knob. Repeat for lifting the pipette up and down using the Z axis control knob. Now see how you can achieve all four movements (i.e. left, right, up and down) using only the joystick lever. This lever will eventually be the only lever you have to use to inject sperm.

It is easier to immobilise sperm that are swimming perpendicular to the injection pipette tip. Slightly lift the injection pipette off the bottom of the dish using the Z-axis control knob on the joystick lever. The amount you lift the pipette off the bottom of the dish is so slight that the pipette should only be slightly out of focus. Move the pipette to an area where there are sperm and wait till a sperm starts to swim underneath it. Using a single twist of the Z-axis
control knob brings the injection pipette tip down onto the sperm. You will note that the sperm will be immobilised under the pipette. It is important to only use the tip of the pipette, as sperm are more likely to get stuck on the pipette shaft as you go closer to the elbow. Repeat this for at least ten sperm.

You now know how to club a sperm. For successful ICSI fertilisation, you need to totally immobilise the sperm and “score” the tail in order to induce membrane changes essential for fertilisation. When immobilizing and scoring the tail, you must keep away from the head and mid-piece region. Damage to the head may prevent head de-condensation and hence pronuclei development. The mid-piece contains the proximal centriole, which is required by the oocyte to participate in bipolar spindle formation for the first mitotic division.

Scoring of the sperm tail entails the trapping of a sperm under the injection pipette and then a quick movement of the injection pipette away from the sperm tail (to the right using the joystick lever). This visually looks like the sperm is rotating around its own axis, some crushing of the sperm and possibly the kinking of the tail. It is so important not just to immobilise the sperm but to continue to score the sperm tail even after the sperm has stopped moving. A high number of single pronucleate zygotes (1pn, haploid) will indicate that you have not scored the sperm tail properly. Practice this technique until you can trap and score a sperm within 30 seconds.

You are now ready to pick up the sperm into your injection pipette. First, check that the air / medium meniscus in the injecting pipette is ¾ up the length of the pipette shank, closer to the elbow. If you don’t allow enough space between the sperm and the air segment, you will risk injecting the oocyte with air and thus damaging it.
Orient the sperm so that the tail is lying parallel to the pipette and its tail tip is at the far right. Move the pipette opening close to the sperm tail and aspirate the sperm up the pipette by using negative pressure on the microinjector control knob. As soon as the sperm starts to move into the pipette, slow down its movement by applying slight positive pressure on the same control knob. Adjust the final position of the sperm near the mouth of the pipette making sure it has finally stopped moving up / down the pipette. Lift the pipette in the Z-axis direction so that the pipette goes slightly out of focus. You can now safely move the pipette into a different drop. Bring the pipette down to the bottom of the new drop and expel the sperm using positive pressure. Repeat this with at least ten other sperm and pool them all into one drop. Take at least ten minutes each day to practice and improve your immobilising technique.

APPENDIX XV

OOCYTE MANIPULATION

1. First, check the holding pipette has ample medium in it so that when you eventually want to release the oocyte, air bubbles will not be injected into the 5µl drop.

2. Lift it completely above the oil.

3. Locate the first polar body of the oocyte and orientate it using your injection pipette so that the polar body is roughly at the 12 or 6 o’clock position.

4. Bring the holding pipette down into the drop (using the Z axis control knob or course control manipulator) being careful not to crush the oocyte below.

5. The opening of the holding pipette should be parallel and close to the oocyte. The closer you are to the oocyte before inducing negative pressure, the less
rotation will occur and more likely the polar body will remain in its correct position.

6. Use gently suctions to secure the oocyte onto the pipette. If the polar body is not in the correct position, lessen the suction and move the oocyte with the aid of the injection pipette. This technique must be demonstrated to you by a trained scientist.

7. This manipulation must be practiced with old oocytes and should become second nature in order to minimise the length of time the oocytes are kept outside the incubator.

8. Ova should be injected in the center of each 5-µl droplet where the optics are optimal. They should never be dragged on the bottom of the dish once attached to the holding pipette. Lift the pipette and oocyte until they go slightly out of focus before moving the pipette / oocyte around.

The holding pipette must be either parallel or slightly pointing downwards to the bottom of the ICSI dish. This will allow the suction of the oocyte in the same focal plane. If the pipette tip is pointing upwards, there will be a delay in the oocyte suction and the oocyte may appear to slightly spin before securing onto the holding pipette.

APPENDIX XVI

SPERM INJECTION INTO THE OOCYTE
One of the most important aspects of ICSI is finding and injecting the center of the oocyte. Cytoplasmic leakage and later degeneration is caused by inserting the injection pipette into an area other than the center of the oocyte. It is thus important for you to take your time to find the center of the zona / oolemma (plasma membrane) as it will result in higher fertilization rates.

Follow these steps when finding the center of the oolemma and injecting the egg:

1. Locate a sperm. Immobilize and score its tail. Aspirate it up into the injection pipette, stabilizing it at the tip.

2. Lift the pipette up slightly so that it does not drag on the bottom of the dish and move to a drop, which contains the oocyte.

3. Rotate the oocyte so that the polar body is at the desired position and attach it onto the holding pipette. NB: Make sure the injection pipette is not in front of the mouth of the holding pipette when it is aspirating as it may drag the sperm out of the injection pipette. Re-adjust the oocyte if necessary.

4. Focus on the oolemma (not the zona) until the membrane is outlined by a distinct dark line/pink.

5. Bring the injection pipette into the same focus as the oolemma by using the Z-axis control knob on the joystick.

6. Adjust the position of the sperm so that it is at the tip of the injection pipette.

7. Advance the injection pipette into the oocyte and note that the oolemma will invaginate as a distinct line. If you have not centered the oocyte properly,
the oolemma will invaginate as a shadow and not as a distinct line. If this occurs, abort the injection and repeat steps 4 to 7 until the centre of the oocyte is found.

8. Once you have advanced into the zona and you are happy that you have found the centre of the oolemma, you are ready to inject the oocyte. Push the zona with the injection pipette in a fast smooth motion until it “pops” open and you are sitting in the perivitelline space. You will find that the pipette is already invaginating into the oolemma.

9. Check the PVP level in the injection pipette before sucking the sperm /media level in the holding pipette before sucking the oocyte.

10. Immediately aspirate the cytoplasm into the injection pipette using small movements on the control knob on the microinjector. The cytoplasm encased within the oolemma will creep up the shank of the injection pipette until the oolemma finally breaks. Stop aspirating further and immediately unravel the sperm with the cytoplasm back into the oocyte. Only insert the minimum amount of media / PVP back into the oocyte.

11. When the sperm has been injected into the centre of the cytoplasm, you may exit the oocyte using a slow, smooth movement to the right. Take your time before exiting so that the sperm does not exit along with the injection pipette.

12. Decrease the suction of the holding the oocyte and with the aid of the injection pipette, move the oocyte off the pipette. Push a turn of PVP as you drag out the injection pipette to make sure the sperm stays and do not move close to oolemma.
13. Move the holding pipette out of the drop making sure not to drag the oocyte along with it. Make a note of how the injection went and the morphology of the oocyte on the laboratory data / proforma sheet.

14. Lift the injection pipette off the bottom of the dish and enter the central drop of sperm and PVP to select another sperm.

The fertilization checks should be performed no later than 16 to 18 hours post injection. Check all zygotes for pronuclei development. It is not uncommon to have some 3PN formation due to the retention of the second polar body. Regardless, treat these zygotes as abnormal and do not freeze or transfer the resulting embryos.
APPENDIX – XVII

We .......................................................... and ......................................... agree to use follicular fluid and blood and semen for research purpose. We are aware that the samples are strictly used for research work and not for any clinical use. We also understand that the samples will be discarded after completion of work.

WITNESS

SIGNATURE

RESEARCH SCHOLAR

GUIDE / SUPERVISOR

Dr. P. Chinnasamy  Ph.D: MAACC,FIFCC,FACB (USA)
Director, Institute of laboratory medicine
Kovai medical centre and hospital
Coimbatore – 14
Tamilnadu
INDIA
APPENDIX - XVIII

MALE PARTNER

NAME : 
AGE : 
MONTHLY INCOME : 
SMOKING/ALCOHOL : 
MALE FACTOR :
OTHER FACTORS : a) ED/Varicocele/Testicular factors  
b) Diabetic  
c) Hypertension  
d) Surgical history  
e) Mumps

FEMALE FACTOR

NAME : 
AGE : 
MONTHLY INCOME :
DURATION OF INFERTILITY :
INFERTILITY : PRIMARY/SECONDARY
MENSTRUAL CYCLE : REGULAR/IRREGULAR
LMP :
PREVIOUS HISTORY : a) PCO  
b) ENDOMETRIOSIS  
c) BILATERAL TUBAL BLOCK  
d) UTERINE FACTORS
IUI : YES /NO  
IF YES HOW MANY

PREVIOUS CYCLE DETAILS

Assessment of Oocyte Quality with AMH, Inhibin B in Serum and Follicular Fluid and Predicting Pregnancy outcome

Sperm DNA Fragmentation in Art Cycles
Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome

Sperm DNA Fragmentation in ART Cycles