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Project 1: Assessing the Inter-, Intra- Observer and Longitudinal Variability in Routine and Advanced Semen Parameters.

Rationale/ Study hypothesis:

Restoring natural fertility to the individual or the couple is the main goal of infertility evaluation. Male infertility is recognized as the primary or contributory factor in more than 40% of infertile couples. Measurement of routine semen parameters is the cornerstone in the evaluation of the infertile male. All andrology laboratories around the world are involved in the measurement of routine semen parameters such as sperm count, motility and morphology. However, there is a huge disparity among various laboratories, and there is inherently a wide range in the measures of each parameter. There is a strict need for strict quality control and quality assurance. Only a handful of these laboratories are accredited by the various crediting agencies such as CLIA (Clinical Laboratory Improvement Act) and CAP (College of American Pathologists), and few laboratory directors are certified as a High Complexity Lab Director (HCLD) by the ABB (American Association of Bioanalysts).

In addition to the inherent variability of the semen sample, external variables also contribute to the acceptable or the 'normal range' of each measure. High turnover in the laboratory staff, lack of uniform training of staff, semen preparation methods, staining techniques and the scoring techniques all account for the lack of an established in acceptable range for the various semen parameters test being evaluated by each lab.

All these factors account for the wide range of measures reported by each lab. Therefore there is a need for implementing strict quality control – both external and internal. External quality control can be achieved by participating in the 'proficiency testing' where an independent agency, such as one of the andrology accrediting agencies, distributes testing material to the participating labs. The labs perform the analyses and return the results to the agency. The agency informs each lab

as to whether their results fall within the acceptable norms of the test group. Laboratory participation occurs twice a year and its implementation is legally mandated for semen analysis in the United States. Internal laboratory control involves the routine incorporation of positive and negative controls as well as rigorous cross-checking of semen analysis results within one particular laboratory, and is performed as frequently as deemed necessary. The experienced staff member verifies the results of each test and examines the mean, standard deviation and the coefficient of variation of each test with 5 readings and these are plotted. Quality assurance demands continuous laboratory refinement and improved performance. It involves self-correction, remedial steps, and implementing new teaching and training material to ensure that all staff members in each lab are well trained and checked off for each test that they are conducting.

The goal of this study is to evaluate the variability in each measure being tested both in the routine semen measures as well as the advanced tests performed in our laboratory, which is accredited by CLIA and CAP, under the direction of a laboratory director, who is certified for high complexity testing by the ABB.

We will examine 3 specific aims:

Aim 1: Establish the inter-assay, intra-assay, inter-observer and intra-observer variability in the routine semen measures such as sperm concentration, sperm motility, vitality and morphology.

Aim 2: Establish these variables in 3 advanced sperm tests – reactive oxygen species (ROS), total antioxidant capacity (TAC) and DNA damage.

Aim 3: Examine the longitudinal variability of these measures over a course of 6 weeks.

Hypothesis	Methodology	Expected outcome
There is a significant variation in the measures of routine semen parameters as well as between the various observers.	<ol style="list-style-type: none"> 1. Complete liquefaction of semen sample. 2. Determine sperm concentration and total sperm count, percent motility, sperm vitality and sperm morphology. 3. Five observers will take 3-4 readings of each parameter. 	Establish the range of variability (inter-intra-assay, inter-and intra-observer variability) of each measure of routine semen parameters.
There is both assay and observer variability in the advanced semen parameters.	<ol style="list-style-type: none"> 1. Measure ROS, TAC and DNA damage. 2. Five observers will take 3-4 readings of each parameter. 	Establish the range of variability (inter-intra-assay, inter-and intra-observer variability) of advanced semen parameters.
Each specimen exhibits variability in different measures of sperm parameters over a period of time.	Same subject will be asked to provide a sample twice a week over a course of 6 weeks.	Establish the longitudinal variability in the semen parameters. This will help establish the reference range of the tested measures.

Significance of the study:

This study will help identify the extent of assay and observer variability in both routine and advanced measures of semen parameters, and document longitudinal changes, necessary to establish acceptable reference ranges.

Experimental Design and Methodology:

Following the approval of the study by the Institutional Review Board of the Cleveland Clinic, semen samples will be collected from healthy male volunteers. Measurement of volume, pH, cell concentration, motility and round cell count will be carried out manually as described in World Health Organization guidelines (WHO, 2010) before specific interventions will take place. All specimens will be subjected to the following tests:

1. Sample collection

Semen samples will be collected from 20 donors and evaluated – following a period of complete abstinence of 48 – 72 hours. Collection will take place by masturbation into sterile containers at the Andrology Laboratory of the Cleveland Clinic. Semen specimens will be allowed to liquefy

completely for 15-30 minutes at 37 °C before further processing. All samples will be discarded, at the latest, by the end of the experiment.

2. Standard semen analysis

Following liquefaction, semen specimens will be evaluated for:

1. Physical characteristics such as: Presence of coagulum, Liquefaction time.
2. Macroscopic characteristics such as: Color, pH, viscosity, ejaculate volume.
3. Microscopic characteristics such as: Sperm concentration, percent motility, velocity, vitality sperm morphology, and presence of round cells.

A total amount of 5 μ L of the sample will be used for manual evaluation of concentration and motility using a Microcell counting chamber (Vitrolife, San Diego, CA). The same volume will be used to assess these parameters using computer assisted semen analysis (CASA)

3. Sperm vitality

Sperm vitality will be assessed in those samples that present with <30% motility. This will be performed by using one-step eosin-nigrosin staining. At least 200 sperm will be scored in 10-20 different fields per sample at 400x magnification. The percentage of dead (colored pink) and live (unstained) cells will be evaluated. This stain produces a dark background that provides contrast to the light color of sperm. Normal live sperm, with intact plasma membranes, do not take up the eosin-nigrosin stain and appear white (unstained), while dead sperm allows the dye to pass through the cell membrane and stain the nucleus pink.

4. Sperm morphology

Smears of the semen will be stained using a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology. The morphological abnormalities will be examined according to Kruger's strict criteria. (WHO 2010).

5. Measurement of extracellular reactive oxygen species

ROS levels will be measured by the conventional chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis, MO). Luminol (10 μ L, 5 mM) prepared in dimethylsulphoxide (DMSO) will be added to 400 μ L of the liquefied seminal

ejaculate. Negative controls will be prepared by adding 10 μL of 5 mM luminol to 400 μL of PBS. Positive control will consist of 50 μL of hydrogen peroxide (37%). The chemiluminescent signal will be monitored for 15 min using a luminometer (Autolumat plus 953; Oakridge, TN) and results will be expressed as RLU/s/ $\times 10^6$ sperm.

Measurement of TAC

Seminal plasma total antioxidant measurement was done using the antioxidant assay kit (Cat # 709001; Cayman Chemical, Ann Arbor, Michigan). The Cayman chemical antioxidant assay will be used to measure the antioxidant capacity of the seminal fluid samples. The principal of the assay is the ability of aqueous- and lipid-antioxidants in the seminal plasma specimens to inhibit the oxidation of the 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS+. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree which is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent ABTS® oxidation will be compared with that of standard - Trolox, a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. This assay measures the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, etc.

All seminal plasma samples will be diluted 1:10 with the assay buffer before assaying to avoid variability due to interference by the plasma proteins or sample dilution. All reagents and samples will be equilibrated to room temperature before beginning the assay. Samples as well as Trolox standards will be assayed in duplicate. Trolox standards and reagent will be prepared as per the manufacturer's instructions at the time of the assay. After the plate configuration, 10 μL of Trolox standard and samples will be loaded on to the corresponding wells of a 96 well plate. Then 10 μL of metmyoglobin and 150 μL of chromogen will be added to all standard/sample wells. The reaction will be initiated by adding 40 μL of hydrogen peroxide (H_2O_2) as quickly as possible. The plate will be covered and incubated for 5 minutes on a shaker at room temperature. Absorbance will be monitored at 750 nm using Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont).

Calculation of assay result

Determination of the reaction rate will be done by calculating the average absorbance of each standard and sample. The average absorbance of the standards as a function of the final Trolox concentration (μM) will be plotted for the standards curve in each run, from which the unknown samples will be determined. The total antioxidant concentration of each sample will be calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant } (\mu\text{M}) = \frac{\text{Unknown average absorbance} - Y/\text{intercept}}{\text{Slope}} = X \text{ dilution} \times 1000$$

6. Measurement of sperm DNA damage

Semen will be washed twice in PBS, resuspended in 1-3% paraformaldehyde at a concentration of $2-4 \times 10^6$ cells/mL and placed on ice for 15 to 30 minutes. Sperm samples will be then washed and resuspended in 70% ice-cold ethanol by centrifugation at X300g for 5 minutes. The ethanol supernatant will be removed, and the sperm pellets will be washed twice in wash buffer. The samples will be resuspended in 100 μL of the staining solution for 1 hour in the dark at room temperature. All cells will be washed using rinse buffer, resuspended in 250 μL and incubated for 30 minutes in the dark on ice for flow cytometry measurements.

Terminal deoxynucleotidyltransferase (Tdt) catalyzes a template-independent addition of brominated deoxyuridine triphosphatase to the 3'-hydroxyl (OH) termini of double and single stranded DNA. Sperm DNA strand breaks will be evaluated using a flow cytometric terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct, BD Biosciences).

A total of 10,000 cells will be examined for each assay at a flow rate of 100 cells/second. The FITC (log green fluorescence) will be measured on FL1 channel. Data acquisition will be performed within 30 min on a flow cytometer equipped with a 515-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). Data will be processed using Flow Jo v4.4.4 software (Tree Star Inc., Ashland, OR).

We will examine the following variability:

1. **Inter-observer Variability:** (Multiple observers on the same day with the same sample)

Average pairs of measures within observer and experiment. The mean difference in measures between observers across the 5 experiments will be reported along with its mean (\pm SD) and 95% CI.

2. **Intra-observer Variability:** (Multiple readings of the same sample by same observer)

3. **Inter-assay Variability:** (Same sample observed at different times by the same observers)

Pairs of measures within observer and experiment (20 averages with 5 per observer) will be averaged, and the coefficient of variation $[(SD/Mean) \times 100]$ will be calculated overall and for each observer.

4. **Longitudinal study:** Same subject(s) providing semen sample over a period of 6 weeks.

Figure 1. Flow diagram showing the Training and Experimental schedule

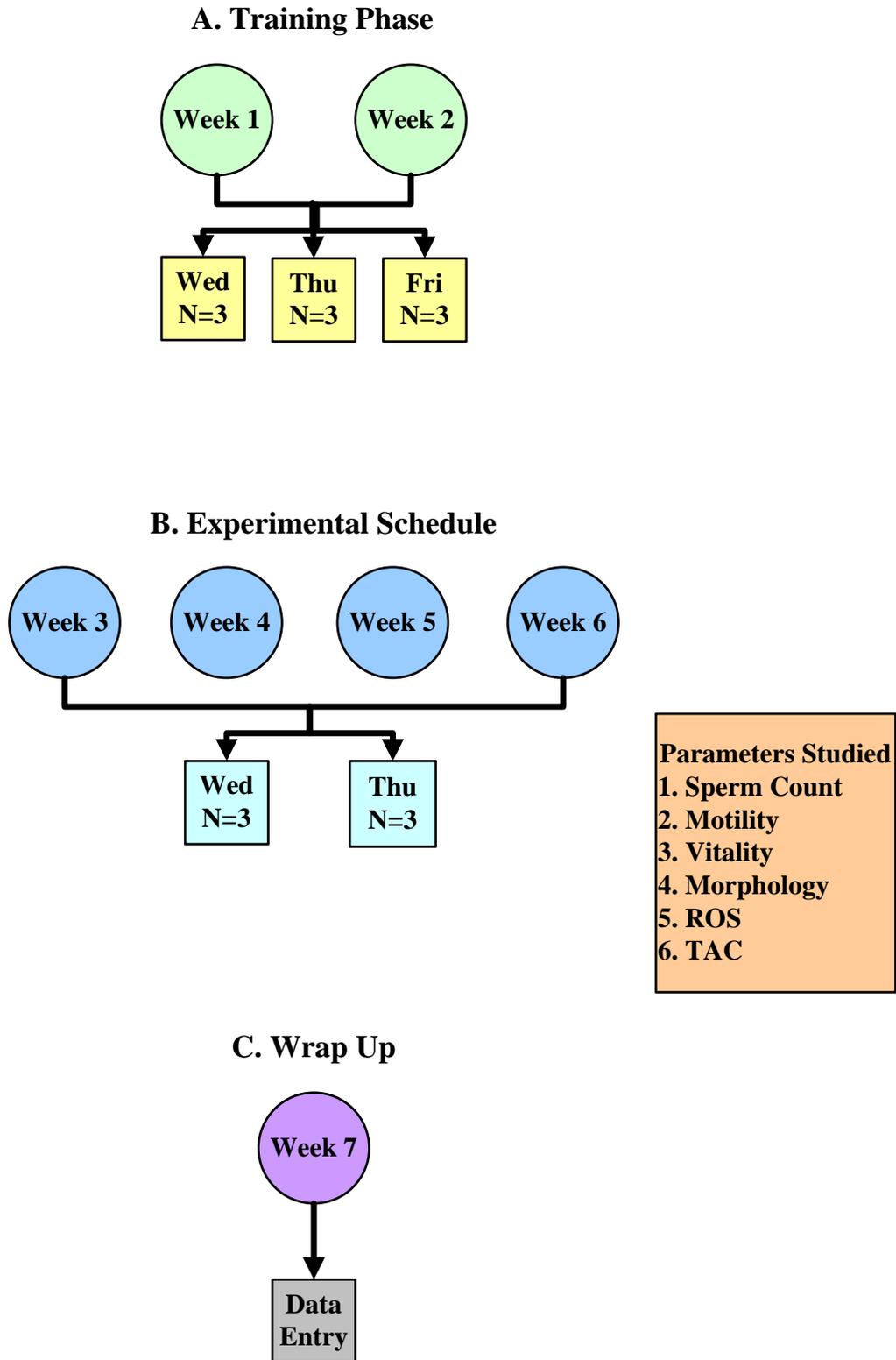


Figure 2. Measure Intra-Assay and Intra-Observer Variability

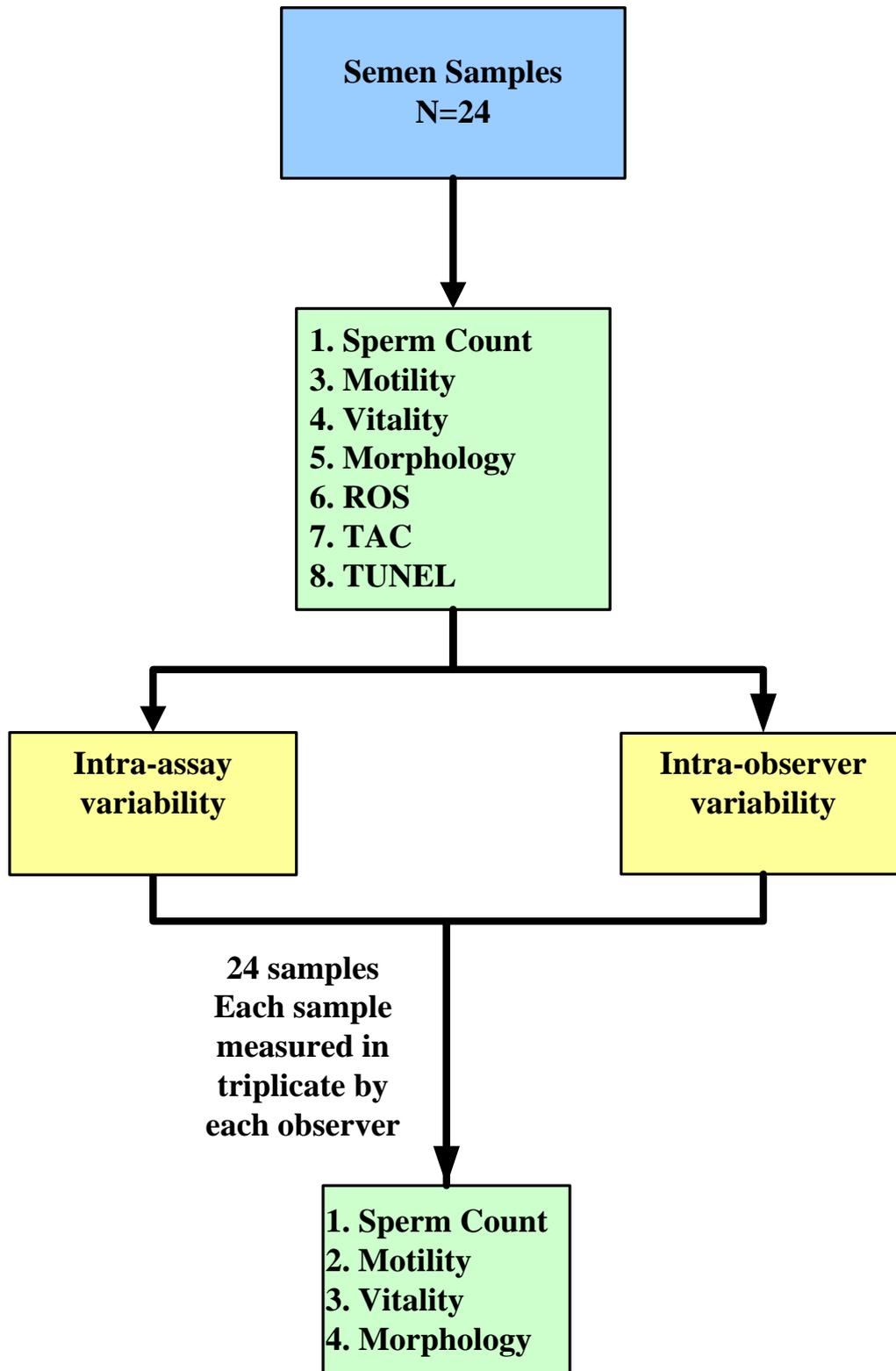


Figure 3. Measure Inter-Assay and Inter-Observer Variability

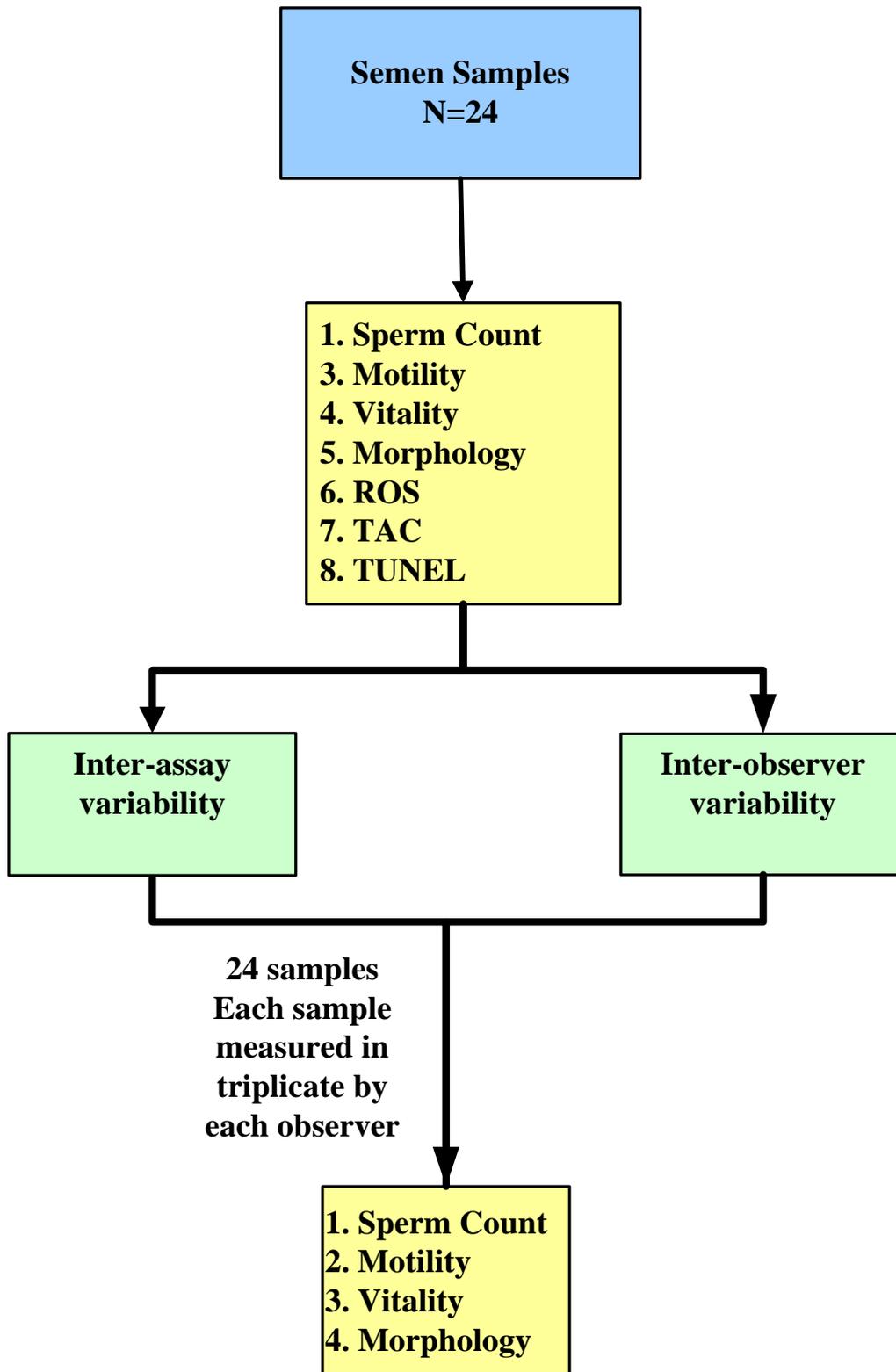
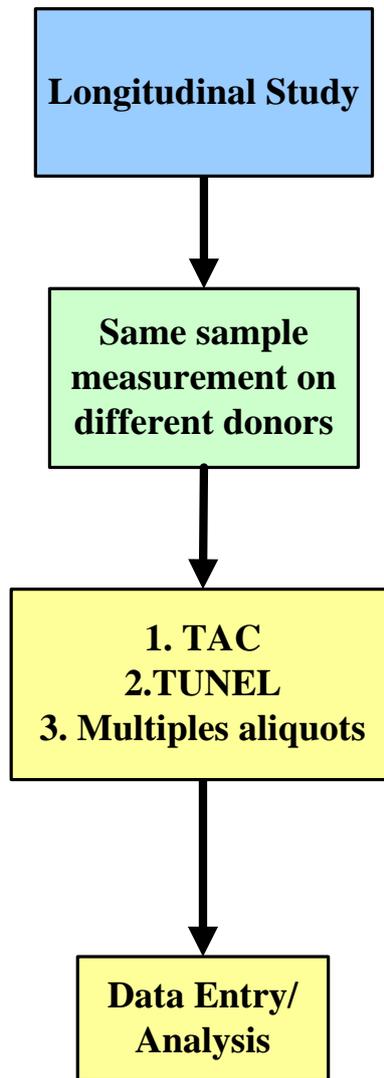


Figure 4. Longitudinal Study and Variation in Semen Parameters



Time Line: 5 interns

7 weeks (Wed and Thursday)

Weeks 1 and 2: Training in different techniques required for the project.

Weeks 3 - 6:

1. Measurement of sperm count, motility– **(All observers)**
2. Measurement of viability, morphology - **(All observers)**
3. Measurement of reactive ROS - **(All observers)**
4. Measurement of reactive TAC – preparation only (Interns 1-5)
5. Measurement of DNA damage - preparation only

Week 7: Data entry (2 interns)

1. **PowerPoint presentation: Highlights of the study**

Materials needed:

A. Equipment

1. Centrifuge
2. Vortex
3. Incubator
4. Microscope
5. Luminometer
6. Slide boxes

B. Reagents /chemicals

1. Donors
2. Sperm wash medium (HTF)
3. Phosphate buffer saline (PBS)
4. Dimethyl sulfoxide (DMSO)
5. Luminol stock solution
6. Hydrogen peroxide
7. Millipore water
8. Diff Quick stain
9. Eosin stain
10. Nigrosin stain
11. Antioxidant assay kit
12. TUNEL kit

C. Disposables

1. Pipettes (1-10 μ L, 10-100 μ L, 100-1000 μ L)
2. Tips (1-10 μ L, 10-100 μ L, 100-1000 μ L)
3. Serological pipettes (2 and 5 mL)
4. Plastic tubes (5 mL and 15 mL centrifuge tubes)
5. Centrifuge tubes (25 mL)
6. MicroCell slides
7. TAC plates

8. Frosted and plain slides
9. Coverslips
10. Transfer pipettes
11. Gloves

7. Training Requirements for Study Participant:

No.	Test	No of Readings (N)	Acceptable Result	Data Entry
1.	Sperm Count	10	<20% difference	Excel
2.	Sperm Motility	10	<20% difference	Excel
3.	Vitality	10	± 2 SD <	Excel
4.	Morphology	10	± 2 SD	Excel
5.	ROS	4	± 2 SD	Excel
6.	TAC and TUNEL	3	Check with mentor for preparation and labeling of samples	

Note

All raw results must be entered in the lab note book
 All results must be verified by the mentor
 Results should be entered daily into the Excel sheet

8. Sample Size Calculation and Statistical Methodology:

Measurements of count, motility, viability, morphology, ROS, TAC, and TUNEL will naturally vary due to several sources. The various sources of variability will be analyzed as follows:

A. Intra-donor variability (which can also be viewed as the longitudinal variability) will be assessed by summarizing and graphing the individual donor results across the four weeks. Within each week, we will consider the overall average measurement for the donor across all observers, as well as within individual observers. In each circumstance, the standard deviation and coefficient of variation among the measurements for the four weeks will be reported. Combining all data and subtracting out donor averages will allow us to use linear regression to test for a general trend over time for a given sperm parameter.

B. Inter-donor variability will be assessed using the observed standard deviations and coefficients of variation within individual weeks and across all weeks for measurements averaged over all observers. Inter-donor variability will also be assessed within individual observers to assess its consistency across observers.

C. Inter-observer variability will be assessed using the observed standard deviations and coefficients of variation among observer averages on measurements after subtracting out the specific donor averages within each week. Inter-observer variability will also be assessed within individual donors and weeks in order to assess its consistency.

D. Intra-observer variability will be assessed using the observed standard deviation and coefficients of variation after subtracting out for observer, donor, and week averages. Intra-

observer variability will also be assessed within individual donors, weeks, and observers in order to assess its consistency.

We will also use a random effects model to quantify the sources of variability, assuming the above more simplistic analyses do not reveal any special inconsistencies or trends in measurements.

The planned total number of samples, which is 24, will ensure with 95% confidence for each standard deviation estimate, that the true standard deviations and coefficients of variation for inter-observer and intra-observer are no more than double the observed values.

Study Budget

Lab Supplies and Reagents	Cost/unit (\$)	Quantity	Total Cost (\$)
Phosphate Buffer 500 mL without calcium and magnesium, ph 6.8	1 X 500 mL	\$25.94 ea	\$25.94
Dimethyl Sulfoxide 100 mL	1 X 100 mL	\$119.50/ btl	\$119.50
Luminol 5G	1 X 5G	\$51.80	\$51.80
Sperm Wash HTF 100 mL	1 X 100 mL	\$30.40 ea	\$30.40
TAC kit	\$225.00	1	\$225.00
TUNEL kit	\$395.00	1	\$395.00
Donors	\$50.00	20	\$1,000.00
Statistical charges (\$70.00/hr) X 10h			\$700.00
Miscellaneous Disposable/Laboratory Supplies Disposable Gloves Pipetting reservoirs Micropipette Tips (5, 10, 1000 microliters) MicroCell slides Serological Pipettes (1, 2, 5 mL) Eppendorf microfuge tubes (1.5 mL) Tubes 12 X 75 mm Centrifuge tubes (25 mL) Cryoboxes (2bxs) Cryovials 1.2 mL Centrifuge tubes (15 mL) Cryogenic markers Shipping labels Transfer pipettes Eosin Nigrosin			\$1,000.00
		Total Cost	\$6,245.00

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Project 2: Comparative Dynamics of Cryopreservation Induced Sperm DNA Damage between Semen Samples Collected Onsite versus Samples Remotely Collected and Shipped

Rationale/ Study hypothesis:

Cryopreservation of human spermatozoa has evolved as an important area in assisted reproductive technology programs and oncology programs. Despite several efforts to improve the sperm cryosurvival, decline in motility of up to 25%–75%, poor cryosurvival, sperm DNA fragmentation, and reduction in vitality and membrane integrity are commonly observed after thawing. Loss of sperm function and DNA fragmentation during cryopreservation has been linked to the production of reactive oxygen species during the freeze-thaw process. Reports suggest an increase in DNA damage (up to 15%) in normozoospermic semen samples after freezing.

Apoptosis and oxidative stress as a result of increased production of reactive oxygen species or reduced antioxidant reserves is mainly responsible for DNA damage. We develop a specialized sperm collection and transport kit (NextGenSM). It is a first-of-its kind product evaluated in a clinical setting and specially designed primarily for men with cancer who are about to undergo treatment (surgery, chemotherapy, radiation therapy, which can render them infertile. In addition men with underlying sub fertility; men who are about to under a vasectomy but who do not want to totally rule out the option of fathering a biological child in the future and military personnel going on long-term deployment whose partners may need a sperm sample for an assisted reproductive procedure while they are gone can also benefit from this kit. Patients collect a semen sample in the privacy of their own homes, place the sample into a vial containing preservative/transport media, and ship the samples overnight to the Cleveland Clinic Andrology Laboratory and Reproductive Tissue Bank for storage. Collecting semen at home and transporting the same overnight reduces emotional anxiety, need to travel from geographically distant places (different cities/states), and is cost-effective.

Our study aims to compare the DNA damage in samples collected on site versus those shipped by NextGen. Two markers of DNA damage will be used. 1) Terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit and 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay kit. Samples will be collected from healthy male volunteers and from men presenting for infertility on site (controls) and samples collected offsite and shipped overnight to the Clinic by NextGen kit (~ 18 hours). Samples will be cryopreserved using our standard freezing protocol. The kit allows samples to be shipped from geographically remote areas, where banking facilities are not available. The study objective is to compare the percent cryosurvival and increase in DNA damage in these two groups post cryopreservation.

We will examine 3 specific aims:

Aim 1: To assess the baseline levels of sperm DNA damage in donors and infertile men collecting onsite.

To achieve this aim, we will measure baseline measures of semen parameters (Count, motility and vitality), DNA damage (by both TUNEL and 8-OHdG) before cryopreservation.

Aim 2: Compare sperm motility, percent cryosurvival and sperm DNA damage levels induced by cryopreservation in samples collected onsite versus those collected offsite and shipped by NextGen.

We will assess the sperm DNA damage levels by TUNEL and 8-OHdG, post cryopreservation in the 2 groups. This will help identify the effects of cryopreservation on semen samples of donors.

Aim 3: Measure the extent of DNA damage induced as a result of oxidative stress alone or oxidative stress + cryopreservation as measured by 8-OHdG. Compare the extent of DNA damage measured by 8-OHdG with the Gold standard - the TUNEL assay.

Group 1 will include semen samples from donors who will collect onsite. Group 2 will include semen samples from the same donors collected offsite and transported in the temperature regulated conditions of the NextGen kit.

Hypothesis	Methods	Expected Outcomes
The extent of sperm DNA damage before cryopreservation is different in semen samples collected onsite by two specific markers of DNA damage TUNEL and 8-OHdG.	<ol style="list-style-type: none"> 1. Semen samples from healthy male volunteers (n = 20). 2. Measure baseline DNA damage in samples before cryopreservation. 3. DNA damage determined by TUNEL and by 8-OHdG assay kits. 	<p>Establish the baseline DNA damage (before by two specific markers.</p> <p>Validate extent of DNA damage measured by 8-OHdG with TUNEL.</p>
Remote collection and transportation of spermatozoa in NextGen preserves the sperm vitality and membrane integrity.	Measure pre- and post-freeze motility and total motile sperm and vitality in the two groups.	Similar levels of sperm motility and vitality with onsite and offsite collection.
Oxidative stress induced DNA damage de novo and due to cryopreservation can be measured with the two markers of DNA damage.	Measure pre and post-freeze levels of sperm DNA damage by TUNEL and by 8-OHdG assay kits.	<p>Compare the extent of DNA damage by oxidative stress and cryopreservation.</p> <p>Examine the efficacy and sensitivity of two assays of DNA damage.</p>

Significance of the Study:

Results of this controlled study will help establish if sperm DNA damage induced by cryopreservation is comparable in samples collected offsite and transported to the andrology lab utilizing the Novel NextGen kit developed by our program versus samples collected onsite and subjected to cryopreservation without the transportation time lag period. We want to ascertain if DNA integrity is preserved when semen samples are transported from geographically remote places (different parts of the country) utilizing the NextGen kit. In addition we want to examine the suitability of the two markers of DNA damage in measuring the oxidative stress induced DNA damage. This kit will allow patients who do not have access to fertility centers and desiring of preserving their future fertility to utilize this kit and ship semen samples without the need to actually travel to fertility center on multiple occasion.

Experimental Design and Methodology:

Semen samples from healthy male volunteers (n = 20) collected onsite and collected at home and shipped overnight via NextGen kit will be obtained after 2-3 days of sexual abstinence. Semen samples from the will be divided into two aliquots. One aliquot will be

used for DNA damage by TUNEL and 8-OHdG test and stored at -80 degrees. The 2nd aliquot will be cryopreserved using the Andrology lab cryopreservation protocol. The work flow will consist of examining sperm motility, concentration, cryopreservation of samples utilizing Andrology lab protocol and measurement of sperm DNA integrity by TUNEL and 8-OHdG in the 2 study groups before and after addition of cryoprotectants (**Flow diagram 1**).

1. Semen analysis

Following incubation of semen samples for liquefaction, manual semen analysis will be performed using a MicroCell counting chamber (Vitrolife, San Diego, CA) to determine sperm concentration and motility. Semen analysis will be performed according to WHO guidelines (2010) to evaluate sperm count, motility and presence of round cells.

2. Measurement of sperm vitality

Vitality will be evaluated by eosin-nigrosin staining. An aliquot from each sample will be mixed with an equal volume of 0.05% eosin and twice the volume of nigrosin to improve the contrast. After 2 min, smears will be prepared and air dried. A total of 100 spermatozoa in duplicate will be evaluated at x 100 under a light microscope. The dead sperm will appear pink; sperm without the dye will be counted as viable.

3. Shipping samples using NextGen kit

Sample collection and shipping instructions are provided to the patient. NextGen kit consists of a collection cup, ice pack, freezing sleeve, and refrigeration media. These are placed in a freezer for at least 12 hours. On day of collection, prior to semen collection, the refrigeration medium and collection cup is removed from freezer and allowed to thaw to room temperature for 60 minutes. It is important that the media is at room temperature prior to use. The seal is broken from the sterile collection cup and semen sample is deposited via manual masturbation. Use of lubricating gels is not recommended. After collection of sample, the entire content of the refrigeration media (5.0 mL) is added to the collection cup. The cup is sealed securely and gently swirled to mix the contents. The cup is placed in the kit – and the cup is surrounded with silver sleeve and placed in-between foam layers. The ice bricks are placed on the on the outside of the foam layers and the kit is sealed (see illustrated instructions). The completed kit is placed inside cardboard container and sealed. The sample is shipped overnight and received by the Andrology laboratory the next morning.

4. Sperm cryopreservation

The liquefied semen samples will be mixed with equal volume of 10% glycerol-based cryoprotectant (glycerol–egg yolk–citrate medium) in 4 equal supplements. The samples will

be equilibrated with the cryoprotectant with mixing cycle of 5 minutes for each supplement of the added cryoprotectant. The equilibrated samples will then be transferred to cryovials and subjected to static cooling at -20°C for 8 minutes and then vapor-phase cooling for 2 hours before being plunged into liquid nitrogen. After 24 hours, thawing will be accomplished at 37°C . The samples will be centrifuged at 1,600 rpm for 7 minutes to remove all cryoprotective medium before further analysis.

5. Measurement of Reactive Oxygen species:

Fresh completely liquefied seminal ejaculate will be used (neat semen). ROS levels will be measured by chemiluminescence assay using luminol (5-amino-2, 3- dihydro-1, 4- phthalazinedione). Test samples will consist of luminol (10 μL , 5 mM) and 400 μL of semen. Negative controls will be prepared by replacing sperm suspension with phosphate buffered saline. Chemiluminescence will be measured for 15 min using a Berthold luminometer (Autolumat Plus 953). Results will be expressed as relative light units (RLU)/sec/ $\times 10^6$ sperm.

6. Measurement of sperm DNA damage

Sperm will be washed twice in PBS, resuspended in 3.7% paraformaldehyde at a concentration of $2-4 \times 10^6$ cells/ mL, and placed on ice for 15 to 30 minutes. Sperm samples will be then washed and resuspended in 70% ice-cold ethanol by centrifugation at X300g for 5 minutes. The ethanol supernatant will be removed, and the sperm pellets will be washed twice in wash buffer. The samples will be resuspended in 100 μL of the staining solution for 1 hour at room temperature in dark. All cells will be washed using rinse buffer, resuspended in 250 μL and incubated for 30 minutes in the dark on ice for flow cytometry measurements.

7. Evaluation of sperm DNA fragmentation by 8-hydroxy-2-deoxyguanosine assay.

Oxidative damage to DNA can be evaluated using the flow cytometric OxiDNA assay kit (Calbiochem, San Diego). It consists of the Wash concentrate and the FITC-conjugated concentrate that binds to the 8-oxoguanine moiety present in the 8-oxoguanosine of oxidized DNA. The probe in the OxyDNA kit is specific for 8-oxoguanine. 8-oxoguanine (as part of the oxidized nucleotide 8-oxyguanosine) is formed during free radical damage to DNA and is a sensitive and specific indicator of oxidative DNA damage. 8-oxoguanine is a particularly important biomarker of oxidative DNA damage as it is formed in relatively large quantities. The kit utilizes a binding protein with high avidity and specificity for 8-oxoguanine, and provides a simple, convenient, sensitive fluorescence method for detecting for oxidative DNA damage. The FITC-Conjugate is added and binds to the 8-oxoguanine moiety present in the 8-oxoguanosine of oxidized DNA. Negative control will be prepared without FITC staining,

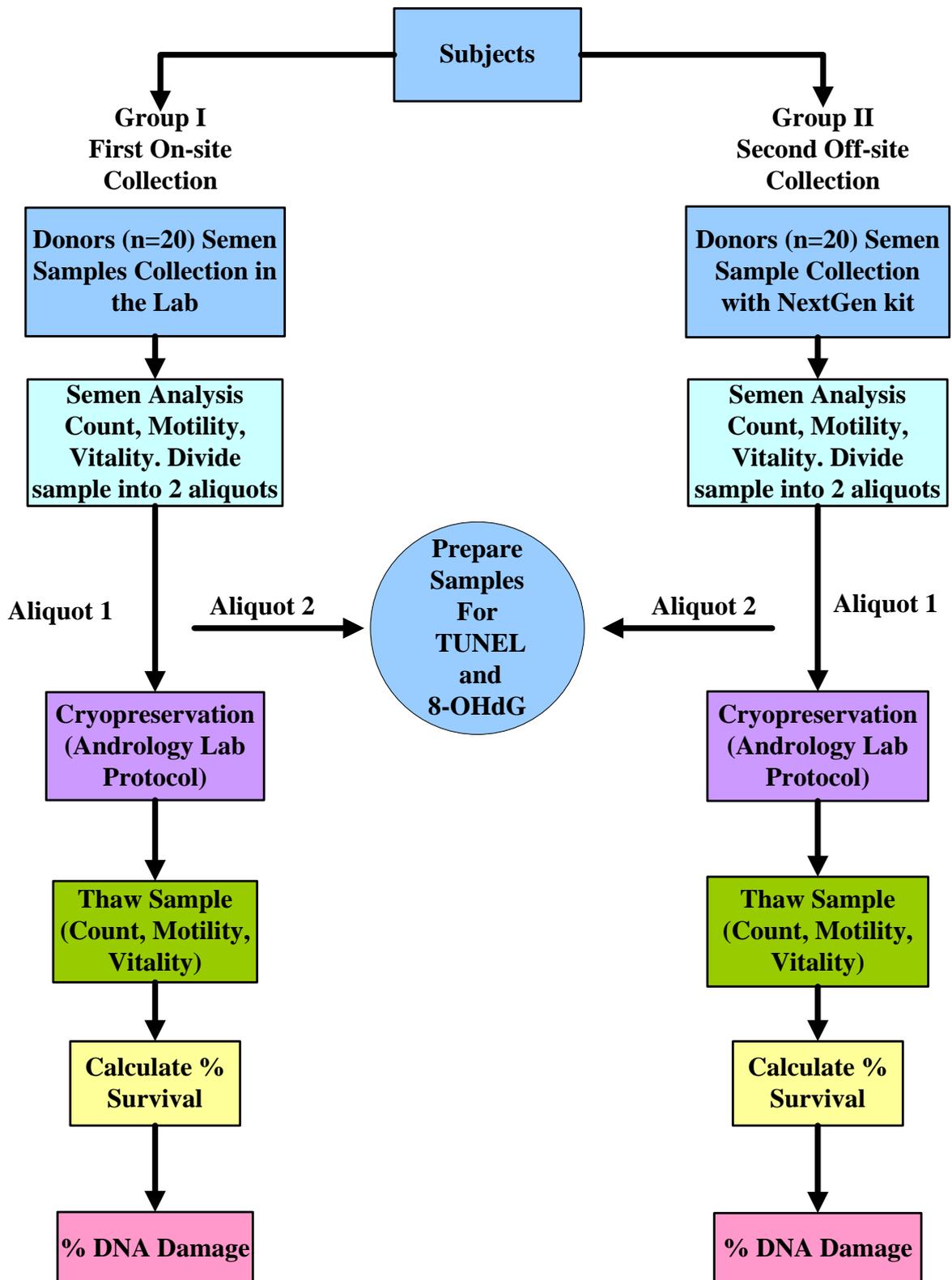
while the positive control will be prepared in the presence of 0.4 mM H₂O₂ and 0.2 mM FeSO₄. The presence of oxidized DNA is indicated by a green/yellow fluorescence that can be read using flow cytometry.

8. Evaluation of sperm DNA fragmentation by terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick end labeling assay

Terminal deoxynucleotidyltransferase (Tdt) catalyzes a template-independent addition of brominated deoxyuridine triphosphatase to the 3'-hydroxyl (OH) termini of double and single stranded DNA. Sperm DNA strand breaks will be evaluated using a flow cytometric terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct, BD Biosciences).

A total of 10,000 cells will be examined for each assay at a flow rate of 100 cells/second. The FITC (log green fluorescence) will be measured on FL1 channel. Data acquisition will be performed within 30 min on a flow cytometer equipped with a 515-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). Data will be processed using Flow Jo v4.4.4 software (Tree Star Inc., Ashland, OR).

Figure 1. Flow Diagram Showing Measurement of Semen Parameters and DNA Damage with Two Methods of Sperm Collection



Time Line and Study Participants: 5 interns

7 weeks (Wed and Thursday)

Week 1 and 2:

1. Training in different techniques required for the project.

Weeks 3 - 6:

1. Sperm motility, vitality, morphology measurement – **(Interns 1 - 3)**
2. Measurement of reactive oxygen species - **(Intern 4)**
3. Cryopreservation of onsite and NextGen samples **(Intern 5)**
4. Measurement of DNA damage by TUNEL and 8-OHdg **(Dr. Sharma)**

Week 7:

1. Data entry **(2 Interns)**
2. PowerPoint presentation: Highlights of the study

Materials needed:

A. Equipment

1. Centrifuge
2. Vortex
3. Incubator
4. Microscope
5. Luminometer
6. Flow core facilities
7. Slide boxes
8. Cryomarkers

B. Reagents /chemicals

1. Donors
2. NextGen Kits
3. Sperm wash medium (HTF)
4. Phosphate buffer saline (PBS)
5. Dimethyl sulfoxide (DMSO)
6. Luminol stock solution
7. Hydrogen peroxide
8. Diff Quick stain
9. Eosin stain
10. Nigrosin stain
11. Freezing media
12. Refrigeration médium
13. TUNEL kits
14. 8OH-dg kits

B. Disposables

1. Pipettes (1-10 μ L, 10-100 μ L, 100-1000 μ L)
2. Tips (1-10 μ L, 10-100 μ L, 100-1000 μ L)
3. Serological pipettes (2 and 5 mL)
4. Plastic tubes (5 mL and 15 mL centrifuge tubes)
5. MicroCell slides
6. Frosted and plain slides

7. Coverslips
8. Transfer pipettes
9. Gloves
10. Cryovials

9. Training Checklist

No.	Test	No of Readings (N)	Acceptable Result	Data Entry
1.	Sperm Count	10	<20% difference	Excel
2.	Sperm Motility	10	<20% difference	Excel
3.	Vitality	10	± 2 SD <	Excel
4.	Morphology	10	± 2 SD	Excel
5.	ROS	4	± 2 SD	Excel
6.	Sperm Cryopreservation	3	Check with mentor for correct preparation and labeling of samples	
7.	Post Thaw sample analysis	3	TMS and % cryosurvival verified by mentor	
8.	TUNEL & 8-OHdG	3	Check with mentor for correct preparation and labeling of samples	

Note:

All raw data must be entered in the lab note book.
 All results will be verified by the mentor.
 Results should be entered daily into the Excel sheet.

10. Sample Size Calculations and Statistical Methodology:

The mean differences between on-site and off-site samples with respect to count, motility, vitality, and %DNA damage (both by TUNEL and 8-OHdG) will be assessed using confidence intervals based on corresponding paired T-tests. Log transformations of the original measurements may be necessary for some parameters to achieve approximate normality.

Differences between methods will be assessed with respect to samples prior to cryopreservation, post-thaw samples, and the changes following cryopreservation. Equivalence between the two sampling methods with respect to %DNA damage change resulting from cryopreservation will be concluded if the 95% confidence interval for the difference in means between sampling methods falls entirely within an interval of $\pm 10\%$. The sample size of 20 subjects will provide 90% power to detect such equivalence if the two methods truly yield identical results on average, and if the standard deviation of differences between sampling methods with respect to the change in %DNA damage resulting from cryopreservation is no more than 10%. This standard deviation assumption will be assessed when complete data is available on 10 donors, in order to ensure that the analyses of change in %DNA damage following cryopreservation will be carried out with high power to detect equivalence.

Budget

Lab Supplies and Reagents	Cost/unit (\$)	Quantity	Total Cost (\$)
NextGen Kits	\$50.00/kit	20	\$1,000.00
Donors	\$50.00	20	\$1,000.00
TUNEL Assay Kit	\$380.00/kit	4 Kits	\$1,520.00
8-Hydroxy-2- deoxyguanosine (8-OHdg) Assay Kit	\$205.00/kit	5 Kits	\$1,025.00
Statistical charges (\$70.00/hr) X 10h			\$700.00
Flow core charges (\$70.00/hr) X 10h			\$700.00
Miscellaneous Disposable/Laboratory Supplies Disposable Gloves Pipetting reservoirs Micropipette Tips (5, 10, 1000 microliters) Serological Pipettes (1, 2, 5 mL) Eppendorf microfuge tubes (1.5 mL) Centrifuge tubes (15 mL) Round bottom tubes (7 mL) Cryoboxes (2bxs) Cryovials 1.2 mL Centrifuge tubes (15 mL) Cryogenic markers Shipping labels Transfer pipettes TYB media			\$1,000.00
		Total Cost	\$6,245.00

NextGenSM Home Sperm Banking Kit for Men From Geographically Remote Sites Seeking Fertility Preservation Services: An Exciting Development

Ashok Agarwal, PhD and Edmund Sabanegh, MD

Infertility is a stressful experience for many couples, a situation made particularly difficult by the need to produce a semen sample in a clinical setting. This is even more traumatic for men who are still in their reproductive years and diagnosed with cancer but desire to start a family. Cancer therapies can be significantly gonadotoxic, causing long-term deterioration in semen quality that often results in azoospermia. These patients are generally instructed to bank multiple specimens in a short period of time, before the start of cancer treatment, in order to have adequate numbers of sperm that can be used for procreation by assisted reproductive techniques. These patients sometimes travel great distances (from other cities or neighboring states) to find a sperm bank, take time off from work and incur significant travel expenses.

We set out to identify and develop a system that allows semen specimens to remain viable through a short transport cycle and to retain adequate viability prior to cryopreservation at our sperm bank. This system is of particular value for 1) men with cancer or underlying subfertility, 2) pre-vasectomy patients who may want to cryopreserve in advance of their vasectomy, 3) men with a desire to ensure potential future fertility and 4) military personnel going on long-term deployment.

Twenty-two samples were subjected to preliminary testing to optimize transport media, temperatures and most ideal kit constructions. Once the appropriate kit components were formalized, nine additional samples were subjected to overnight shipment and analyzed for motility, count and viability on the basis of normal semen analysis according to the World Health Organization (WHO) guidelines.

We standardized the transport media used in the kit as well as the proper cooling components necessary during overnight transportation that could take up to 24 hours and mimic a temperature of 37 degrees Celcius. Results were compared to determine the sustainability of media and establish an optimized cooling environment. Semen samples were analyzed before treatment (0 hour) to establish a baseline on which to compare the overnight shipping (24h). Average pre-shipment total motile sperm (TMS) was 59.46 million \pm 42.76, and post-shipment TMS was 26.97 million \pm 22.73. The percent recovery of TMS was 41.28 percent \pm 19.46 percent. Sperm motility and viability showed a decrease of approximately 50 percent during in

Key Point:

Results from our pilot study indicate that the NextGenTM kit is successful in maintaining sperm motility and viability in samples shipped from different parts of the country under prevailing temperature conditions. Results show that the NextGenTM kit provides a safe, reliable and convenient method for men to ship their samples to Cleveland Clinic from any part of the United States.

vitro incubation via overnight shipment. This decrease was consistent with the percentage change in motility seen in samples collected on site. Overnight shipment of sperm in transport media in the NextGenSM Home Banking Kit preserves sperm motility and viability for securing future fertility.

Cancer and infertile patients who live in the U.S. can easily ship semen samples for subsequent banking. Furthermore, the NextGenTM banking kit allows the patients the privacy of their home to collect the semen sample and make this experience convenient and less stressful.

More information can be obtained about the NextGenSM program by visiting clevelandclinic.org/nextgen.

Tel: 800-223-2273 or 866-9BANKIN

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Project 3: Proteomic Analysis of Differential Protein Expression in Mature and Immature Spermatozoa**Rationale/ Study hypothesis:**

Different sperm populations obtained from the same ejaculate have been shown to vary not only morphologically, but also functionally. Under physiological conditions free radicals are necessary for capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion. The free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) can act as important second messengers during signal transduction pathways and cascades that are involved in these functional parameters. When free radicals reach pathological levels it can induce lipid peroxidation, DNA damage and apoptosis of spermatozoa. Morphologically abnormal sperm produce increased levels of ROS and therefore significantly affect sperm parameters. Density gradient separation is commonly used technique in many ART programs. Sperm preparation techniques allow the separation of highly motile, morphologically normal spermatozoa.

Spermatozoa are terminally differentiated cells and both their protein content and composition is completed during the process of spermatogenesis and their time spent in the epididymis. It is hypothesized that a fully functional, mature, highly motile, morphologically normal and nonapoptotic spermatozoa capable of fertilization will have a different protein constitution where several proteins are differentially expressed compared to an immature, abnormal spermatozoa that is apoptotic and functionally incompetent.

Immature and mature spermatozoa and apoptotic versus nonapoptotic spermatozoa express proteins that are unique and responsible for their functional differences.

We will examine 2 specific aims:

Aim 1: Compare differentially expressed proteins between mature and immature spermatozoa and apoptotic versus nonapoptotic spermatozoa. Immature apoptotic

spermatozoa produce higher levels of ROS compared to mature nonapoptotic spermatozoa.

Aim 2: Relate the differences in functional parameters (such as motility, morphology, vitality and acrosome reaction) between immature and mature apoptotic versus nonapoptotic spermatozoa to the differences in major proteins as determined by proteomics.

Hypothesis	Methodology	Expected outcome
Immature apoptotic sperm express proteins that are unique and different from nonapoptotic mature spermatozoa.	<ol style="list-style-type: none"> 1. Separation of immature and mature sperm. 2. Measure ROS levels in mature and immature fraction. 3. Separate immature and mature into nonapoptotic and apoptotic sperm. Determine proteome of mature nonapoptotic and immature apoptotic spermatozoa by 2-DIGE, protein identification by LC-MS.	Protein content differs between mature nonapoptotic and immature apoptotic spermatozoa.
Different proteins affect the functional parameters in immature and mature spermatozoa.	<ol style="list-style-type: none"> 1. Steps 1-2 in aim 1. 2. Measure vitality, morphology and motility in immature and mature spermatozoa. 	Sperm quality is related to the extent of specific proteins expressed.

Significance of the study:

The results of this study may provide a better understanding of the role and effects of various proteins in spermatozoa.

Experimental Design and Methodology:

Following the approval of the study by the Institutional Review Board of the Cleveland Clinic, semen samples will be collected from healthy male volunteers. Measurement of volume, pH, cell concentration, motility and round cell count will be carried out manually as described in World Health Organization guidelines (WHO, 1999) before specific interventions will take place. Samples may be pooled. All specimens will be subjected to the following assays before and after centrifugation:

1. Sample collection

Semen samples will be collected from 20 donors and evaluated for infertility following a period of sexual abstinence of 48 – 72 hours. Collection will take place at the Andrology Laboratory of the Cleveland Clinic by masturbation into sterile containers. They will be allowed to liquefy completely for 15-30 minutes at 37°C before further processing. All samples will be discarded at the latest by the end of the experiment.

2. Standard semen analysis

Following liquefaction, semen specimens will be evaluated for:

1. Physical characteristics such as: Liquefaction time, semen age, split ejaculate.
2. Macroscopic characteristics such as: Physical appearance, color, viscosity, ejaculates volume and presence of round cells.
3. Microscopic characteristics such as: Sperm count, total sperm count, percent motility, velocity, vitality and sperm morphology.

A total amount of 5 µL of the sample will be used for manual as well as computer assisted semen analysis (CASA) evaluation of concentration and motility using a MicroCell counting chamber (Vitrolife, San Diego, CA).

3. Separation of immature and mature sperm

The sperm sample will be loaded onto a 47% and 90% density gradient and centrifuged at 300 g for 20 minutes. The immature spermatozoa will be retrieved from the resulting interface between the 47% and 90% layers and the mature spermatozoa aspirated from the 90% pellet, and transferred to separate test tubes. The pellets from both the fractions will be resuspended in sperm wash medium and the concentration adjusted to 2 million/mL.

4. Sperm vitality

Sperm vitality will be assessed in those samples that present with <30%. This will be done by using one-step eosin-nigrosin staining. At least 200 sperm will be scored per sample by 400x magnification. The percentage of dead (stained pink) and live (unstained) cells will be evaluated. This stain produces a dark background that provides contrast to the light colour of sperm. Normal live sperm do not take on the eosin-nigrosin stain and appear white (unstained), while dead sperm allows the dye to pass through the cell membrane and stain the nucleus pink.

5. Sperm morphology

Smears of the semen will be stained using a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology. The morphological abnormalities will be examined according to Kruger's strict criteria (WHO 5th edition, 2010).

6. Measurement of extracellular reactive oxygen species

ROS levels will be measured by the conventional chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis, MO). Luminol (10 μ L, 5 mM) prepared in dimethylsulphoxide (DMSO) will be added to 400 μ L of the sperm suspension. Negative controls will be prepared by adding 10 μ L of 5 mM luminol to 400 μ L of PBS. The chemiluminescent signal will be monitored for 15 min using a luminometer (Autolumat plus 953; Oakridge, TN) and results will be expressed as RLU/s/ $X10^6$ sperm.

7. Separation of apoptotic and non apoptotic spermatozoa

Apoptotic and non apoptotic spermatozoa can be separated by magnetic activated cell sorting (MACS) technique. During apoptosis, phosphatidyl serine residues are translocated from the inner membrane of the spermatozoa to the outside. Annexin V has a strong affinity for phosphatidyl serine but cannot pass the intact sperm membrane. Colloidal superparamagnetic beads (~ 50 nm in diameter) are conjugated to highly specific antibodies to annexin V and used to separate dead and apoptotic spermatozoa by magnetic activated cell sorting (MACS). Annexin V binding to spermatozoa indicates compromised sperm membrane integrity.

A 100 μ l aliquot of the sperm suspension isolated from the pellet (10 million total spermatozoa) is mixed with 100 μ l of MACS Microbeads (ANMB microbeads) and incubated at room temperature for 15 min. This mixture is loaded on top of the separation column placed in the magnetic field (0.5 Tesla (T) between the poles of the magnet and 1.5 T within the iron globes of the column). The column is rinsed with buffer. All unlabelled (annexin V-negative non apoptotic) sperm pass through the column and all annexin positive (apoptotic) sperm are retained in the column. The column is removed from the magnetic field. The annexin V-positive fraction will be eluted using the annexin V-binding buffer.

$$\text{Sperm recovery} = \frac{\text{number of spermatozoa after separation}}{\text{number of spermatozoa before separation}} \times 100$$

8. Determination of Protein content by proteomics

Immature and mature fractions and non-apoptotic and apoptotic immature and mature sperm fractions will be frozen till ready for preparation for proteomic analysis. Samples will be prepared and submitted to the proteomic core lab for protein identification. Proteomic content will be measured using 2-Dimensional gel electrophoresis followed by identification of proteins using the LC-MS system, Finnigan LTQ linear ion trap mass spectrometer and HPLC (Jupiter C18 reversed-phase capillary chromatography) column for online separation of peptide. Quantitative comparison using normalized spectral count ratio of Immature /apoptotic and mature /nonapoptotic spermatozoa will be conducted along with 2-dimensional differential in-gel electrophoresis (2-DIGE). Spermatozoa will be labelled with Cy3/ Cy5 fluorescent CyDye. Cy2-labeled internal standard (IS) will be prepared by pooling 50 µg of each sample and labelling with Cy2. Duplicate 2-DIGE gels with sperm samples, will be dye swapped, and run to provide more reliable data and allow for statistical analysis. Image analysis will be performed using DeCyder software.

9. Statistical analysis

This is a pilot study where the results will be compared with the control and test aliquots for each parameter. A 20% difference in the ROS production and differences in sperm motility, morphology and other parameters may be statistically and clinically meaningful. Therefore, we will enroll at least 10 samples to detect significant changes in these parameters.

All data will be expressed as mean \pm SEM. Student's *t*-test or One-way analysis of variance (ANOVA) (with Bonferroni post hoc test if $p < 0.05$) will used for statistical analysis. Differences will be regarded statistically significant if $p < 0.05$.

Figure 1. Flow Diagram showing Separation of Immature and Mature Spermatozoa Fractions for Protein Identification

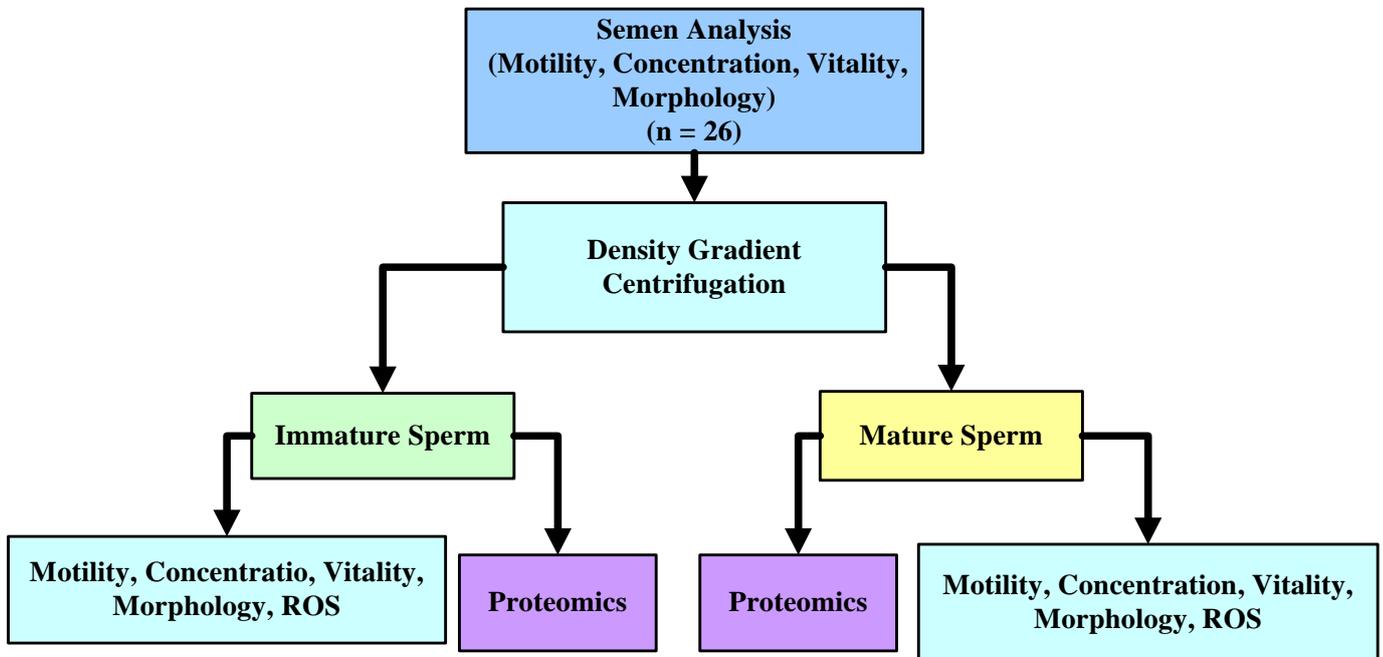
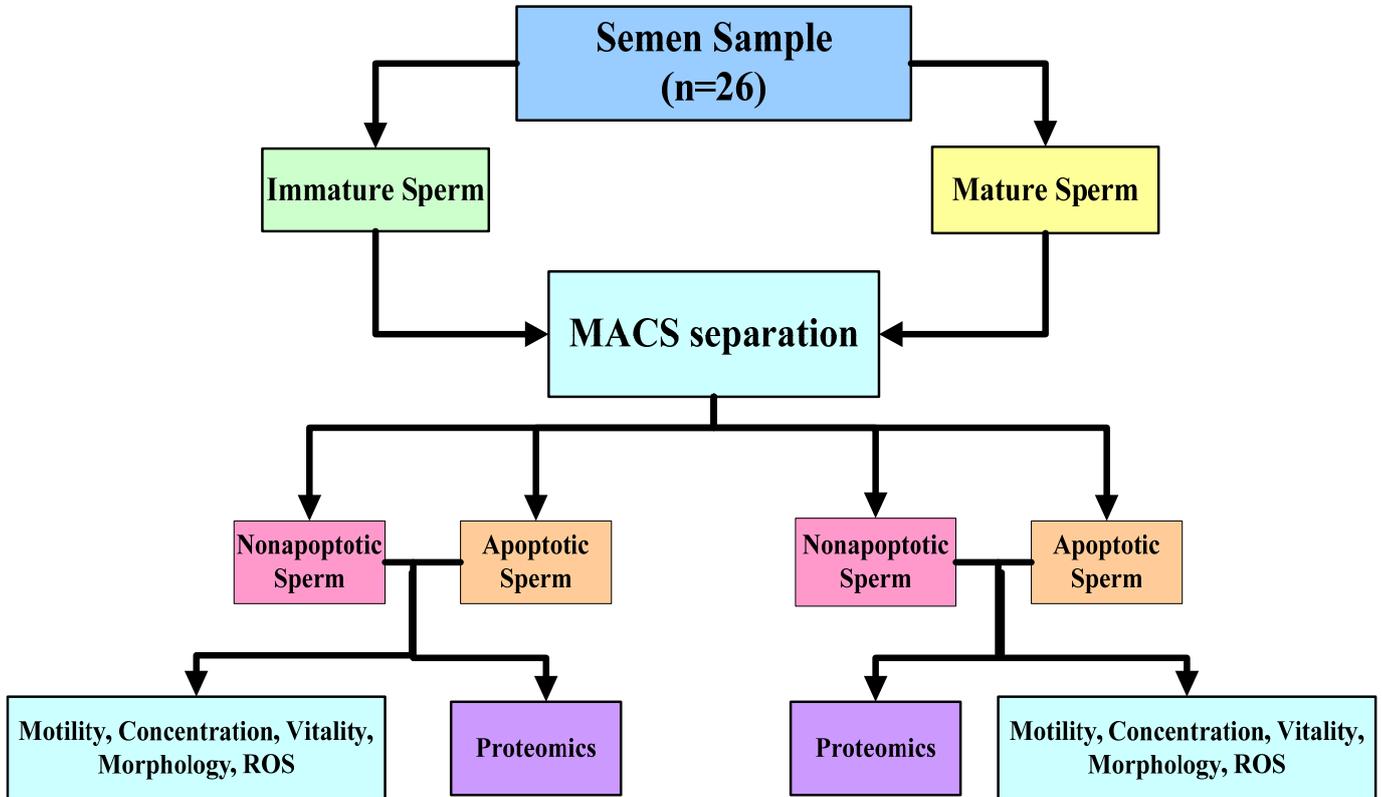


Figure 2. Separation of Non-Apoptotic and Apoptotic Spermatozoa for Protein Identification



Time Line: 5 interns

7 weeks (Wed and Thursday)

Weeks 1 and 2:

Training in different techniques required for the project.

Weeks 3 - 6:

1. Sperm motility, vitality, morphology measurement – **(Interns 1 - 3)**
2. Measurement of reactive oxygen species - **(Intern 4)**
3. Separation of immature and mature sperm - **(Intern 5)**
4. Separation of nonapoptotic and apoptotic sperm - **(2 interns)**

Week 7: Preparation of samples for proteomic study – **(2 interns)**

5. Data entry **(2 interns)**
6. PowerPoint presentation: Highlights of the study

Materials needed:

A. Equipment

1. Centrifuge
2. Vortex
3. Incubator
4. Microscope
5. MACS separator
6. Luminometer
7. Slide boxes
8. Cryoboxes
9. Cryomarkers

B. Reagents /chemicals

1. Donors
2. Density gradients (Upper and lower phase)
3. Sperm wash medium (HTF)
4. Phosphate buffer saline (PBS)
5. Dimethyl sulfoxide (DMSO)
6. Luminol stock solution
7. Hydrogen peroxide
8. Diff Quick stain

9. MACS annexin kit
10. Eosin stain
11. Nigrosin stain

C. Disposables

1. Pipettes (1-10 μ L, 10-100 μ L, 100-1000 μ L)
2. Tips (1-10 μ L, 10-100 μ L, 100-1000 μ L)
3. Serological pipettes (2 and 5 mL)
4. Plastic tubes (5 mL and 15 mL centrifuge tubes)
5. MicroCell slides
6. Frosted and plain slides
7. Coverslips
8. Transfer pipettes
9. Gloves

10. Training Checklist

No.	Test	No of Readings (N)	Acceptable Result	Data Entry
1.	Sperm Count	10	<20% difference	Excel
2.	Sperm Motility	10	<20% difference	Excel
3.	Vitality	10	± 2 SD <	Excel
4.	Morphology	10	± 2 SD	Excel
5.	ROS	4	± 2 SD	Excel
6.	Separation of immature and mature sperm	4	Check with mentor for % motility, TMS and % recovery	
7.	Separation of nonapoptotic and apoptotic sperm	3	Check with mentor for percent motility	
8.	TUNEL	3	Check with mentor for correct preparation and labeling of samples	
9.	Preparation of samples for Proteomics	Check with mentor for correct preparation and labeling of samples		

Note:

All raw data must be entered in the lab note book.

All results will be verified by the mentor.

Results should be daily entered into the Excel sheet.

11. Sample Size Calculation and Statistical Methodology:

The mean differences among the sperm divided first by immature versus mature, then by nonapoptotic versus apoptotic, will be assessed with respect to motility, concentration, vitality, morphology, and ROS using confidence intervals based on corresponding paired T-tests among the four resulting sperm groups. Log transformations of the original measurements may be necessary for some parameters to achieve approximate normality, and this is strongly anticipated for ROS. The individual confidence levels will be set to 99.1% in order to achieve an overall confidence level of 95% by Bonferroni correction among six pair wise group comparisons. For

each of the confidence intervals comparing a pair of groups, the study sample size of 26 subjects will provide 90% power to detect a 75% increase in the mean ROS levels, assuming a log-normal distribution for ROS and a coefficient of variation no more than 50%.

Study Budget

	Quantity	Cost/unit (\$)	Total Cost (\$)
Laboratory reagents			\$858.94
Sperm Wash HTF 100 mL	1 x 100 mL	\$30.40 ea	\$30.40
Phosphate Buffer 500 mL: without calcium and magnesium, ph 6.8	1 x 500 mL	\$25.94 ea	\$25.94
Dimethyl Sulfoxide 100 mL	1 x 100 mL	\$119.50/ btl	\$119.50
Luminol 5G	1 x 5G	\$51.80	\$51.80
Density gradient			
Upper layer	1x100 mL	\$215.65	\$215.65
Lower layer	1x100 mL	\$215.65	\$215.65
MACS kit	\$200.00/ kit	\$200.00	\$200.00
Proteomic analysis – Core Lab			\$2,000.00
2-dimensional gel charges (patients and donors)	\$50.00/gel run	20 x \$50.00	1,000.00
Mass spectroscopy identification	\$100.00/gel	20 x \$100.00	1,000.00
Miscellaneous laboratory supplies			\$600.00
Pipette tips, serological pipettes			
Transfer pipettes			
Tubes 12 X 75 mm			
Eppendorff microfuge tubes			
Falcon tubes (15ml graduated)			
MicroCell slides			
Frosted slides, plain slides, cover slips			
Cryo Tubes			
MACS Columns			
Eosin			
Nigrosin			
Donor payment	20x \$50/donor		\$1,000.00
Biostatistician charges	\$70/h X 10h		\$700.00
		Total	\$5,158.94

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Project 4: Evaluation of Semen Quality following Preparation of Human Semen Specimens for ART: A Controlled Trial**Rationale/ Study hypothesis:**

Approximately 2% to 4% of births in developed countries involve the use of assisted reproductive techniques (ART). With ART, semen samples must first be processed before they can be used for insemination. Specifically, sperm preparation methods seek to replicate *in vitro* the natural process in which viable sperm are separated from other constituents of the ejaculate as they actively migrate through the cervical mucus.

During processing, viable sperm cells are first separated from other constituents of the ejaculate as early as possible to limit damage from leukocytes and other cells present in the semen. Various sperm separation or isolation methods exist to select sperm cells. These include swim-up methods, two-layer discontinuous gradient centrifugation, pentoxifylline wash, test-yolk buffer, sedimentation methods, and polyvinylpyrrolidone (PVP) droplet swim-out, electrophoresis and fluorescence cell sorting methods.

Density gradient separation and the swim up are the two commonly used sperm preparation methods. The density gradient method involves the use of two gradients. The highly motile, morphologically normal, viable spermatozoa form a pellet at the bottom of the tube. Centrifugal force (<300g) and time are kept at the lowest possible values in order to minimize the production of ROS by leukocytes and non-viable sperm cells. Also, non-viable spermatozoa and debris are separated from viable sperm cells as soon as possible to minimize oxidative damage. Media containing silane-coated silica particles are commonly used. Isolate™ (Irvine Scientific, Santa Ana, CA), IxaPrep™, Sperm preparation medium™ and Suprasperm™ (Origio, MediCult, Copenhagen, Denmark), Enhance-S-PLUS™ (Conception Technologies, San Diego, CA), SilSelect™ (FertiPro N.V., Beernem, Belgium) and PureSperm™ (NidaCon Laboratories AB, Gothenburg, Sweden) are commonly used.

Density gradients can either be continuous or discontinuous. Density gradually increases from the top of a continuous gradient to its bottom. There are clear boundaries between layers of discontinuous gradients. Double density gradients (DDG) comprise the commonly used sperm preparation protocol for ART. Although the recovery of highly motile sperm is improved, there are concerns that sperm prepared by density gradient separation still exhibit sperm DNA damage. Compromised sperm showing DNA damage may affect the fertilization and post fertilizations steps. Therefore it is critical to have sperm separation techniques and media that allow the separation of highly motile sperm with minimal DNA damage.

We will examine 2 specific aims:

Aim 1: Evaluate semen quality (% motility, % morphology, total motile sperm (TMS), % recovery, ROS levels, and DNA damage) following sperm preparation by 3 different commercially available density gradient media. We will use the gradient currently used in the Cleveland Clinic’s Andrology lab and test it alongside 2 other similar products in the market.

Aim 2: Evaluate the 3 gradients in terms of their recovery and the extent of DNA damage.

Hypothesis	Methodology	Expected outcome
Total motile sperm and recovery rates are different in the 3 selected density gradients.	<ol style="list-style-type: none"> 1. Complete liquefaction of semen sample. 2. Perform sperm count, percent motility, sperm vitality and sperm morphology. 3. Perform density gradient separation using 3 gradients. 	Determine the most suitable gradient in terms of sperm quality, i.e. total motile sperm, and recovery rates.
Highly motile sperm recovered from the 3 gradients have varying amount of DNA damage.	Measure DNA damage in the highly motile spermatozoa fraction.	Spermatozoa prepared from the gradient with least amount of DNA damage is the gradient of choice.

Significance of the study:

The results of this study will allow us 1) in identifying and validating the performance of some of the popular sperm density gradients in the market and 2) in selecting the best DDG media for sperm preparation protocol used daily for our IUI patients.

Experimental Design and Methodology:

Following the approval of the study by the Institutional Review Board of the Cleveland Clinic, semen samples will be collected from healthy male volunteers. Measurement of volume, pH, cell concentration, motility and round cell count will be carried out manually as described in World Health Organization guidelines (WHO, 1999). 20 semen specimens from unproven donors will be examined before and after separation on 3 different sperm density gradients for total motile sperm, percent recovery and extent of DNA damage. Samples may be pooled if necessary to give adequate number of sperm for separation on the 3 gradients.

1. Sample collection

Semen samples will be collected from 20 donors and evaluated for infertility following a period of sexual abstinence of 48 – 72 hours. Collection will take place at the Andrology Laboratory of the Cleveland Clinic by masturbation into sterile containers. They will be allowed to liquefy completely for 15-30 minutes at 37°C before further processing. All samples will be discarded at the latest by the end of the experiment.

2. Standard semen analysis

Following liquefaction, semen specimens will be evaluated for:

1. Physical characteristics such as:

Liquefaction time, semen age, split ejaculate,

2. Macroscopic characteristics such as:

Physical appearance, color, viscosity, ejaculates volume and presence of round cells.

3. Microscopic characteristics such as:

Sperm count, total sperm count, percent motility, velocity, vitality and sperm morphology.

A total amount of 5 μ L of the sample will be used for manual as well as computer assisted semen analysis (CASA) evaluation of concentration and motility using a MicroCell counting chamber (Vitrolife, San Diego, CA).

3. Sperm vitality

Sperm vitality will be assessed in those samples that present with <30%. This will be done by using one-step eosin-nigrosin staining. At least 200 sperm will be scored per sample by 400x magnification. The percentage of dead (colored pink) and live (unstained) cells will be evaluated. This stain produces a dark background that provides contrast to the light color of sperm. Normal live sperm do not take on the eosin-nigrosin stain and appear white (unstained), while dead sperm allows the dye to pass through the cell membrane and stain the nucleus pink.

4. Sperm morphology

Smears of the semen will be stained using a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology. The morphological abnormalities will be examined according to Kruger's strict criteria.

5. Sperm preparation by density gradient

Components of the density gradient sperm separation procedure include a colloidal suspension of silica particles stabilized with covalently bonded hydrophilic silane supplied in HEPES. There are two gradients: a lower phase (High density gradient) and an upper phase (low density gradient). Sperm washing medium (Modified HTF with 5.0 mg/mL human albumin) is used to wash and resuspend the final pellet.

Below are some of the main steps of the process:

1. Place all components of the upper and lower phase and semen samples in an incubator at 37°C for 20 minutes.
2. Transfer 2 mL of the lower phase into a sterile conical-bottom, disposable centrifuge tube.
3. Layer 2 mL of the upper phase on top of the lower phase using a transfer pipette. Slowly dispense the upper phase lifting the pipette up the side of the tube as the level of the upper phase rises. A distinct line separating the two layers will be observed. This two-layer gradient is stable for up to two hours.
4. Measure semen volume to be loaded using a sterile 2 mL pipette. Remove a drop of semen using sterile technique for count, percent motility and presence of round cells.

5. Gently place up to 3 mL of liquefied semen onto the upper phase (leaving approximately 0.1 mL in original container for a prewash analysis). If volume is greater than 3 mL, it may be necessary to split the specimen into two tubes before processing.
6. Centrifuge for 20 minutes at 1600 rpm.

Note: Occasionally, samples that do not liquefy properly and remain too viscous to pass through the gradient will be encountered. Increasing the centrifugal force up to but no more than 600Xg will aid in separating the sperm in these cases.

1. Using a transfer pipette, add 2mL of HTF and resuspend pellet. Mix gently with pipette until sperm pellet is in suspension.
2. Centrifuge for 7 minutes at 1600 rpm.
3. Again, remove supernatant from the centrifuge tube using a transfer pipette down to the pellet.
4. Resuspend the final pellet in a volume of 0.5 mL using a 1 mL sterile pipette with HTF. Record the final volume. Calculate the total motile sperm and percent recovery in each gradient.

Tips to maximize the sperm yield:

1. It is important to make sure that all components of the gradient and sperm wash medium are at room to body temperature before use. This will protect spermatozoa from “cold shock.” In addition, any condensation on the media bottles will disappear, which aids in the visual detection of contamination. Any bottle whose contents appear in any way cloudy or hazy should not be used.
2. Do not use the same pipette in more than one bottle of media.
3. Prolonged exposure to a 5% CO₂ environment will alter the pH of these products, which may in turn affect their nature and performance.
4. Highly viscous semen usually should be treated with 5 mg of trypsin, dissolved in 1.0 mL of sperm washing media and added to the ejaculate 5 minutes before loading on the upper gradient. This will increase the motile sperm yield without causing any measurable damage to the motile sperm.
5. Avoid overloading the gradient as it causes a phenomenon called ‘rafting’. Rafting is the aggregation of desirable as well as undesirable components of the semen that will be present in the post-centrifugation pellet.
6. Use the gradient within one hour after creating it - eventually the two phases over time blend into each other and a sharp interface will not exist.

6. Measurement of sperm DNA damage

Sperm will be washed twice in PBS, resuspended in 1-3% paraformaldehyde at a concentration of $2-4 \times 10^6$ cells/mL and placed on ice for 15 to 30 minutes. Sperm samples will be then washed and resuspended in 70% ice-cold ethanol by centrifugation at X300g for 5 minutes. The ethanol supernatant will be removed, and the sperm pellets will be washed twice in wash buffer. The samples will be resuspended in 100 μ L of the staining solution for 1 hour at room temperature in dark. All cells will be washed using rinse buffer, resuspended in 250 μ L and incubated for 30 minutes in the dark on ice for flow cytometry measurements.

Terminal deoxynucleotidyltransferase (Tdt) catalyzes a template-independent addition of brominated deoxyuridine triphosphatase to the 3'-hydroxyl (OH) termini of double and single stranded DNA. Sperm DNA strand breaks will be evaluated using a flow cytometric terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct, BD Biosciences).

A total of 10,000 cells will be examined for each assay at a flow rate of 100 cells/second. The FITC (log green fluorescence) will be measured on FL1 channel. Data acquisition will be performed within 30 min on a flow cytometer equipped with a 515-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). Data will be processed using Flow Jo v4.4.4 software (Tree Star Inc., Ashland, OR).

7. Training Checklist

No.	Test	No of Readings (N)	Acceptable Result	Data Entry
1.	Sperm Count	10	<20% difference	Excel
2.	Sperm Motility	10	<20% difference	Excel
3.	Morphology	10	± 2 SD	Excel
4.	ROS	4	± 2 SD	Excel
5.	Separation of immature and mature sperm on 3 media	4	Check with mentor for % motility, TMS and % recovery	
6.	TUNEL	3	Check with mentor for correct preparation and labeling of samples	

Note:

All raw data must be entered in the lab note book
 All results will be verified by the mentor
 Results should be daily entered into the Excel sheet

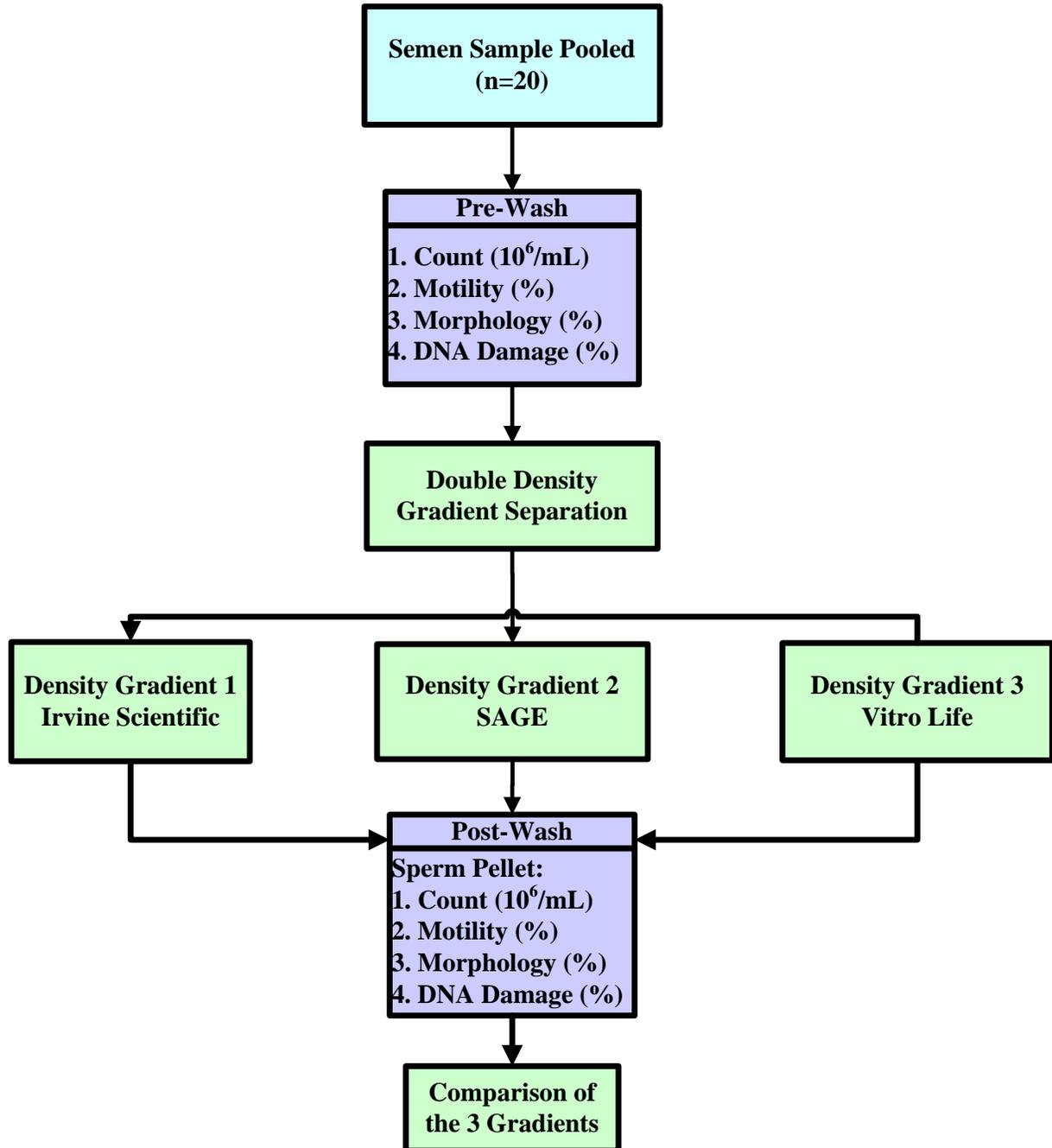
8. Sample Size Calculation and Statistical Methodology:

The mean differences between samples assessed with the three different density gradients with respect to count, motility, morphology, and %DNA damage will be assessed using confidence intervals based on corresponding paired T-tests among the gradients. Log transformations of the original measurements may be necessary for some parameters to achieve approximate normality. The individual confidence levels will be set to 98.3% in order to achieve an overall confidence level of 95% by Bonferroni correction. For each of the confidence intervals comparing a pair of density gradients, the study sample size of 20 subjects will provide 90% power to detect a difference of 12% with respect to the mean post-wash change in %DNA damage, assuming the standard deviation of differences between sampling methods with respect to the post-wash change in %DNA damage is no more than 10%. This standard deviation assumption will be assessed when complete data is available on 10 subjects, as will the variability among other sperm parameters, in order to ensure that the final analyses will be carried out with high power to detect possible differences among the gradient methods.

Study Budget

Lab Supplies and Reagents	Cost/unit (\$)	Quantity	Total Cost (\$)
Donors	\$50.00	20	\$1,000.00
Density Gradient (SAGE)			
Upper Phase	1x100 mL	\$215.65	\$215.65
Lower Phase	1x100 mL	\$215.65	\$215.65
Density Gradient (Irvine Scientific)			
Upper + Phase Lower Phase	1x100 mL	\$553.00	\$553.00
Density Gradient (Vitro Life)	1x 100 mL	\$270.00	\$270.00
Sperm Rinse	4 X 30 mL	49.00	\$196.00
TUNEL kit	\$395.00	1	\$395.00
Sperm Wash HTF 100 mL	1 x 100 mL	\$30.40 ea	\$30.40
Statistical charges (\$70.00/hr) X 10h			\$700.00
Miscellaneous Disposable/Laboratory Supplies			\$1,000.00
Disposable Gloves			
Micropipette Tips (5, 10, 1000 microliters)			
Serological Pipettes (1, 2, 5 mL)			
MicroCell slides			
Centrifuge tubes (15 mL)			
Round bottom tubes (7 mL)			
Transfer pipettes			
Transfer pipettes			
Eosin			
Nigrosin			
		Total Cost	\$4,575.00

Figure 1. Flow Diagram Comparing Semen Parameters Utilizing 3 Different Sperm Preparation Media



Time Line: 4-5 interns

7 weeks (Wed and Thursday)

Weeks 1 and 2:

Training in different techniques required for the project.

Weeks 3 - 6:

1. Sperm motility, vitality, morphology measurement – **(Interns 1 - 3)**
2. Separation of immature and mature sperm - **(Intern 4-5)**
3. Measurement of total motile sperm, percent recovery **(Intern 4-5)**
4. Measurement of DNA damage **(Dr. Sharma)**
5. Data entry **(2 interns)**
6. PowerPoint presentation: Highlights of the study

Materials needed:

A. Equipment

1. Centrifuge
2. Vortex
3. Incubator
4. Microscope
5. Luminometer
6. Slide boxes

B. Reagents /chemicals

1. Donors
2. Sperm wash medium (HTF)
3. Density Gradient medium **(Sage)**
4. Upper phase
5. Lower phase
6. Density Gradient medium **(Vitrolife)**
 - i. Upper phase
 - ii. Lower phase
7. Density Gradient medium **(Irvine Scientific)**
 - i. Upper phase
 - ii. Lower phase
8. Phosphate buffer saline (PBS)
9. Dimethyl sulfoxide (DMSO)
10. Luminol stock solution
11. Hydrogen peroxide
12. Diff Quick stain
13. TUNEL kit

C. Disposables

1. Pipettes (1-10 μ L, 10-100 μ L, 100-1000 μ L)

2. Tips (1-10 μ L, 10-100 μ L, 100-1000 μ L)
3. Serological pipettes (2 and 5 mL)
4. Plastic tubes (5 mL and 15 mL centrifuge tubes)
5. MicroCell slides
6. Frosted and plain slides
7. Coverslips
8. Transfer pipettes
9. Gloves

Rakesh Sharma, Ph.D.

Project Staff & Coordinator
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1. AGEING AND REPRODUCTIVE HEALTH

Introduction/ Background:

The effects of lifestyle and ageing on human health and wellbeing, particularly on cardiovascular and reproductive function is fast becoming critical in clinical practice and the management of the infertile couple in the developed world.

The developing world has its own problems of poor reproductive outcomes including high rates of miscarriage and congenital abnormalities linked to poor nutritional standards and to environmental breakdown. The rapid expansion in research aimed at solving fertility problems in an increasingly ageing, obese and generally unfit population can therefore also be applied to those who live in impoverished conditions that erode reproductive health. Understanding scientific and social issues arising from lifestyle choices associated with ageing, cardiovascular disease, and obesity and acquired STDs is important and how these can impact or influence reproductive function and infertility management. It is important to understand how poor nutrition and lifestyle choices can affect overall health and reproductive function.

Significance:

Fast paced lifestyles, advancing careers, poor eating habits, and sedentary lifestyle can cause physical, emotional stress and significantly affect young couples in reproductive years who are attempting to raise families.

Outline of the planned article:

- Occupational exposure
- Use of lubricants

- Exposure to pesticides
- Exposure to endocrine disruptors
- Eating healthy
- Smoking and drugs. Avoid cigarettes and any drugs that may affect sperm count or reduce sexual function.
- Weight management, obesity may be associated with infertility.
- Get sufficient rest, and exercise moderately but regularly. (Excessive exercise can impair fertility.)
- Stress management, stress may contribute to reduced sperm quality.
- Wearing tight underwear and pants
- Avoid hot baths, showers, and steam rooms
- Future strategies/ directions
- Conclusions

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Urologists, Gynecologists, REIs, primary care physicians

Suggested literature sources: Pub med, Science Direct

Deadline: Aug 15, 2012

2. AGING SPERMATOZOA AND MALE INFERTILITY

Introduction/ Background:

Age influences semen quality. Comparing 30 year-old men to 50-year, reduced semen quality was seen, notably a decline in volume (3-22%); motility (3-37%); morphology (4-18%). Male age may also affect miscarriage rate. Link between older men and pregnancy loss has been reported when the father was over the age of 35. Miscarriage rates were nearly 17% if the father was over 34 years old; around 20% if the father was between the ages of 35 and 39 and over 32% if the father was older than 44 yr.

We will address the following: 1) How age affects spermatogenesis 2) Role of declining testosterone concentrations and the male biological clock 3) Identify the link between male biological clock and pregnancy outcomes and subsequent developmental effects in children.

Significance:

Advanced paternal age may affect functional quality of sperm and pregnancy outcome.

Outline of the planned article:

- Effects of age on sperm
- Decrease of sperm parameters with age
- Decrease of male hormones with age
- Histological changes in testes
- DNA damage (both mitochondrial and nuclear)
- Telomere shortening
- Apoptosis
- Genetic syndrome with advanced age
- 47XXY syndrome
- Aneuploidy
- Down's syndrome
- Schizophrenia
- Numerical and structural abnormalities
- Fetal anomalies
- Future strategies/ directions
- Conclusions

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Andrologists, Male infertility specialists

Journal: Review article (TBD)

Deadline: Aug 15, 2012

3. SPINDLE AND CHROMOSOMAL ALTERATIONS IN METAPHASE II OOCYTE

Introduction/ Background:

The meiotic spindle is a crucial structure to oocyte development during meiosis I and meiosis II that results in a metaphase II oocyte that has the potential to be fertilized. Proper spindle formation and function are required for normal chromosome alignment and segregation of maternal chromosomes. Alterations in the spindle structure during normal meiotic process can be caused by excess oxidative stress and cryopreservation. Compromised oocytes are at increased risk for a variety of chromosomal abnormalities, such as aneuploidy, and numerous diseases due to improper chromosomal segregation. This article will highlight factors that contribute to spindle damage and elucidate current methods of dealing with the resulting problems of this damage.

Significance:

Alterations in the spindle structure can affect subsequent quality. Effect of spindle alterations on pregnancy outcome is not clear.

Outline of the planned article:

This article is already written, but it needs some revision and editing.

- Overview of oogenesis and follicular growth
- Meiotic progression
- Spindle structure, composition, and function
- Factors affecting spindle structure
- Effect of oxidative stress on spindle damage and resulting consequences
- Oxidative Stress and Its Implications for ART
- Clinical implications in spindle damage
- Oxidative stress and *in vitro* maturation
- Minimizing spindle damage
- Spindle location and relevance to the 1st polar body

- Conclusions

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Gynecologists, Embryologists, Reproductive Endocrinologists

Journal: Review article (TBD)

Deadline: August 15, 2012

4. SPERMATOGENESIS

Introduction/ Background:

Spermatogenesis is the most extensive series of events that results in the transformation of a diploid non-differentiated cell into a highly specialized fully functional, haploid spermatozoon that is designed to transfer the genetic information. This chapter will highlight the hypothalamic and pituitary axis, hormonal regulation and the neurological pathway. Various steps involved in the reproductive tract, including cellular processes and transformations that occur during spermatogenesis will be examined to provide a clear understanding of the most complex cellular metamorphosis that occurs in the human body. The overview also includes information on many cyto- and microanatomical structures, the regulatory processes, and the fertilization process.

Significance:

It is critical to understand how the various steps in spermatogenesis occur, its importance and how alterations, abnormalities during the process of spermatogenesis can result in compromised sperm quality.

Outline of the planned article:

- Organization of the testis
- Structure of the seminiferous tubule
- Spermatogenesis

- Hormonal control of spermatogenesis
- Efficiency of spermatogenesis
- Regulation of spermatogenesis
- Intrinsic regulation
- Extrinsic influences
- Disturbances of spermatogenesis
- Sperm transport
- Duration of spermatogenesis
- Sperm entry into cervical mucus
- Capacitation
- Acrosome reaction
- Fertilization
- Concluding remarks

Literature review: Resources of Cleveland Clinic Alumni Library

Book chapter: Physiology of Male Gametogenesis. In: Clinical Reproductive Medicine and Surgery, (Editors: Falcone T and WW. Hurd), Elsevier, 2nd edition

Deadline: August 1, 2012

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5. OVARIAN ENDOMETRIOMA: EFFECTS ON ART OUTCOME

Introduction/ Background:

Endometriosis is classified as an estrogen-dependant, benign gynecological disease in which endometrial cells and stromal tissue reside in anatomical locations outside of the uterus. However, there are no well-designed RCT's that have specifically assessed the effect of ovarian endometrioma treatment on IVF outcomes (for obvious ethical reasons). As a result, there is no consensus or standard as to which treatment option is the most effective.

In this chapter, the specific focus will be on ovarian endometrioma, a subcategory of this complex disorder in which the misplaced endometrial tissue is present in one or both ovaries. These endometriotic implants have been conventionally termed “chocolate cysts” because they are lined with endometrial connective tissue as opposed to true ovarian cysts, which are insulated with epithelial cells. Thus, these pseudo cysts are filled with old blood contents that may be brown in color. The symptoms of endometriosis include dysmenorrhea, chronic pelvic pain, dyspareunia, and infertility. However, symptomatology and imaging such as transvaginal sonography are not sufficient to diagnose endometriosis. While many controversies exist as to the best way to treat endometriosis, there is consensus that the only way to diagnose endometriosis definitively is through laparoscopic surgery and biopsy. We will discuss the treatments that are available to minimize the disease before an IVF cycle is started and the effect those treatments have on IVF outcomes in women with ovarian endometriomas in one or both ovaries.

Significance:

Infertility is closely associated with ovarian endometrioma. Currently, there is not a cure for ovarian endometrioma and this disorder continues to afflict women with symptoms of chronic pelvic pain, dysmenorrhea, menorrhagia, dyspareunia, dysuria, dyschezia, and infertility. 17-44% of women with endometriosis have ovarian endometriomas and endometriosis is present in approximately 30% of women suffering from unknown subfertility. Because endometriosis affects women of reproductive age, many women turn to IVF/ICSI to achieve pregnancy. Although there are many medical therapies and surgical techniques that can be used to manage the disease before an IVF or ICSI cycle is started, there is no consensus or standard for the most effective treatment option. After an extensive review of the literature, we recommend that multiple factors be considered when creating an advantageous plan of action.

Outline of the planned article:

- Introduction
- Pathogenesis of endometriosis
- Ovarian endometrioma: prevalence and complications
- Effects of ovarian stimulation in women with endometrioma
- Impact of endometriosis on IVF outcomes
- Surgical techniques: an Overview
- Aspiration
- Combined surgical techniques
- Cystectomy
- Loss of ovarian reserve with surgery for endometrioma
- Predictors of ovarian response subsequent to endometrioma surgery
- Nonintervention for ovarian endometrioma and responsiveness to ovarian hyperstimulation
- Benefits and disadvantages of surgery for endometriomas
- Role of fertility preservation with surgical interventions
- Conclusions

- Future directions and studies

Literature review: Preliminary literature search has been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive medicine, assisted reproduction, infertility, gynecologists and family practitioners.

Journal: Expert Reviews of Obstetrics and Gynecology – Invited Review

Deadline: Aug 1, 2012

6. ENDOMETRIOSIS AND OVARIAN CARCINOMA: UNDERSTANDING THE COMMON MECHANISTIC PATHWAYS

Introduction/ Background:

Endometriosis and EAOC have several common risk factors such as early menarche, more frequent periods, low parity, infertility, late menopause, hormonal factors, persistent inflammatory status, immunological dysregulation, genetics, and exposure to environmental agents. The risk of developing ovarian cancer among women with endometriosis was also evaluated. An endometrioma diameter of 9 cm or more and postmenopausal status is independent predictive factor in patients for developing EAOC.

Significance:

Endometriosis is a condition which commonly causes pelvic pain and infertility. It affects 5-15% of women of reproductive age and current estimates suggest that more than 5.5 million women in the US have the disease. Recent studies have provided much evidence to support that endometriosis is actually a neoplasm that is related to ovarian cancer. These carcinomas have been termed as endometriosis associated ovarian cancer (EAOC). The histologic subtypes of EAOC are clear cell carcinomas (CCC) (40-55%), endometrioid carcinomas (EAC) (20-40%), and less than 10% of them are serous and mucinous subtypes. This chapter will discuss the

evidence that suggests a correlation between endometriosis and EAO and analyze how the body's own reaction may exacerbate the transition.

Outline of the planned article:

- Introduction
- How do we know endometriosis associated cancer (EAO) exists?
- Physical factors (Risk factors)
- Pathogenesis of endometriosis: various theories
- Screening for ovarian cancer
- Epidemiology of endometriosis and ovarian cancer
- SCSOCS
- Inflammation and the immune system in endometriosis
- Preventive measures against inflammation in endometriosis:
- The immune system, endometriosis and ovarian cancer - overall connection:
- Histology and genetics
- PTEN
- Loss of heterozygosity
- Epigenetics
- Transcriptomic profiling
- Proteomic profiling
- Conclusions
- Future directions and Research

Literature review: Preliminary literature search has been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive medicine, assisted reproduction, infertility, Gynecologists and Family practitioners.

Journal: TBD

Deadline: Sep 1, 2012

7. LIFE STYLE FACTORS AND OXIDATIVE STRESS IN FEMALE REPRODUCTION: IS THERE AN EVIDENCE BASE TO SUPPORT THE LINKAGE

Introduction/ Background:

Optimal amounts of reactive oxygen species (ROS) are necessary for physiological functioning. Physical activity causes an increase in ROS, which in turn heightens antioxidant response, thus providing protection from future attacks. The 21st century has been burdened with a sharp increase in the use of several substances of abuse. This problem significantly affects the younger generations, which encompass the female reproductive years. Cigarette smoking, alcohol use, and recreational drug use have been implicated in the pathogenesis of perturbed female reproductive mechanisms, leading to increased times to conception and infertility. Even moderate alcohol use during pregnancy can result in IUGR and LBW, and increase the risk for congenital anomalies. Physical exercise produces an oxidative state due to excessive ROS generation. Any type of extreme aerobic or anaerobic activity (i.e. marathon running, weight training) may contribute to cellular damage.

Significance:

A person's time to pregnancy and their chance of having a healthy, live birth may be affected by factors such as weight, vitamin and iodine intake, alcohol and caffeine consumption, smoking, substance abuse, stress, environmental pollutants, vaccinations and oxidative stress.

Outline of the planned article:

- Introduction
- Free radical generation - homeostasis and generation of oxidative stress
- Redox pathways in the control of physiological events in female reproduction
- Reactive oxygen species (ROS) - induced programmed cell death in gametes and embryos
- Antioxidants: Exogenous and endogenous
- Regulatory role of ROS in the endometrial cycle

- Role of oxidative stress in pregnancy and associated complications
- Early pregnancy loss
- Recurrent pregnancy loss
- The effect of paternal factors on pregnancy outcomes
- Preeclampsia
- Hydatiform mole
- Role of oxidative stress in female infertility
- Lifestyle and environmental Factors
- Obesity/ overnutrition
- Underweight/ malnutrition
- Exercise
- Cigarette smoking
- Alcohol use
- Recreational drug use
- Link between oxidative stress and lifestyle factors
- Environmental and occupational exposure
- Organochlorine pesticides (OCPs)
- Polychlorinated biphenyls (PCBs)
- Organophosphate pesticides (OPCs)
- Impact of lifestyle factors on assisted reproductive techniques
- IVF/ICSI
- IVM
- Conclusions
- Future research and recommendations

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive medicine, assisted reproduction, infertility, Gynecologists and Family practitioners.

Journal: To be decided

Deadline: Sep 1, 2012

PLESSIS, Stefan S. Du, Ph.D.

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8. DIET, MALNUTRITION AND NUTRIENT DEFICIENCIES: EFFECT ON MALE FERTILITY

Introduction/ Background:

Malnutrition that leads to deficiencies in certain vitamins, such as vitamin C, folate (folic acid), selenium, and zinc can contribute to male infertility. In addition, when malnutrition leads to being severely underweight, men may have issues with low sperm counts and diminished sperm quality and motility (the ability to effectively move through the women's reproductive system and implant into an egg). Deficiencies in certain nutrients, such as vitamin C, selenium, zinc, and folate, may be particular risk factors for low sperm count and male infertility.

Nutritional deficient diets lacking antioxidants, vitamins and synergistic minerals are unable to eliminate excessive amounts of reactive oxygen molecules. For example Vitamin C and Vitamin E are essential antioxidants that protect the body's cells from damage from OS and free radicals. Vitamin C is the most abundant antioxidant in the semen of fertile men, and it contributes to the maintenance of healthy sperm by protecting the sperm DNA from free radical damage (Song et al. 2006).

Vitamin E is a fat-soluble vitamin that helps protect the sperm's cell membrane from damage. Studies have shown that Vitamin E improves sperm motility and morphology. Vitamin C functions to regenerate Vitamin E, thus these vitamins may work together to improve sperm function (Eskenazi et al., 2005; Fraga et al., 1991; Song et al., 2006; Therond et al. 1996). Selenium on the other hand is a mineral that also functions as an antioxidant. Selenium supplements have been shown to increase sperm motility, and a combination of selenium and

Vitamin E has been shown to decrease damage from free radicals and improve sperm motility in infertile men (Hawkes & Turek, 2001).

Significance:

The best way to prevent infertility caused by being malnourished and underweight is to maintain a healthy weight and normal amounts of fat stores through a healthy diet that includes sufficient calories and nutrients and exercise that is not excessive. It is recommended that malnourished and underweight men who want to father children ensure they are eating a healthy diet with sufficient vitamin intake and gain weight before attempting a pregnancy.

Outline of the planned article:

- Introduction
- Defining malnutrition and underweight and the effects on fertility
- Specific nutrients that affect male fertility
- Conclusion

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written. Manuscript 60% written in 2011 – need to be finalized

Intended audience: The article would be of interest to researchers working in reproductive biology, infertility, cell and molecular biology.

Book Chapter: In: Lifestyle and Environmental Factors Influencing Male Fertility (Eds. Stefan Du Plessis, Edmund Sabanegh & Ashok Agarwal).

Suggested literature sources: Pubmed, Science direct

9. HEAT STRESS MALE INFERTILITY DUE TO OCCUPATION, SEDENTARY LIFESTYLE AND CLOTHING

Introduction/Background:

Scientists warn that welders, taxi drivers and office workers are at risk of decreased male fertility due to heat stress. Heat can raise the temperature of the testicles which can cause a decline in sperm levels.

It is well known that the testicles should be cooler than the rest of the body for optimal spermatogenesis. Just as the harmful effect of a varicocele on sperm production is believed to result from the excessive warming of the testicular area (caused by dilated veins), similarly various recreational (e.g. cycling, hot tubs or prolonged baths) and occupational (e.g. long distance driving, furnace operators) activities can lead to increasing testicular temperature. Welders may be risking infertility because of the high temperatures associated with their work. Taxi drivers and long-haul lorry drivers are thought to be at risk from sitting in the same position for long periods of time. This can raise the temperature of the testicles, causing sperm levels to drop. A study of 200 Italian taxi drivers in 2001 found they had much lower sperm counts than normal levels. Office workers who spend hours sitting in front of a computer are also at risk from an increase in testicular temperature. Scientists say you should take a break every 20 to 30 minutes to get away from your desk and help regulate the temperature of the testicles.

Significance:

It is generally assumed that these raised temperatures lead to high rates of oxidative DNA damage and hence more mutations in the resulting spermatozoa. Furthermore, obesity and the accompanying accumulation of adipose tissue within the groin region also results in raising testicular temperature. This has been linked to the development of OS in the testis and reduced sperm quality.

Outline of the planned article:

- Introduction

- Evidence that occupation and lifestyle factors, that lead to heat stress, does influence male fertility
- Mechanisms through which heat stress affect fertility
- Conclusion

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive biology, infertility, cell and molecular biology.

Book Chapter: Lifestyle and Environmental Factors Influencing Male Fertility (Eds. Stefan Du Plessis, Edmund Sabanegh & Ashok Agarwal).

Suggested literature sources:

1. Banks, King, Irvine, & Saunders, 2005
2. Perez-Crespo, Pintado, & Gutierrez-Adan, 2008
3. Ishii et al., 2005
4. Ivell, 2007

10. EFFECT OF SMOKING ON MALE INFERTILITY

Introduction/Background:

Tobacco smoke contains nearly 4000 harmful substances (e.g. alkaloids, nitrosamines, nicotine, hydroxycotinine etc.). Many of these substances generate ROS and RNS (Cross et al., 1987; Traber et al. 2000). It is well established that smoking has detrimental effects on the male reproductive system and has been significantly correlated with lower sperm count, motility and morphology (Saleh et al. 2002).

Saleh demonstrated that cigarette smoking leads to an increase in ROS levels and decreases in ROS-TAC scores. It was furthermore reported that smokers have high levels of leukocytospermia and suggested that OS develops due to ROS generation by activated leukocytes (Saleh et al., 2002). It was also reported that smokers have decreased levels of seminal plasma antioxidants such as Vitamin C and Vitamin E.

Various compounds of cigarette smoke (i.e. polycyclic aromatic hydrocarbons) and smoking metabolites may act as chemotactic stimuli and thereby induce an inflammatory response, leading to the recruitment of leukocytes with subsequent generation of ROS (Richthoff et al. 2008). Sperm motility correlates negatively with the amount of cotinine and hydroxycotinine in seminal plasma (de Lamirande & Gagnon, 1992).

A recent study showed that motility is one of the first sperm parameters affected and asthenozoospermia may be an early indicator of reduced semen quality in light smokers. The incidence of teratozoospermia was also significantly higher in heavy smokers when compared to non-smokers (Gaur et al. 2007). A study of three smokers who were followed for 5-15 months after stopping smoking reported that their sperm counts rose 50-800%, suggesting that toxic chemicals in the smoke are responsible and any reduction in sperm count is reversible.

Significance:

Smoker's sperm counts are on average 13%-17% lower than non-smokers. This is most likely due to higher levels of OS induced by smoking. Semen of smokers shows a 100-fold increase in OS and up to 5x higher cadmium levels (Saleh et al. 2002).

Outline of the planned article:

- Introduction
- Prevalence of smoking and content of cigarette smoke
- Effect of smoking on male infertility
- Possible mechanisms through which smoking affects male reproduction
- Conclusion

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive biology, infertility, cell and molecular biology.

Book Chapter: In: Lifestyle and Environmental Factors Influencing Male Fertility (Eds. Stefan Du Plessis, Edmund Sabanegh & Ashok Agarwal).

Suggested literature sources:

1. Traber MG, van der Vliet A, Reznick AZ, Cross CE. Tobacco-related diseases. Is there a role for antioxidant micronutrient supplementation? *Clin Chest Med.* 2000 21(1):173-87.
2. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. *Fertil Steril.* 2002 Sep;78(3):491-9.
3. Richthoff J, Elzanaty S, Rylander L, Hagmar L, Giwercman A. Association between tobacco exposure and reproductive parameters in adolescent males. *Int J Androl.* 2008 Feb;31(1):31-9.
4. de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl.* 1992 Sep-Oct;13(5):379-86.
6. Gaur DS, Talekar M, Pathak VP. Effect of cigarette smoking on semen quality of infertile men. *Singapore Med J.* 2007 Feb;48(2):119-23.

11. OXIDATIVE PHOSPHORYLATION VS. GLYCOLYSIS: WHAT FUEL DOES SPERM USE?

Introduction/ Background:

Mammalian spermatozoa expend energy, generated as intracellular ATP, largely on motility. If the sperm cell cannot swim by use of its flagellar motion, it cannot fertilize the egg. Studies of the means by which this energy is generated span a period of six decades. This review gives an overview of these studies, which demonstrate that both mitochondrial oxidative phosphorylation, for which oxygen is friend, and glycolysis, for which sugar is friend, can provide the energy, independent of one another. In mouse sperm, glycolysis appears to be the dominant pathway; in bull sperm, oxidative phosphorylation is the predominant pathway. In the case of bull sperm, the high activity of the glycolytic pathway would maintain the intracellular pH too low to allow sperm capacitation; here sugar is enemy. The cow's oviduct has very low glucose concentration, thus allowing capacitation to proceed. The choice of the pathway of energy generation in vivo is set by the conditions in the oviduct. The phospholipids of the sperm plasma membrane have a high content of polyunsaturated fatty acids represented in their acyl moieties, rendering them highly susceptible to lipid peroxidation; in this case oxygen is enemy. But the susceptibility of the sperm membrane to lethal damage by lipid peroxidation allows the female oviduct to dispose of sperm that have overstayed, thereby keeping in balance sperm access to the egg and sperm removal once this has occurred.

Significance:

In the literature there is lots of conflicting information regarding the preferred source of energy used by human spermatozoa. It appears that spermatozoa can make use of forms of energy under different conditions and specifically with regards to different functions. A complete review highlighting and logically addressing all the information is currently outstanding and would contribute significantly to the body of knowledge.

Outline of the planned article:

- Introduction

- Defining oxidative phosphorylation and glycolysis
- Sperm functions: which source is utilized?
- Conclusion

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to researchers working in reproductive biology, infertility, cell and molecular biology.

Journal or Book: Review Manuscript – Journal to be decided

Suggested literature sources: Pub med, Science direct

1. Hereng TH, Elgstøen KB, Cederkvist FH, Eide L, Jahnsen T, Skålhegg BS, Rosendal KR. Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. *Hum Reprod.* 2011 Dec;26(12):3249-63. Epub 2011 Sep 23.
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12. IMPACT OF DIABETES MELLITUS ON MALE SEXUAL FUNCTION AND INFERTILITY

Introduction/ Background:

Diabetes mellitus (DM) is a chronic metabolic disease associated with a wide range of complications affecting most organ systems. It is characterized by changes in plasma insulin, glucose, lipid, triglyceride and ketone levels. DM (especially Type 2) is also often accompanied by obesity and the metabolic syndrome and their cluster of co-morbidities. Significant quantities of data show a relationship between diabetic /obese individuals and subfecundity, thereby establishing it as a further co-morbidity. DM basically impinges on the male reproductive system and fertility through its effects on erectile dysfunction (ED) and impaired semen parameters. In the industrialized world, both Types 1 & 2 DM is on the increase due to predominantly lifestyle factors. This is a common phenomenon amongst especially younger

people, with onset before and during their reproductive years, which not only impacts directly on their fertility, but also has devastating psychological effects.

As the mechanisms via which DM can impact on fertility is via hyperinsulinemia, changes in reproductive hormonal levels, oxidative stress as well as adipokines and adipocyte derived hormones such as resistin and leptin, lifestyle factors should be seriously considered as possible treating modalities. By addressing controllable factors such as diet/nutrition, smoking and exercise, individuals with DM can improve their fertility status.

Significance:

DM and the detrimental effects of lifestyle factors are on the increase and affect male fertility directly. A review of the mechanisms via which DM affect male reproductive function combined with lifestyle changes that can enhance fertility is lacking in the literature. This will help physicians treating diabetic patients trying to become pregnant.

Outline of the planned article:

- Introduction
- Overview of DMI and DMII
- Mechanisms via which DM affect male reproduction.
- Treatments and solutions for the infertile DM patient: Lifestyle changes
- Conclusion

Literature review: Preliminary literature searches have been done. An exhaustive literature review needs to be done before the article is written.

Intended audience: The article would be of interest to clinicians (endocrinologists, urologists) and fertility specialists as well as researchers working in reproductive biology.

Journal or Book: Review Article – Journal to be decided

Suggested literature sources: Pubmed, Science direct

1. Bener A, Al-Ansari AA, Zirie M, Al-Hamaq AO. Is male fertility associated with type 2 diabetes mellitus? *Int Urol Nephrol*. 2009 Dec;41(4):777-84. Epub 2009 Apr 21.
2. Mallidis C, Agbaje I, McClure N, Kliesch S. The influence of diabetes mellitus on male reproductive function: a poorly investigated aspect of male infertility]. *Urologe A*. 2011 Jan;50(1):33-7. German.
3. La Vignera S, Calogero AE, Condorelli R, Lanzafame F, Giammusso B, Vicari E. Andrological characterization of the patient with diabetes mellitus. *Minerva Endocrinol*. 2009 Mar;34(1):1-9.
4. Navarro-Casado L, Juncos-Tobarra MA, Cháfer-Rudilla M, de Onzoño LÍ, Blázquez-Cabrera JA, Miralles-García JM. Effect of experimental diabetes and STZ on male fertility capacity. Study in rats. *J Androl*. 2010 Nov-Dec;31(6):584-92. Epub 2010 Mar 4.
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6. Mallidis C, Agbaje I, O'Neill J, McClure N. The influence of type 1 diabetes mellitus on spermatogenic gene expression. *Fertil Steril*. 2009 Dec;92(6):2085-7. Epub 2009 Jul 8.
7. Delfino M, Imbrogno N, Elia J, Capogreco F, Mazzilli F. Prevalence of diabetes mellitus in male partners of infertile couples. *Minerva Urol Nefrol*. 2007 Jun;59(2):131-5.
8. Agbaje IM, Rogers DA, McVicar CM, McClure N, Atkinson AB, Mallidis C, Lewis SE. Insulin dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod*. 2007 Jul;22(7):1871-7. Epub 2007 May 3.

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13. STATUS OF THE HUMAN EMBRYO: “PERSONHOOD”

Introduction/Background:

Use of human embryos for research, including creation of embryos for investigative studies, along with the future of the more than 500,000 frozen embryos have led to issues that span ethical, moral, societal, religious, legal, and legislative concerns. Paramount to these concerns is the status of the human embryo and the definition of “personhood”.

Significance:

The status of the human embryo has long been considered an area of controversy with opinions as varied as: 1) a human with all the rights and respects accorded humans; 2) reproductive tissue without moral or legal status; or 3) reproductive tissue that is not human, but that has a special status, deserving respect above other human body tissues.

Understanding the positions of various groups and stakeholders on the status of the human embryo and the definition of “personhood” are essential when addressing this significant issue and making recommendations for policy.

Outline of the planned article:

- Introduction
- Bioethics
- Principles and systems
- Beginning of life
- Science

- Religion
- Stakeholders
- Frozen embryo storage facilities
- Research scientists
- Industry supporting embryonic stem cell research
- Genetic parents of frozen embryos
- Recipient parents of donated embryos
- Special interest groups
- National Embryo Donation Academy
- 'Snowflake'
- Other
- Current and proposed legislation in U.S.
- Laws in effect internationally regarding storage of cryopreserved embryos and embryonic stem cell research
- Recommendations for consideration
- decreasing number of embryos in storage
- embryos currently in storage
- alternatives to creation of embryos for research

Literature review: Extensive literature review required

Intended audience: The article would be of interest to a large and diverse audience including assisted reproductive technology (ART) professionals, research scientists, industry involved in stem cell research, bioethicists, stakeholders, policy makers, and special interest groups,

Journal(s): Fertility and Sterility, Reproductive Biomedicine OnLine, Other (bioethics journal)

Suggested literature sources: Pubmed, International Federation of Fertility Societies (IFFS) reports, 'Snowflake' website, American Society for Reproductive Medicine (ASRM) website, National Embryo Donation Academy courses (no charge).

14. GENE EXPRESSION IN EMBRYOS CREATED BY ASSISTED REPRODUCTIVE TECHNOLOGY

Introduction/Background:

The use of assisted reproductive technology (ART) continues to expand with usage increasingly linked to a range of adverse effects and consequences, including complications of pregnancy and parturition, and a range of birth defects in offspring. The cause of these effects is not well defined with potential contributors being: 1) the underlying infertility disease; 2) ART procedures, such as intracytoplasmic sperm injection (ICSI); 3) drugs used for ovarian stimulation; and 4) ART methodology, including skills of technologists performing the procedures. Both human studies and animal models has been used to distinguish factors contributing to the negative outcomes of ART.

Significance:

Knowledge of detrimental effects of ART procedures, as determined by genetic studies, including early embryonic gene expression in animal models, may be used to aid in the diagnosis of infertility and the selection of ART treatment. For example, recent work has shown that procedural-induced gene expression in a murine model is different for IVF-versus ICSI-IVF and that these changes in genes regulating specific biological pathways reveal some consistency to known adverse consequences (i.e. aberrant growth and development or cleft palate in offspring). Furthermore, it is suggested that chemical activation of oocytes at ICSI effectively mimics, at the genetic level, a proportion of the events initiated by sperm penetration with gene expression more similar to IVF than ICSI without activation is to IVF. The majority (>50%) of the nearly 150,000 ART cycles performed in the United States annually utilize ICSI with ICSI increasing in use and routinely being prescribed over IVF regardless of

the infertility etiology. Knowledge of procedural gene expression due to ICSI could suggest a decrease in use of the procedure or the addition of the activation step.

Outline of planned article:

- Introduction
- Genetic diseases and traits associated with infertility
- ART offspring
- Genetic diseases and traits
- Alterations in gene expression
- Epigenetic changes
- Animal studies
- Gene expression resulting from ART procedures
- Differential gene expression due to procedure
- Genetic tests and procedures
- Diagnostic
- Preimplantation genetic screening and diagnosis
- Other
- Research
- Microarray
- Future directions

Literature review: Extensive literature review completed; updating required.

Intended audience: The review article would be of interest to ART practitioners, reproductive scientists, and policy-makers addressing use of ART procedures.

Journal(s): Fertility and Sterility, Reproductive Biomedicine OnLine, Assisted Reproduction and Genetics

Suggested literature sources: Pubmed

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15. ROLE OF ENDOMETRIUM IN IVF SUCCESS**Introduction/ Background:**

IVF success not only depends on the oocyte or sperm quality but the quality of the endometrium. There is no special tool to assess endometrial quality except ultrasound. Patients with endometriosis or inflammation have elevated oxidative stress markers both in ectopic and eutopic endometrial tissue. These inflammatory markers may be associated with poor pregnancy outcomes. Patients who have hyperstimulation syndrome may also have poor endometrial quality affecting IVF outcomes. It has been demonstrated by few studies that frozen embryo transfers do better compared to fresh transfers which indirectly indicate a dominant role of the uterine factor.

Significance:

To explore various current evidences involving the uterine involvement and parameters in patients with various causes of infertility. Endometrial profiles in patients with endometriosis, inflammation, uterine fibroids and correlation with pregnancy outcomes.

Outline of the planned article:

- Introduction
- Physiological changes in the endometrium throughout a normal cycle
- Pathological changes in endometriosis or pelvic inflammatory states including oxidative stress markers, cytokines and gene regulation.
- Anatomical alterations contributing to infertility, recurrent miscarriages and IVF success
- Endometrial pattern and correlation with IVF success
- Uterine fibroid and infertility / IVF

- Endometrial changes in ovarian hyperstimulation syndrome
- Comparing fresh vs frozen success attributing to endometrial factor

Literature review: Extensive reading will be required for the above topics before starting to write.

Intended audience: Clinicians and researchers involved in reproductive biology and medicine

Suggested literature sources: Pubmed, Science Direct

16. SIGNIFICANCE OF OVARIAN STEM CELLS IN FERTILITY PRESERVATION

Introduction/Background:

Fertility preservation is of utmost importance in young females undergoing chemotherapy or radiotherapy for management of cancer. Various methods including in vitro maturation, GnRH agonist suppression, ovarian cortex or whole ovary cryopreservation, surgical repositioning has been proposed. Most of the techniques are experimental and only IVF prior to therapy followed by embryo freezing is practically utilized. Newer research shows that ovarian stem cells can be extracted from ovaries, grown in culture and implanted in ovaries of older women which can generate new cohort of oocytes having better mitochondrial function. This modality is very new and it will be interesting to explore the existing data.

Outline of the planned article:

- Oogenesis- basic physiology
- Ovarian stem cells.
- Methods of fertility preservation and success.
- Oocyte ageing, oocyte quality with age.
- Ovarian stem cell implantation- mouse and human studies.
- Controversies

Literature review: Extensive reading on oocyte biology, Oogenesis and ovarian stem cells will be required prior to writing.

Intended audience: Reproductive biologists

Suggested literature sources: Pub med, science direct.

17. OOCYTE QUALITY- FACTORS AFFECTING, METHODS OF ASSESSMENT, ROLE OF OXIDATIVE STRESS AND ANTIOXIDANTS.

Introduction/Background:

Oocyte quality is a vital factor governing fertility and IVF success. Various parameters in a mature oocyte represent the quality of the oocyte. Any alteration of these parameters may affect the quality of the oocyte contributing to adverse reproductive outcomes. It is important to explore the factors which may contribute to poor oocyte quality emphasizing on the role of oxidative stress. To explore if any existing method can be utilized to assess the quality of the oocyte.

Significance:

Identifying factors altering oocyte quality and possible remedies which may improve reproductive outcomes. Reactive oxygen species, cytokines can be identified to contribute to oocyte damage or accelerate oocyte ageing. It will be important to explore whether any antioxidant could improve oocyte quality and whether clinical or scientific studies have been successful to find markers of oocyte quality damage.

Outline of the planned article:

- Introduction
- What is oocyte quality
- Factors affecting oocyte quality

- Factors contributing to accelerated oocyte ageing.
- Oxidative stress and oocyte quality
- Parameters/ markers to assess oocyte quality.
- Factors which can prevent or reverse oocyte quality damage.

Literature search: Extensive reading about oocyte quality, parameters defining oocyte quality, diseases involved with poor oocyte quality and infertility will be required before writing this project.

Intended audience: reproductive biologists, reproductive endocrinologists, embryologists

Suggested literature sources: Pub med, science direct

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18. NOVEL STRATEGIES AND BIOLOGICAL FOUNDATIONS FOR SELECTING HUMAN GAMETES AND EMBRYOS IN ASSISTED CONCEPTION TECHNOLOGIES

Introduction/Background:

Assisted reproductive techniques (ART) created a new milestone in the history of mankind and served as a major breakthrough in fertility treatment, resulting in the birth to date of an estimated 3 million children across the world. Since its inception in 1978, there has been a remarkable increase in the numbers of IVF cycles worldwide. Approximately 1 in 50 births in Sweden, 1 in 60 births in Australia, and 1 in 80 to 100 births in the United States now result from IVF. The emphasis has been on improving every aspect of ART: oocyte recovery, fertilization rates; embryo culture techniques; a better understanding of embryo-uterine interactions which are all aimed at improving take-home baby rates. ART techniques also have undergone changes from some less-successful techniques such as gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT) and round spermatid injection (ROSNI) to those that are now routine, such as intracytoplasmic sperm injection (ICSI), which revolutionized the treatment of male infertility. However, in spite of several notable improvements to these ART procedures, the occurrence of multiple pregnancies and the related premature low birth weight babies is still considered a major problem. To comply with regulations, mandates and guidelines and to meet patient and social demands to limit the impact of multiple pregnancies, it is imperative that reliable methods are devised that allow for selection of the embryo with maximum implantation potential.

Significance:

To meet patient and social demands and to limit the impact of multiple pregnancies, it is imperative that reliable methods are devised allowing for selection of the embryo with maximum implantation potential. There are various scoring systems for embryo selection in IVF. However, there is no single review article bringing all of them together. This review article should present a comprehensive collection of recent research about the reliable prediction of embryo viability based upon molecular biology techniques or noninvasive selection criteria.

Outline of the planned article:

- Approaches for selecting the right conceptus for embryo transfer
- Factors involved in embryo selection
- Perifollicular vascularity
- Follicular fluid – the “biological window”
- Growth factors and cytokines in follicular fluid milieu
- Oocyte competence
- Polscope based spindle and zona birefringence
- Respiration rate
- Metabolic markers
- Time lapse imaging
- Genomics, proteomics and metabolomics
- Investigating the genome
- Investigating the secretome
- Investigating the metabolom
- Sperm separation and manipulations - selecting the male gametes for ART
- Annexin v-conjugated microbeads (ANMB) - magnetic-activated
- Cell sorting (MACS)
- Microfluidic based sperm processing
- Electrophoretic sperm processing
- Sperm selection for ICSI

- Intracytoplasmic morphologically selected sperm injection (IMSI)
- Hyaluronic acid (HA) bound sperm selection
- Sperm birefringence by polscope
- Avenues for improving sperm quality and selecting best gamete for assisted conception
- Conclusion

60% work completed. Need revision and addition of latest literature from 2008 to present

Literature review: Preliminary literature searches completed. An exhaustive literature review needed.

Intended audience: Infertility specialists, embryologists, reproductive researchers

Journal: Human Reproduction Update

Deadline: Sep 15, 2012

Important recent references:

1. Wang SX. The past, present, and future of embryo selection in in vitro fertilization: frontiers in reproduction conference. *Yale J Biol Med.* 2011 Dec;84(4):487-90.
2. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. *Hum Reprod Update.* 2011 Nov-Dec;17(6):719-33.
3. Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. *Fertil Steril.* 2012 Feb 22
4. Stylianou C, Critchlow D, Brison DR, Roberts SA. Embryo morphology as a predictor of IVF success: an evaluation of the proposed UK ACE grading scheme for cleavage stage embryos. *Hum Fertil (Camb).* 2012 Mar;15(1):11-7.

5. Hashimoto S, Kato N, Saeki K, Morimoto Y. Selection of high-potential embryos by culture in poly(dimethylsiloxane) microwells and time-lapse imaging. *Fertil Steril*. 2012 Feb;97(2):332-7.
6. Nel-Themaat L, Nagy ZP. A review of the promises and pitfalls of oocyte and embryo metabolomics. *Placenta*. 2011 Sep;32 Suppl 3:S257-63.
7. Vozdova M, Kasikova K, Oracova E, Prinosilova P, Rybar R, Horinova V, Gaillyova R, The effect of the swim-up and hyaluronan-binding methods on the frequency of abnormal spermatozoa detected by FISH and SCSA in carriers of balanced chromosomal translocations. *Rubes J. Hum Reprod*. 2012 Mar;27(3):930-7.

19. SOCIAL EGG FREEZING- CURRENT AND FUTURE PERSPECTIVE

Introduction/ Background:

Until recently, there was little to offer young women with cancer facing chemotherapy, radiotherapy or surgery and the probability of premature menopause and sterility. The first 'frozen egg' baby was born in 1986, but success rates were so low that egg freezing was neglected. Three technological developments in assisted reproduction treatment (intracytoplasmic sperm injection, dehydro-cryoprotectants and vitrification) have transformed this picture and now young women with frozen eggs have the same probability of a live birth per embryo transfer as women undergoing conventional IVF. For many women it is not cancer but the passage of time that denies them a chance of motherhood. Social, educational and financial pressures often lead them to delay starting a family until their late thirties, by which time the chance of success is compromised by low fecundity rates and an increased risk of miscarriage if they become pregnant. Donor eggs are not an option for many because of supply constraints and ethical concerns. Freezing a woman's eggs at age 30 literally 'freezes in time' her fertility potential and gives her the chance of a healthy pregnancy at a time of her choosing. The possibility for healthy women to cryopreserve their oocytes in order to counter future infertility has gained momentum in recent years. Women with a history of premature ovarian insufficiency, ovarian cysts, living in an area with high exposure to pesticides or heavy metals, or undergoing exposure to chemical or biological warfare due to military service may

also consider egg freezing, the largest numbers of women considering social egg freezing are likely to be those who either desire or foresee delaying their childbearing years.

Significance:

Social egg freezing generally arises because a woman chooses to delay bearing children. This could be because they wish to further their career before parenthood or have not found a partner with whom they wish to share parenthood. There is a second category of 'social' egg freezing: the donation of oocyte for paying customers. Failure to produce a pregnancy in these cycles has no impact on the donor since their transaction is purely financial.

Since there are a number of institutions offering social egg freezing around the world, there is a need to have a critical assessment of the technology and some of the concerns that emerge from social egg freezing.

Outline of the planned article:

- Ageing process and egg quality
- New technologies in egg freezing
- Pregnancy rate in women above 40 year old by IVF
- Worldwide status on pregnancy rate when they opt for donor eggs,
- Social awareness on egg freezing
- Present worldwide status of social egg freezing:
 - North America
 - South America
 - Europe
 - Asian Countries
 - Future perspectives

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Infertility specialists, reproductive scientists

Journal: Human Reproduction Update

Deadline: August 1st

Some recent publications:

1. Liu KE, Greenblatt EM. Oocyte cryopreservation in Canada: a survey of Canadian ART clinics. *J Obstet Gynaecol Can.* 2012 Mar;34(3):250-6.
2. Mertes H, Pennings G. Elective oocyte cryopreservation: who should pay? *Hum Reprod.* 2012 Jan;27(1):9-13.
3. Mertes H, Pennings G. Social egg freezing: for better, not for worse. *Reprod Biomed Online.* 2011 Dec;23(7):824-9.
4. Shkedi-Rafid S, Hashiloni-Dolev Y. Egg freezing for non-medical uses: the lack of a relational approach to autonomy in the new Israeli policy and in academic discussion. *J Med Ethics.* 2012 Mar;38(3):154-7.
5. Lockwood GM. Social egg freezing: the prospect of reproductive 'immortality' or a dangerous delusion? *Reprod Biomed Online.* 2011 Sep;23(3):334-40.
6. Stoop D, Nekkebroeck J, Devroey P. A survey on the intentions and attitudes towards oocyte cryopreservation for non-medical reasons among women of reproductive age. *Hum Reprod.* 2011 Mar;26(3):655-61.
7. Jona K, Gerber A. MyOncofertility.org: a web-based patient education resource supporting decision making under severe emotional and cognitive overload. *Cancer Treat Res.* 2010;156:345-61. Review.

20. FERTILITY PRESERVATION IN CANCER PATIENTS: CRYOBIOLOGICAL ADVANCES

Introduction/ Background:

As a result of a remarkable improvement in the survival rates of cancer patients, there has been an increased interest in the long-term effects of cancer treatment on quality of life. In particular, infertility is one of the major sequelae of cancer therapy and may be of considerable distress to cancer survivors. In female patients, risk of menopause-related complication and infertility at a very young age due to cancer treatment may be more devastating and be

considered as a loss of their essential femininity. The freezing of ovarian tissue is currently being proposed with the primary purpose of preserving ovarian function in these patients. Currently, the major challenge of groups working with preservation of fertility is the use of

cryopreserved ovarian tissue after disease remission. The main alternatives presented today are the implantation of hetero- or orthotopic tissue and isolation of immature follicles from ovarian tissue followed by in vitro maturation and assisted reproduction procedures.

Significance:

The ovarian tissue cryopreservation are either experimental or have not been fully evaluated and thus can not be proposed to patients at this time. Cryopreservation/ transplantation of ovarian tissue and in vitro maturation (IVM) of follicles/ oocytes are two such emerging techniques. The aforementioned treatment regimes are opening up more alternatives and allow for more suitable choices to preserve fertility according to the patient's specific situation. For these technologies to be recognized and carried out routinely, they must be safe, easy to perform and deliver successful results. As these technologies are still quite novel, the goal of this article is to offer an in-depth look at the challenges that must be overcome with the cryopreserving and transplanting of ovarian tissue to ultimately lead to successful fertility preservation.

Outline of the planned article:

- Introduction
- Effect of chemo-, radio therapy on ovarian tissues
- Fertility preservation options in young cancer patients
- Cryobiology of organs
- Cryo preservation of ovarian tissues by slow freezing
- Multi thermal gradient (directional freezing)
- Vitrification strategies for ovarian tissues
- Whole ovary cryopreservation
- Ovarian tissue transplantation
- Current clinical scenario of ovarian tissue freezing
- Ethical concerns

- Future aspects
- Conclusion

Journal: TBD

Literature review: Use Cleveland Clinic Alumni Library for literature search

Important references (recent):

1. Varghese AC, du Plessis SS, Falcone T, Agarwal A. Cryopreservation/transplantation of ovarian tissue and in vitro maturation of follicles and oocytes: challenges for fertility preservation. *Reprod Biol Endocrinol*. 2008 Oct 2;6:47. Review. PMID: 18828928
2. Michaeli J, Weintraub M, Gross E, Ginosar Y, Ravitsky V, Eizenman E, Mitrani E, Lebovich M, Laufer N, Kennedy S, Revel A. Fertility preservation in girls. *Obstet Gynecol Int*. 12; 2012:139-193. Epub 2012 Feb 16. PMID: 22496695
3. Gracia CR, Chang J, Kondapalli L, Prewitt M, Carlson CA, Mattei P, Jeffers S, Ginsberg JP. Ovarian tissue cryopreservation for fertility preservation in cancer patients: Successful establishment and feasibility of a multidisciplinary collaboration. *J Assist Reprod Genet*. 2012 Apr 1. PMID: 22466745
4. Rodriguez-Wallberg KA, Oktay K. Recent advances in oocyte and ovarian tissue cryopreservation and transplantation. *Best Pract Res Clin Obstet Gynaecol*. 2012. PMID: 22301053
6. Sarah Posillico, Amr Kader, Tommaso Falcone, Ashok Agarwal. Ovarian Tissue Vitrification: Modalities, Challenges and Potentials. *Current Women's Health Reviews*, 2010, 6, 352-366
7. Target: invited book chapter:
<http://www.intechopen.com/welcome/cdd9872a05001212b3583bff95bae979/seanglin.tan@muhc.mcgill.ca>

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21. FROM CONCEPTION TO BIRTH: HOW ENDOMETRIOSIS AFFECTS EACH STAGE OF LIFE DEVELOPMENT

Introduction/ Background:

Endometriosis affects approximately 10% of reproductive-age women and almost 40% are infertile. (Carvalho et al, 2012) The exact pathophysiology underlying infertility in the presence of endometriosis is poorly understood. Endometriosis may affect fertility via:

- Abnormal folliculogenesis
- Changes in ovarian steroid enzymes
- Impaired oocyte maturation leading to poor oocyte quality and consequently reduced fertilization rate or poor embryo quality
- High levels of caustic peritoneal fluid components such as oxidative stress and inflammatory interleukins and cytokines
- Tubal obstruction or other distorted pelvic anatomy particularly with excessive scarring and adhesions present in advanced stages of endometriosis
- Sperm dysfunction given eutopic endometrial environment; and implantation defects
Gupta et al 2008; de Ziegler et al, 2010; Carvalho et al 2011

Significance:

Given the high prevalence of endometriosis in women with infertility, it can be assumed that there is an association between these two conditions. However, a definitive cause and effect relationship has yet to be established. It is not completely clear as to how endometriosis affects fertility.

Outline of the planned article:

- Possible factors contributing to reduced fertility in patients with endometriosis and adenomyosis.
- Oocyte quality (steroid enzymes/maturation)
- Disturbed Ovulation
- Egg fertilization/Insemination
- Embryo quality in patients with endometrioma
- Transport through fallopian tube/ utero-tubal transport sperm
- Implantation defects (adenomyosis/endometriosis)
- Early gestational risk (increase risk of miscarriage)
- Pre Term Delivery

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Specialist in Reproductive Medicine

Journal: Reproductive Biology

Suggested literature sources:

1. Carvalho L, Podgaec S, Bellodi-Privato M, Falcone T, & Abrao, M. S. (2011). Role of eutopic endometrium in pelvic endometriosis *Journal of Minimally Invasive Gynecology*, 18(4), 419-427.
2. Coccia, M E, & Rizzello, F (2008). Ovarian reserve. *Annals of the New York Academy of Sciences*, 1127, 27-30
3. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. *Lancet*. 2010 Aug 28;376 (9742):730-8. Review. PubMed PMID: 20801404
4. Garrido N, Navarro J, Remohí J, Simón C, Pellicer A. Follicular hormonal environment and embryo quality in women with endometriosis. *Hum Reprod Update*. 2000 Jan-Feb;6(1):67-74. Review.

5. Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal A. Pathogenic mechanisms in endometriosis-associated infertility. *Fertil Steril*. 2008 Aug;90 (2):247-57. Review. PubMed PMID: 18672121
6. Martínez-Conejero JA, Morgan M, Montesinos M, Fortuño S, Meseguer M, Simón C, Horcajadas JA, Pellicer A. Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. *Fertil Steril*. 2011 Oct;96(4):943-50. Epub 2011 Aug 6.
7. Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohí J, Simón C. The follicular and endocrine environment in women with endometriosis: local and systemic cytokine production. *Fertil Steril*. 1998 Sep;70(3):425-31.
8. Pellicer A, Albert C, Garrido N, Navarro J, Remohí J, Simón C. The pathophysiology of endometriosis-associated infertility: follicular environment and embryo quality. *J Reprod Fertil Suppl*. 2000;55:109-19. Review

22. OVARIAN ENDOMETRIOMA AND IN VITRO FERTILIZATION: IS LAPAROSCOPIC SURGERY HARMFUL?

Introduction/ Background:

Endometriomas are benign ovarian tumors and surgery is the most acceptable approach for endometrioma > 4cm. However repeated laparoscopic surgeries can cause scarring and adhesion formation and may diminish ovarian reserve. The conservative surgical management of endometrioma is a topic of constant debate regarding ovarian preservation and recurrence for many years. There are plenty of studies in literature about this issue but still the answer is questionable.

Significance:

There is a constant controversy regarding the cyst wall removal. Aggressive removal of cyst wall may damage the ovarian cortex. Surgery near the hilum of the ovary cause more damage to the ovary. Inadequate removal of cyst wall and simple drainage of a cyst may cause recurrence. We will explore, how laparoscopic endometrioma cystectomy affects the ovary reserve and IVF outcomes.

Outline of the planned article:

- Introduction
 - Endometrioma: Surgery and ovarian Reserve
 - Endometrioma and IVF
 - After Surgery
 - Before Surgery
- Laparoscopy techniques - Ovarian reserve after surgery
- Endometrioma and IVF outcome
- Tables and Diagram
- Conclusion

Literature review: Resources of Cleveland Clinic Alumni Library

Intended audience: Specialist in Reproductive Medicine

Journal: Gynecological Surgery

Suggested literature sources:

1. Benaglia L, Somigliana E, Vighi V, Ragni G, Vercellini P, Fedele L. Rate of severe ovarian damage following surgery for endometriomas. *Hum Reprod.* 2010 Mar;25(3):678-82.
2. Vercellini P, Chapron C, De Giorgi O, Consonni D, Frontino G, Crosignani PG. Coagulation or excision of ovarian endometriomas? *Am J Obstet Gynecol.* 2003 Mar;188(3):606-10. Review.
3. Pellicano M, Bramante S, Guida M, Bifulco G, Di Spiezio Sardo A, Cirillo D, Nappi C. Ovarian endometrioma: postoperative adhesions following bipolar coagulation and suture. *Fertil Steril.* 2008 Apr;89(4):796-9.
4. Hart RJ, Hickey M, Maouris P, Buckett W. Excisional surgery versus ablative surgery for ovarian endometriomata. *Cochrane Database Syst Rev.* 2008 Apr 16;(2):CD004992. Review.

5. de Carvalho BR, Rosa e Silva AC, Rosa e Silva JC, dos Reis RM, Ferriani RA, Silva de Sá MF. Ovarian reserve evaluation: state of the art. *J Assist Reprod Genet.* 2008 Jul;25(7):311-22.
6. La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Artenisio AC, Stabile G, Volpe A. Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update.* 2010 Mar-Apr;16(2):113-30.

23. SURGERY FOR DEEP INFILTRATIVE ENDOMETRIOSIS INCREASES THE ODDS OF IN VITRO FERTILIZATION: WHAT IS THE EVIDENCE?

Introduction/ Background:

Endometriosis is an estrogen-dependant pelvic inflammatory disease which affects approximately 10% of women in the United States. It is the underlying cause of infertility in roughly 50% of all infertile women. Researchers have started to assess the use of surgical techniques in conjunction with IVF as a way to improve fecundity in women with endometriosis. In a recent Meta-analysis, laparoscopic surgery for the treatment of subfertility related to minimal and mild endometriosis concluded that patients with Stage I/II and endometriosis had a lower pregnancy rate, however, only few published studies have examined the impact of surgery on stage III/IV on pregnancy outcome.

Significance:

While there has been an increasing link between infertility and advanced stage of endometriosis, the evidence from the literature is unclear. We aim to review and grade the evidence in the literature.

Outline of the planned article::

- Introduction
- Endometriosis
 - What is deep endometriosis?

- How endometriosis affect fertility
 - Indications of IVF in endometriosis
 - Review of the paper in the literature and the evidence
- Answer the following questions:
 - Surgery Improve IVF outcome in patients with deep infiltrating endometriosis infertility patients?
 - What is the evidence regarding deep infiltrating endometriosis and IVF outcome
- Conclusion

GRADING/SCORING SYSTEM FOR EACH COMPOUND STUDIED:

Evidence Classification	
Ia	Evidence from meta-analysis of randomised controlled trials
Ib	Evidence from at least one randomised controlled trial
IIa	Evidence from at least one controlled study without randomisation
IIb	Evidence from at least one other type of quasi-experimental study
III	Evidence from non-experimental descriptive studies, such as comparative studies, correlation studies and case-control studies
IV	Evidence from expert committee reports or opinions and/or clinical experience of respected authorities

Recommendation Grading	
A	Directly based on category I evidence
B	Directly based on category II evidence, or extrapolated recommendation from category I evidence
C	Directly based on category III evidence, or extrapolated recommendation from category I or II evidence
D	Directly based on category IV evidence, or extrapolated recommendation from category I, II or III evidence

Literature review: PubMed; Lilacs Cochrane Reviews

Journal: International Journal of Gynecology & Obstetrics

Suggested literature sources:

1. Jacobson TZ, Duffy JM, Barlow D, Farquhar C, Koninckx PR, Olive D. Laparoscopic surgery for subfertility associated with endometriosis. Cochrane Database Syst Rev. 2010 Jan 20; (1):CD001398. Review

2. Coccia ME, Rizzello F, Cammilli F, Bracco GL, Scarselli G. Endometriosis and infertility Surgery and ART: An integrated approach for successful management. *Eur J Obstet Gynecol Reprod Biol.* 2008 May;138(1):54-9.
3. Bianchi PH, Pereira RM, Zanatta A, Alegretti JR, Motta EL, Serafini PC. Extensive excision of deep infiltrative endometriosis before in vitro fertilization significantly improves pregnancy rates. *J Minim Invasive Gynecol.* 2009 Mar-Apr;16(2):174-80.
4. Chapron C, Fritel X, Dubuisson JB. Fertility after laparoscopic management of deep endometriosis infiltrating the uterosacral ligaments. *Hum Reprod.* 1999 Feb;14(2):329-32.
5. Vercellini P, Somigliana E, Viganò P, Abbiati A, Barbara G, Crosignani PG. Surgery for endometriosis-associated infertility: a pragmatic approach. *Hum Reprod.* 2009 Feb; 24(2):254-69.
6. Koninckx PR, FAU - Martin DC, Martin DC. Deep endometriosis: a consequence of infiltration or retraction or possibly adenomyosis externa? - *Fertil Steril.* 1992 Nov;58(5):924-928