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Faculty of Health Sciences

School of Medicine

Investigating a novel *in vitro* embryo culture system –
The Walking Egg Affordable Assisted Reproductive Technology

by

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DEDICATION

"The Sciences, unlike the Graces or the Eumenides, are not limited in number. Once born, they are immortal, but, as knowledge increases, they are ever multiplying, and so great is now the dominion of the scientific mind that every few years sees a new one brought into the world. Some spring, fully armed, from the brains of one or two men of genius, but most of them, perhaps, come only gradually to their full development through the labours of many obscure and accurate observers."
Joseph Needham (1931)

This obscure (for now) and hopefully accurate observer would like to thank his loving wife Ilana for her support, assistance and encouragement during the execution and compilation of this research; I am unequivocally grateful to you, without your help I would not have been able to complete this undertaking.

DECLARATION BY CANDIDATE

'I hereby declare that the dissertation submitted for the degree MSc Reproductive Biology, at the Faculty of Health Sciences, University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.'

GERHARDUS MARTHINUS BOSHOFF

       Name in block letter   Signature

       Date

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26/11/2015

Approval Certificate
New Application

Ethics Reference No.: 460/2015

Title: Investigating a novel In vitro embryo culture system - The Walking Egg Affordable Assisted Reproductive Technology.

Dear Gerhardus Boshoff

The **New Application** as supported by documents specified in your cover letter dated 17/11/2015 for your research received on the 17/11/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/11/2015.

Please note the following about your ethics approval:
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- Please remember to use your protocol number (**460/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2015 (Department of Health).

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SUMMARY

Introduction: The desire to have a biological child transcends race, religion and socio-economic status. However for those faced with infertility, the financial resources needed to conceive are often not available. Current research in assisted reproduction has gravitated towards cost reduction to address restricting financial factors, without compromising quality of treatment. One such initiative is the development of a low-cost embryo culture method by The Walking Egg foundation. This method utilizes a standard chemical reaction and simple equipment to equilibrate culture media pH and to regulate temperature; both aspects were investigated in this study. An exploration into the insemination concentration to achieve oocyte fertilization was also undertaken.

Methods: Quality control of temperature regulation on six different heating devices, including a comparison of inter- and intra-variations was carried out. The utilization of citric acid and bicarbonate of soda for carbon dioxide production, which subsequently facilitate setting of pH values, was tested by injecting increasing citric acid volumes (1.2 ml – 3.0 ml in 0.2 ml increments) into set volumes of bicarbonate of soda. Further investigation evaluated gas production at various temperatures (37°C, 25°C and 15°C), at increasing intervals (16 – 30 hours) of equilibration and these were compared by measuring pH of the culture media. The influence of altitude on pH was explored by repeating the chemical reaction experiment at five different locations in South Africa. Furthermore, the addition of water to citric acid before gas generation was explored.

The minimal insemination concentration needed for fertilization was determined by the addition of decreasing numbers of spermatozoa to non-fertilized bisected oocytes. The experiment was repeated with a selected sperm insemination number in 1 ml or 50 µl culture media to compare the tested culture system with conventional culture. Spermatozoa bound to the hemi-zonae were counted with the aid of an inverted phase contrast microscope. Hemi-zonae with bound sperm were also stained with ethidium homodimer and evaluated using a confocal laser-scanning microscopy system. After removal of hemi-zonae, the spermatozoa in culture were isolated for deoxyribonucleic acid fragmentation analyses and reactive oxygen

species presence in the culture media was measured. Additionally, reactive oxygen species generation in simulated culture was measured over time.

Results: All the equipment tested bar one, the warming oven, proved useable with the simplified Walking Egg *in vitro* fertilization culture system. By decreasing the citric acid volumes, it was indicated that 1.8 ml citric acid, diluted with 1.2 ml water, is the optimal volume to facilitate the required culture media pH. Omitting the water dilution from citric acid volumes affected the culture media pH adversely, however reducing the temperature during gas equilibration did not. A change in altitude had no effect on culture media pH.

Lower insemination numbers resulted in decreased sperm binding, with 2×10^3 motile sperm insemination providing the lowest number to still obtain sufficient sperm–zona binding (≥20 sperm bound). Incubation in 1 ml vs. 200 µl culture media indicated decrease in sperm bound. Sperm deoxyribonucleic acid fragmentation and the presence of reactive oxygen species in the culture media were similar in both the test and control groups. A comparison over time revealed less reactive oxygen species in 1 ml culture media, from the simplified Walking Egg *in vitro* fertilization culture system after three days of culture, than 200 µl culture media drops under oil, from conventional culture after 18 hours, however the results were not statistically significant.

Discussion: Purpose-made heating devices provide superior stabilization of culture media temperature. When selecting a heating device, intra-variations should be considered. Culture media can be manipulated to the required pH by carbon dioxide production, with meticulous attention paid to the citric acid volumes used. However, if gas generation is performed at room temperature, equilibration time must be increased.

In conventional culture, the minimum insemination number can be reduced to 2×10^3 motile sperm. Due to lower binding of sperm in large volumes of culture media, $2 - 5 \times 10^3$ motile sperm should be considered for the simplified culture system, depending on a holistic consideration of all sperm parameters. Extended culture for at least three days with the simplified culture system can be performed without

increasing reactive oxygen species present in culture media. Further research of this novel culture method should include the application of the culture method in a South African environment.

Keywords: Affordable assisted reproduction, developing countries, DNA fragmentation, pH, quality control, reactive oxygen species, temperature, The Walking Egg

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LIST OF ABBREVIATIONS

ACCOG	-	Association of Childless Couples of Ghana
ART	-	Assisted reproductive technology
CA	-	Citric acid
CI	-	Confidence interval
CO_2	-	Carbon dioxide
CV	-	Coefficient of variance
DCF	-	2',7'-Dichlorodihydrofluorescein
DCF-DA	-	2',7'-Dichlorodihydrofluorescein diacetate
DNA	-	Deoxyribonucleic acid
ESHRE	-	European Society of Human Reproduction and Embryology
EthD	-	Ethidium homodimer
FSH	-	Follicle stimulating hormone
GMP	-	Good manufacturing procedures
GNI	-	Gross National Income
H^+	-	Hydrogen
HCO_3^-	-	Bicarbonate
HDI	-	Human development index
HEPA	-	High efficiency particulate air
HZA	-	Hemi-zona binding assay
ICSI	-	Intra-cytoplasmic sperm injection
ISO	-	International Organization for Standardization
IUCS	-	*In-utero* culture system
IUI	-	Intra-uterine insemination
IVF	-	*In vitro* fertilization
LH	-	Luteinizing hormone
N_2	-	Nitrogen
O_2	-	Oxygen
RBL	-	Reproductive Biology Laboratory
RFU	-	Relative fluorescence units
ROS	-	Reactive oxygen species
SB	-	Sodium bicarbonate

SD	-	Standard deviation
tWE	-	The Walking Egg
ULPA	-	Ultra-low particulate air
UN	-	United Nations
VOCs	-	Volatile organic compounds
VVOG	-	Flemish Society of Obstetrics and Gynaecology
WHO	-	World Health Organisation

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Chapter 1

Overview of study

Chapter 1 Overview of study

1.1. Motivation for research

Infertility is a worldwide problem with millions of couples in need of assisted reproductive technology (ART). Compromised fertility can be attributed to either the female i.e. bilateral tubal occlusion, anovulation, endometriosis or the male due to varicocele, azoospermia or asthenozoospermia, among others.[1] In some cases a combination of female and male factors can influence the fertility potential of a couple.[2] Due to the diversity of causes of infertility, therapeutic approaches can vary widely, even though the baseline approach remains the same, i.e. oocytes are fertilized with sperm, resulting in cleaved embryos to blastocysts, which can hatch and implant after transfer. During ART, oocytes can either be fertilized *in vivo* i.e. as part of an intra-uterine insemination (IUI) procedure or *in vitro* after the oocytes have been obtained by trans-vaginal oocyte aspiration.[1]

The high cost of ART, comprising 'direct' and 'indirect' costs, can render services inaccessible.[3] Direct fees include consultation, medication, laboratory and medical procedure bills.[3,4] Expenses incurred by the medical facility that are not directly associated with a specific patient, such as preparation and administration are recouped from a surcharge added to patient fees. This surcharge can be as high as 30%.[5] Laboratory fees that are usually fixed amounts based on the ART procedure, can amount to 48% of the total cost per procedure. These laboratory fees are the sum of disposables, media and solutions, as well as the cost of setting up a laboratory, purchasing equipment and remunerating staff.[6] Indirect costs are incurred as a consequence of the ART procedure being performed.[4] These could include the cost of travel for patients who have to visit a medical centre where ART is available, which in developing countries are often by unreliable transport systems,[7] accommodation and even airfares and visas if the patients have to travel vast distances.[6,8] Unplanned, indirect expenses are associated when complications with the ART procedure or pregnancy occurs. Clinical difficulties such as ovarian hyperstimulation syndrome can result in additional hospitalisation[5] and the occurrence of twin pregnancies also cause added expense for the patients.[9,10] Limiting the direct and indirect expenses, without compromising efficiency will economize procedures and increase accessibility to ART.

Affordability of ART is not only measured by the cost of ART, but rather determined by the economic burden placed on patients to fund their own treatment. An ART cycle is expensive, but when subsidized it becomes affordable to a larger number of patients.[3, 11] Economic accessibility to ART may be increased if ART cycles are subsidized by medical aid or government policies, but unfortunately ART subsidies are a concept mainly encountered in developed countries.[11,12] Although ART costs appear to be interrelated, direct cost-drivers can be manipulated to some extent depending on price and procedure flexibility. Reducing direct costs could imply milder ovarian stimulation by lower dosages of medication. In addition, laboratory costs could be decreased if more cost-effective methods are used to perform ART. However, there are many factors that influence *in vitro* embryo development.[1,13] Accurate monitoring of assisted reproduction by a controlled quality control (QC) programme (figure 1.1) is therefore most important.[14,15]
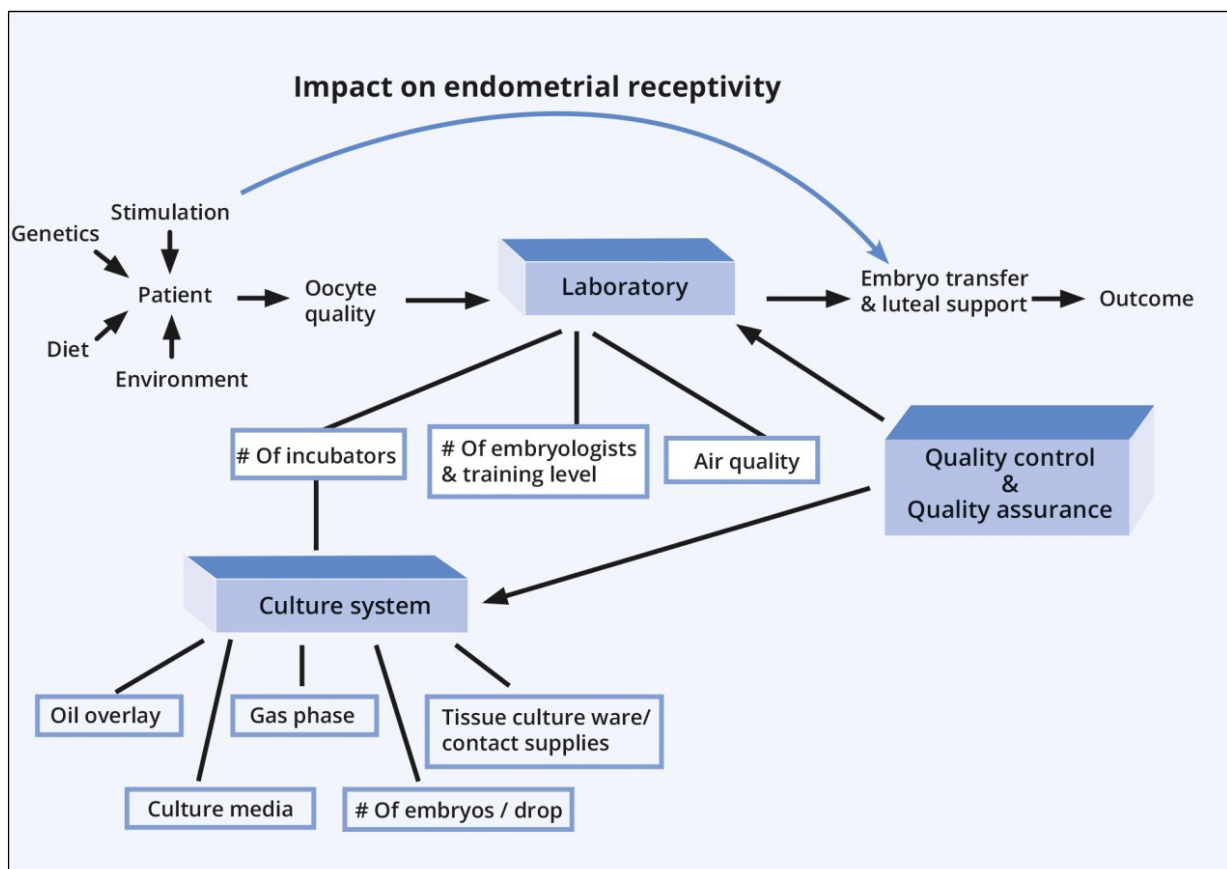
Figure 1.1: Aspects that may affect assisted reproductive outcomes and need to be monitored by laboratory quality control and assurance (originally adapted from Gardner D, Melbourne, Australia).[15]

The Walking Egg (tWE) foundation (tWE, Genk, Belgium)[16] is a non-profit organisation that focuses on providing accessible and affordable ART, specifically in developing countries, and has developed an ART culturing method to provide *in vitro* fertilization (IVF) procedures at lower cost.[17] The culture model, which does not require expensive laboratory equipment such as microprocessor-controlled tissue culture incubators and large area air filtration systems, was investigated by tWE foundation and successfully applied in an established ART programme in Genk, Belgium.[18] A roll-out of the system in developing countries has started, with the first tWE lab established at the Pentecost Fertility Centre in Accra, Ghana.[7,19]

This study investigated aspects of an ART laboratory using a basic culture system designed for human embryo culturing. The culture system was modelled on the simplified tWE IVF culture system and tested thoroughly to ensure that the embryo culture conditions met the required standards.

1.2. Research questions

1. Is it feasible to obtain ideal embryo culture conditions with less equipment than conventionally used during assisted reproduction, i.e. the simplified Walking Egg *in vitro* fertilization culture system vs. conventional assisted reproduction culture procedures?

2. Can a simplified culture system deliver similar outcomes regarding sperm-zona binding, sperm deoxyribonucleic acid damage and reactive oxygen species generation when compared with conventional culture?

1.3. Hypotheses

$H_{A(1)}$: Ideal embryo culture conditions can be obtained with less equipment than used in conventional assisted reproduction technology i.e. using the simplified Walking Egg *in vitro* fertilization culture system.

- Stable temperature (36.9 ±0.1°C) of embryo culture media can be ensured using different ART heating devices.

- The pH of embryo culture media can be manipulated to an ideal standard (7.30 ±0.05) using equipment as described by the simplified Walking Egg *in vitro* fertilization culture system.

$H_{A(2)}$: Minimal concentrations of spermatozoa can provide sufficient sperm-zona binding to result in oocyte fertilization.

- Insemination with low numbers ($<50 \times 10^3$) of motile sperm in large volumes of culture media (1 ml) will show sufficient sperm-zona binding.

- Insemination with low numbers ($<50 \times 10^3$) of motile sperm in large of culture media volumes (1 ml) will result in lower concentrations of reactive oxygen species and less sperm deoxyribonucleic acid damage.

1.4. Aim

The aim of this study was to investigate the use of an alternative and more affordable *in vitro* pre-implantation human embryo culture system by validation of culture conditions through the use of scientific testing.

1.5. Objectives

- Investigating different heating devices with the simplified Walking Egg *in vitro* fertilization culture system to determine which can reliably maintain culture media temperature at the desired level of 37°C;

- Determining the ideal volume of citric acid to establish a pH of 7.30 (±0.05) while using the simplified Walking Egg *in vitro* fertilization culture system in different environmental conditions i.e. at different altitudes; 37°C vs. ambient temperature; with and without the addition of water to citric acid;

- Determining with the use of a bio-test, the lowest number of motile spermatozoa that would provide sufficient sperm-zona binding to predict oocyte fertilization; and

- Comparing the percentage of sperm with deoxyribonucleic acid damage obtained after using the simplified Walking Egg *in vitro* fertilization culture system vs. conventional culture and measuring the amount of reactive oxygen species that are generated using either culture system.

Chapter 2

Literature review

Chapter 2 Literature review

2.1. Introduction

"All animals are equal, but some animals are more equal than others."[20] This quotation from James Orwell's Animal Farm is a satirical observation on the inequality of power, provided by government, but it could similarly have stated the inequality of assisted reproductive treatment in various populations in the world. Although motives for having a biological child vary, these can stem *inter alia* from a search for fulfilment and happiness, continuity of the family line, social status, religious beliefs and security in old age. However, notwithstanding the motives, the expectation to have children is global and transcends race, religion and socioeconomic status.[6,21,22] This is supported by the United Nations (UN) Declaration of Human Rights, Article 16:1 which states: "Men and women of full age, without any limitation due to race, nationality or religion have the right to marry and found a family".[23-25] However, despite the rapid growth of research in the field of reproductive medicine, infertility remains a global problem of which the needs have not been fully addressed.[21]

This is mirrored in the immense number of couples worldwide who experience infertility, ranging from 45 to 185 million.[8,21,26] The burden of infertility is probably underestimated. According to Hammarberg & Kirkman the international prevalence of infertility is rated at 9% on average, while infertility in some developing countries can be as high as 30-40%.[27] Vayena *et al.* report less than 20% of the global need for infertility treatment and 1% of the projected requirement in developing countries are being met.[28] The inequality in fertility treatment in developing countries originates largely from a shortage of health-care resources and the perception that high population growth rates do not merit expenditure on assisted reproductive technology (ART).[27,29] As a result, health-care facilities offering fertility diagnosis and treatment are largely absent, inaccessible or unaffordable in most developing countries.[8] Unfortunately, this sends a clear message that a woman in a developing country who is unable to fall pregnant is not eligible to receive infertility treatment as her neighbour has five or six children. This perception is completely contradictory to the UN Declaration of Human Rights, Article 16:1.

Accessibility and affordability of infertility treatment in developing countries cannot be separated. Since the expenditure associated with ART often counterbalances the treatment, if at all available, it remains inaccessible to the patient.[30] A drive towards more accessible and affordable infertility treatments has fuelled the quest to develop more economical alternatives to conventional ART.[8,17,31] This include standardized and comprehensive, yet cost-effective, fertility workup of patients.[17,32] The couple's medical history, combined with a one-day diagnostic evaluation of both partners can be used to aim for a treatment option that addresses the couple's specific needs.[17,24] This will avoid expensive ART treatments, when alternative options are available.[17] One of the alternatives to *in vitro* fertilization (IVF), depending on the patient profile, is to perform an intra-uterine insemination (IUI) or timed intercourse in a natural menstrual cycle or combined with mild ovarian stimulation via clomiphene citrate instead of gonadotropins.[26,33]

The most common reason for infertility in developing countries stems from sexually transmitted diseases and pregnancy-related infections.[17] Two-thirds of infertile women in the "infertility belt" in Central and Southern Africa are diagnosed with sterilizing reproductive tract infections that have resulted in blocked fallopian tubes.[29] This condition is two to four times more prevalent here than in the rest of the world.[29] Male infertility can be caused by infections of testicular, accessory glands and urethral tissue.[24,34] Sexually transmitted diseases could result in the presence of anti-sperm antibodies and sperm parameters can be affected.[34] These include, but are not limited to, sperm deoxyribonucleic acid (DNA) damage as well as sperm count, motility and morphology.[34,35]

Female infertility due to bilateral fallopian tube blockage or severe male infertility can often be treated only by oocyte aspiration followed by IVF or intra-cytoplasmic sperm injection (ICSI).[26,32] This need for affordable and accessible ART in developing countries, where infections are extensively prevalent and funds are scarce, underlines the necessity for research into procedural cost reductions, without compromising treatment quality.

2.2. A historical journey through the rise of assisted reproduction

Assisted reproduction comprises of multiple fields of medicine and science. Gynaecology, laparoscopy, sonography, microscopy, andrology, physiology, anatomy, cell culture, micromanipulation, cryopreservation and other sciences combine to assist in the reproduction of the human species.[36,37] As a result of co-operative efforts in these fields, it is almost impossible to define exactly where the history of ART began. Figure 2.1 provides a timeline with a selection of events in the evolution of ART as discussed in this section.

Early times and the search for the origin of life

Research on reproduction dates to ancient times, with possibly the first description of human conception in the ancient Hindu text *Garbha Upinandas* (1416 BC) entailing the combination of semen and the mother's blood to bring an embryo into existence.[38] Around 400 BC, Hippocrates wrote that an embryo is formed from semen of both the male and the female,[39] after which it develops inside a membrane while being fed by the mother's blood.[38] Circa 350 BC, Aristotle cracked open chicken eggs to follow the development of embryonic growth.[38,40] He also theorised that a child does not take on just any form, but is given form by his parents' seeds, which combine to form the child.[41]

Throughout history more theories surrounding reproduction, reproductive anatomy and embryology have been formulated by researchers such as Claudius Galenus (~200 AD), Albertus Magnus (~1250 AD), Leonardo da Vinci (~1500 AD), Gabriel Fallopius (~1550 AD), William Harvey (~1600 AD) and Renier de Graaf (~1650 AD).[38-40,42] Most of these theories were observational in nature. However, after the development of the microscope, more concrete data became available. Antoni van Leeuwenhoek, a Dutch merchant and craftsman by trade, was so fascinated by the magnifying "weaver's glass" used for counting threads, that he was inspired to develop the first microscope, applying principles that are in use up to date.[43] Acknowledged as the father of optic microscopy, in 1677 he became the first person to describe spermatozoa.[42-44] Van Leeuwenhoek and De Graaf brought into view the two gametes needed for fertilization.[44] Unfortunately, dissimilarity on the subject was reported as Van Leeuwenhoek opined that nothing as big as a Graafian follicle could pass through a passage as small as a fallopian tube.[44]
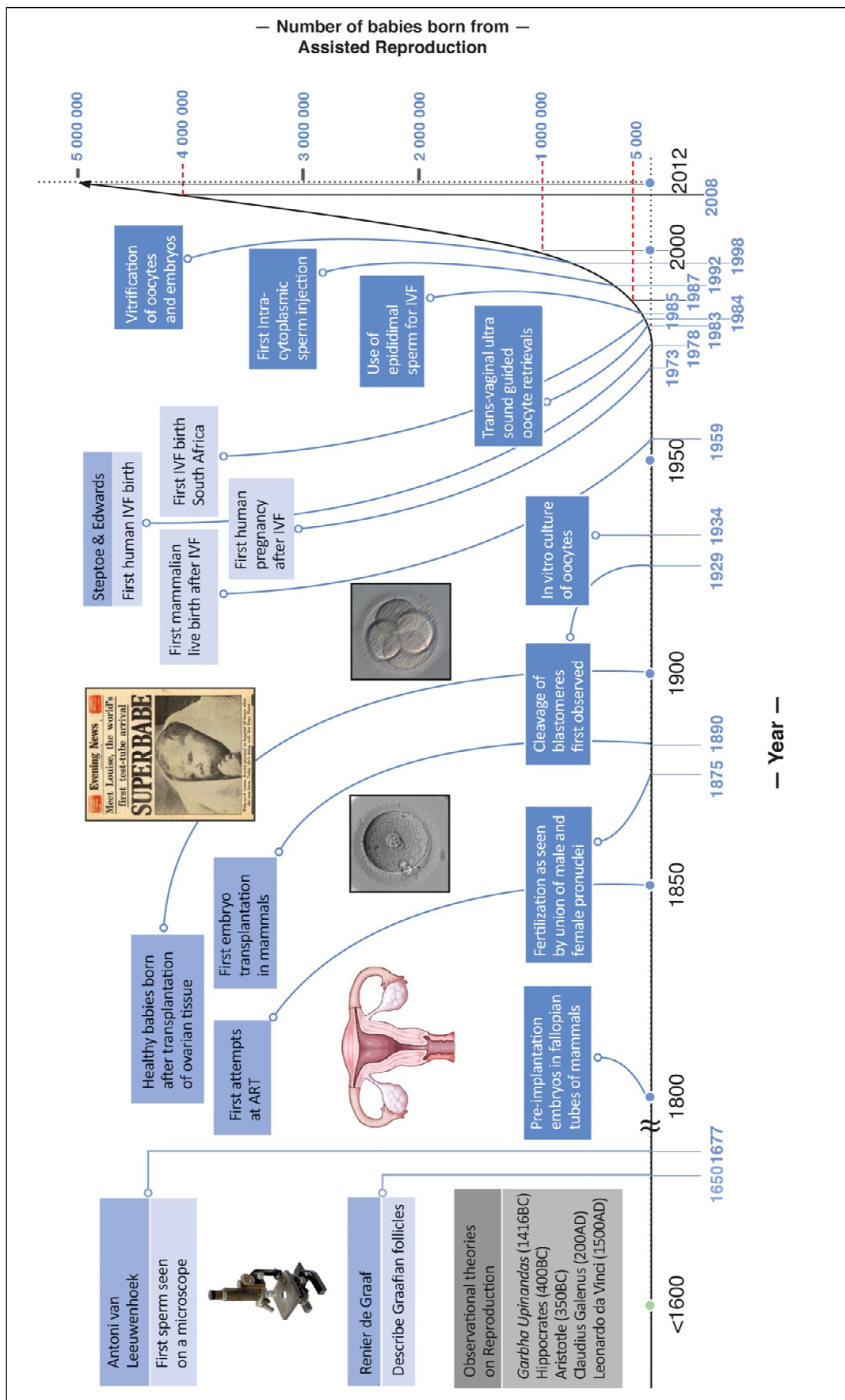
Figure 2.1: Timeline of major historic events related to the advancement of assisted reproductive treatment (references for each time-point in text).

Milestones during the establishment of assisted reproduction treatment

The discovery of pre-implantation embryos in the fallopian tubes of mammals (mid 1800s)[45] and the first known case of embryo transplantation by Walter Heape (1890s), albeit in rabbits,[37,38] were milestones in the field of reproductive research. Based on the former breakthrough, bilateral fallopian tube occlusion was inferred as being a cause of infertility. In The Lancet (1849), W Tyler Smith described one of the first possible infertility treatments as a procedure where blocked fallopian tubes were catheterized with a whalebone "bougie" and a silver catheter.[45,46] Met with criticism from peers, this operation faded into the pages of history.[45] Later attempts at ART around the turn of the 19th century showed more success, with healthy born babies reported after the transplantation of ovarian tissue into the uterus or at the stump of a severed fallopian tube.[45,47] Although the mechanism of oocyte fertilization was not yet known, the use of transplanted ovarian tissue was frowned upon by some observers due to ethical and legal considerations of who the natural parents were after such a procedure and whether such a child could conceivably have three parents.[45,47]

Parallel to the work done during the late 1800s on occluded fallopian tubes and on-going to the mid-1900s, major breakthroughs were made in the field of IVF of mammalian oocytes and the subsequent culture of embryos. Several groups were working on this topic and the different steps of fertilization were described by scientists such as Heape, Schenk, Onanoff, Krasovskaja and Pincus.[38,48] In 1875 and 1876, Van Beneden and Hertwig observed the formation and union of pronuclei in rabbit and sea-urchin eggs respectively and establishing the modern concept of fertilization.[49] Lewis and Wright were the first to film and report on the cleavage of blastomeres in 1929 (on mice embryos),[38] while Pincus and Enzmann reported in 1934 on their attempts at IVF,[50] which gave considerable insight into the field of *in vitro* culture of oocytes.[45,49]

First successes and the development of modern assisted reproduction

While the first report on a mammalian live birth after IVF was written by Chang in 1959,[37,45,51] the first pregnancy in humans was reported in 1973.[52] Several groups were in competition during the 1970s to achieve the first human IVF derived life birth.[52] In 1978 Patrick Steptoe and Robert Edwards were able to report that they

had indeed won this race (figure 2.2),[37,53] an accomplishment for which Edwards was awarded the Nobel Prize in Physiology or Medicine in 2010.[54] More births followed quickly after this triumph and *in vitro* achieved births were reported in Australia in 1979,[52] the USA in 1981,[37,45] Sweden and France in 1982,[55] South Africa (University of Stellenbosch) in early 1984[6,56] and at the ART unit of the University of Pretoria later in 1984.[6,57] By 1986, 140 IVF programmes had been recorded world-wide in the registry of the Journal of *In Vitro* Fertilization and Embryo Transfer,[58] and by 1987 more than 5000 babies had been born through IVF,[59,60] establishing ART firmly as a growing new field.

Figure 2.2: A: Cover of the Evening News (27 July 1978), announcing the arrival of Louise Brown, the first *in vitro* fertilization derived life born baby[61] and B: Robert Edwards with Louise (age 30), her mother Lesley, and son Cameron.[62]

The field of assisted reproduction and specifically *in vitro* embryo culture has come a long way since the birth of Louise Brown on July 25, 1978. The beginnings of refined embryo culture media started with the investigation of scientists such as Hope and Whitten who reported on the use of albumin in a modified simple Krebs-Ringer bicarbonate solution.[63] The B2 culture media, developed by Menezo,[64] preceded the well-known human tubal fluid media from the team of Quinn,[65] who continued to search for better results by modification of this embryo culture media.[66] The continual development of culture media in work of scientists such as David Gardner,[67] John Biggers,[68,69] Patrick Quinn,[66,67] Simon Cooke,[70,71] Thomas Pool[67,69] and others[13,67] paved the way to availability of the multitude of commercial embryo culture media that are currently available.[13,72]

Further developments in the field of assisted reproduction followed with the inclusion of embryo freezing,[52] trans-vaginal ultra-sound guided oocyte retrievals,[73] the use of epididymal sperm for IVF,[74] micro-injection of sperm under the zona pellucida[75] and ICSI.[76,77] Pregnancies after biopsy and pre-implantation genetic testing have been reported since 1995,[76,78] as well as after the vitrification of oocytes and embryos in 1998.[79] All these developments occurred within twenty years of the first IVF birth, before the end of the twentieth century. With numerous advancements, more than one million IVF babies had been reported by 2002, a number that increased to more than five million by 2012.[37,80] The science of reproduction that is growing exponentially remains one of the most interesting and perplexing fields in biology with so much left to be discovered.

2.3. A selected overview of embryo culture

From oocyte aspiration to consecutive *in vitro* embryo culture, there are many factors that influence the development of the embryos.[1,13] Selected areas of *in vitro* embryo development and culture, that pertains to the current research will be discussed further, including gamete interaction during oocyte fertilization and the micro-environment in which the embryos are cultured i.e. temperature and pH stability of culture media, as well as the macro-environment of the laboratory.[1,15]

2.3.1. In vivo maturation and in vitro interaction of gametes

Oocyte development and maturation

Fertilization of oocytes by spermatozoa involves a cascade of events (figure 2.3).[81-83] Oogenesis initiates during foetal development in humans, however goes into arrest at prophase I of meiosis.[15,84] At this maturational stage the nucleus is termed a germinal vesicle, which can be observed *in vitro* as a round structure with a distinctive single nucleolus inside (figure 2.3A).[83,85] The oocyte remains in meiotic arrest for years, until the onset of ovulatory cycles when the female reaches puberty.[86] Once an antral follicle is recruited to develop further, the oocyte undergoes cytoplasmic development in preparation for ovulation.[15,87] After a pre-ovulatory surge in luteinizing hormone (LH) and follicle-stimulating hormone (FSH), the oocyte resumes the steps of meiosis I.[84,86] Germinal vesicle breakdown occurs and during metaphase I the oocyte is a large single cell with no nuclear material visible (figure 2.3B).[83,87] Meiosis I culminates in asymmetrical cell division, giving rise

to the large functional oocyte and a small first polar body (figure 2.3C).[85,86] After meiosis I has been completed, the oocyte proceeds into the second meiotic division until another meiotic arrest at metaphase II occurs.[15,83]
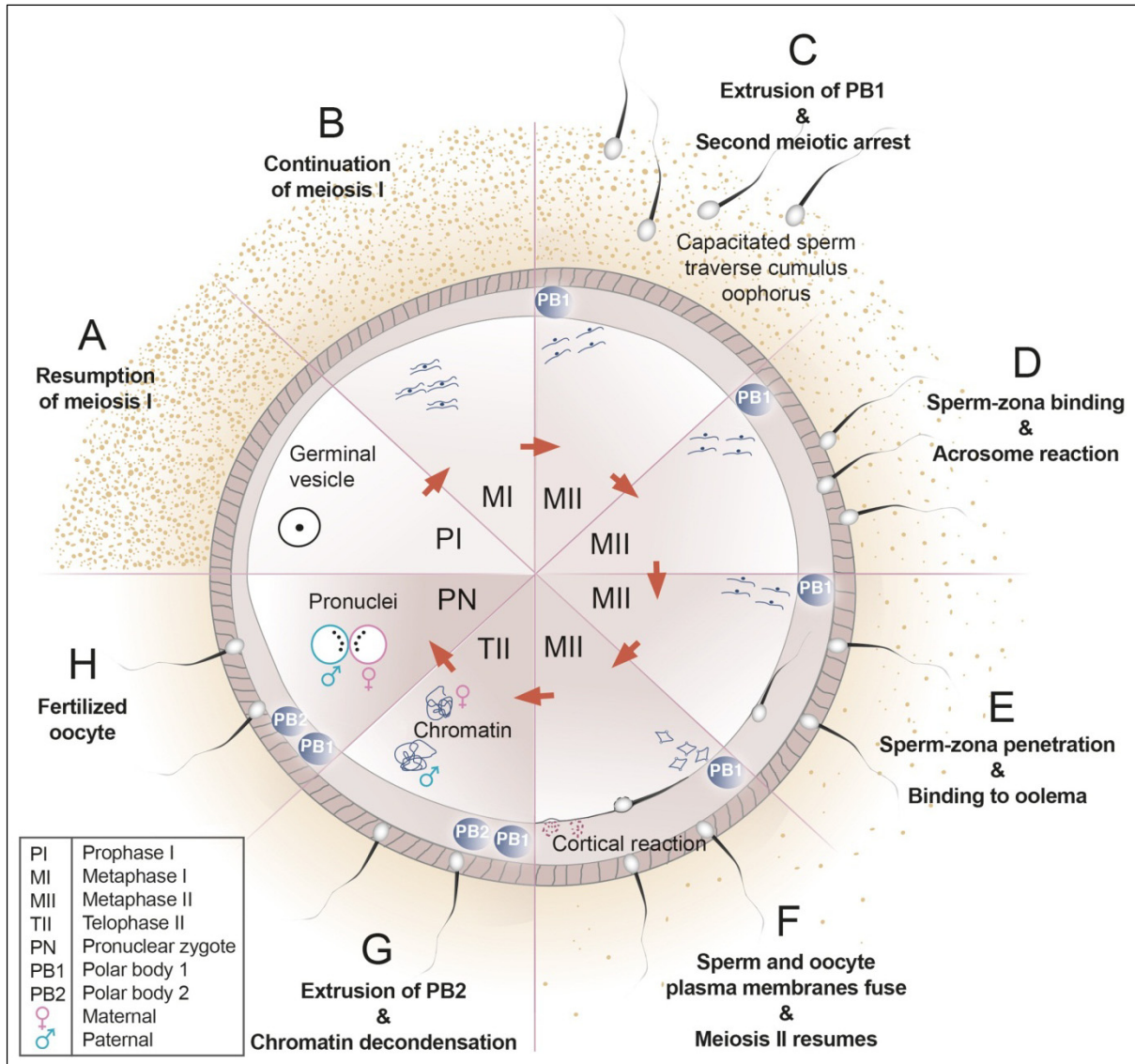
Figure 2.3: The cascade of events leading to fertilization, starting with oocyte maturation from A: prophase I with a germinal vesicle present, past B: metaphase I to C: a mature metaphase II oocyte with capacitated sperm present. D: indicates the binding of sperm to the zona pellucida and acrosome reaction, with E: showing a single sperm that has entered the zona pellucida and bound to the oolema. F: depicts the fusing of sperm and oocyte plasma membranes, cortical reaction and re-activation of meiosis II, G: the decondensation of genetic material and extrusion of a second polar body, culminating in H: a fertilized oocyte with both male and female pronuclei visible (references for each event in text).

The arrested meiosis II oocyte is ready for fertilization approximately 38-40 hours after the LH and FSH surge, with meiosis continuing only when fertilization occurs.[15,85] During ART procedures, trans-vaginal oocyte aspirations usually takes place approximately 36 hours after a human chorionic gonadotropin or an LH injection triggers the LH/FSH surge.[37,86] This can often lead to the cumulus-oocyte-complex containing an oocyte not at the ideal stage of maturation for fertilization.[84] The desired stage should however be reached within 3-4 hours after aspiration.[37,86]

Sperm capacitation and acrosome reaction

Following ejaculation, a series of physiological changes need to occur for the sperm within the seminal fluid to be able to bind and fertilize an oocyte.[54,88,89] The capacitation process is required for oocyte binding and occurs *in vivo* over several hours while the sperm passes through the female reproductive tract.[54,88] The progression of capacitation can be induced *in vitro* by additives to the semen sample, such as albumin or heparin.[81,89] Density gradient centrifugation used during sperm preparation for IVF or IUI also induces sperm capacitation, allowing embryologists to use the prepared sperm for insemination without further manipulation.[90,91]

When the capacitated sperm encounters a cumulus-oocyte-complex, the sperm will pass through the cumulus oophorus surrounding the oocyte propelled by the hyperactivated movement of the sperm and the digestive action of hyaloronidase found on the surface of the sperm head (figure 2.3C).[81,92] Subsequently the acrosome reaction takes place, of which the precise activation is not completely understood, with commencement signified when the capacitated sperm binds to the zona pellucida surrounding the oocyte (figure 2.3D).[54,81,88,93] Some researchers theorise that the acrosome reaction can be induced earlier by contact with the cumulus cells.[88,94] Irrespective of when the acrosome reaction begins, once the sperm is bound to the oocyte, the acrosomal content is released, digesting a pathway for the sperm through the zona pellucida (figure 2.3E).[83,92]

Physiological gamete interactions leading to fertilization

Once the inner acrosomal membrane of the sperm makes contact with the oocyte's outer membrane, a fusion of the membranes occurs (figure 2.3F),[15,92] allowing the complete sperm head i.e. the sperm nucleus to enter the oocyte.[83,93] Subsequent to

this event, cortical granules that lie peripherally in the oocyte fuse with the oocyte plasma membrane (figure 2.3F).[85,87,95] The expulsion of the cortical granule content into the perivitiline space between the oocyte and zona pellucida elicits a zona reaction.[83] A zona reaction is defined as a chemical hardening of the zona pellucida that prevents supernumerary sperm from penetrating the zona,[83,85] thereby inhibiting many sperm from fusing with the oocyte.[83,87,95] Sperm bound to the zona pellucida at the time of fertilization will remain there.[85,96] The number of sperm bound to the zona pellucida is considered to be indicative of fertilization potential.[96,97]

After fusion of the sperm and oocyte, the chromatin from the naked sperm nucleus decondenses and develops into the male pronucleus.[15,83] The increase in intra-cellular calcium, triggered by sperm penetration, activates the oocyte to continue with meiosis to telophase II,[15] culminating in another asymmetrical cell division into a large oocyte and a small second polar body (figure 2.3G).[15,83,86] Once meiosis II has been completed, the female nuclear material decondense and a pronucleus starts forming and becomes associated with the male pronucleus.[15,83] The male and female pronuclei can be visible from as early as four hours after gamete fusion.[15] The presence of two polar bodies in the perivitiline space and two pronuclei in the oocyte is an indication of normal fertilization (figures 2.3H and 2.4).[15,86] After pronuclear association has taken place (approximately 18-24 hours after gamete fusion), the pronuclear envelopes will break down and parental chromosomes will come together in syngamy to form the embryonic genome.[15,83]

Figure 2.4: Fertilized human oocyte, with male and female pronuclei showing nucleoli and two polar bodies visible in the perivitiline space (Photo Library, Reproductive Biology Laboratory, University of Pretoria).

During conventional IVF, a cumulus-oocyte-complex is cultured in a micro-drop of ~50 – 250 µl culture media with the addition of ~25 – 100 x 10^3 motile sperm,[15,86] followed by an incubation period of ~16 – 18 hours. After the incubation period, denudation of the oocyte and transfer to unused, pre-equilibrated culture media occurs. Once isolated from the cumulus cells, an evaluation of the oocyte is performed to confirm fertilization.[15]

2.3.2. Factors impacting on embryo culture

2.3.2.1. Temperature

Stable control of temperature fluctuations throughout human embryo culture is imperative.[36,98,99] The human oocyte and pre-implantation embryo are very sensitive to variation in temperature outside the physiological range of approximately 37°C.[100,101] Temperature, along with culture media pH, is one of the few parameters that can be manipulated during embryo culture.[36] This section will discuss temperature regulation during human embryo culture in detail, along with a discussion on culture media pH in the following section.

Temperature sensitivity is due to the spindle microtubules within the oocytes and embryos that attach to chromosomes and chromatids during meiosis and mitosis, respectively.[15,100] These microtubules are responsible for fixing chromosomal position and segregating chromatids in anaphase during mitotic cell division,[15,102] with any decrease in temperature initiating degradation of these cellular structures.[15,103] A minor decrease in temperature of less than 2°C will allow the microtubules to reform, once temperature has returned to the physiological range.[36,103] However, with a major reduction in temperature (<35°C) the changes to the spindle could possibly be irreversible.[15,36] Should the microtubules be damaged, the movement of genetic material to daughter cells will be compromised.[36,99,102] Culture temperatures above 37°C also pose a risk to embryo development through the formation of heat-shock proteins, which have been shown to affect embryo development negatively.[36,104] Although the amount of temperature fluctuation that oocytes and embryos can withstand is unknown, heat loss or overheating can often be traced when compromised embryo development is suspected.[36,105,106] A study by Sherbahn reports follicular fluid temperature during oocyte retrieval leads to significantly better

blastulation, pregnancy and live birth rates when performed at 36.4-36.9°C rather than at lower (<36.4) or higher (>36.9) temperatures.[107]

From the time a gamete leaves the human body, whether a sperm cell during ejaculation or an oocyte during oocyte aspiration, the temperature at which the cell is kept needs to be strictly controlled.[36] The semen sample should be placed in a warming oven or on a heated surface set at 37°C and all equipment used during processing of the semen must be set to 37°C using a standard measuring instrument.[36,108] During trans-vaginal oocyte aspiration, warming blocks or tube heaters are used to maintain follicular fluid at 37°C.[36,37] Once the oocytes have been identified in the follicular fluid, care must be taken to minimize temperature fluctuations when transferring between culture media dishes.[15,37] After the oocytes have been moved to an embryo culture incubator, all the consecutive steps of embryo culture will be performed according to standard operative procedures. The appropriate culture dish will be removed from the embryo culture incubator, the necessary procedure performed and returned to the incubator.[37] When the culture dish is removed from the incubator, fluctuations in temperature are minimized if the dish is returned without delay.[37,100,102]

Placing dishes on surfaces heated to the appropriate temperature when out of the incubator similarly diminish the chance of a loss in temperature.[37,100] Equipment that is commonly used during embryo manipulations to curtail temperature variations includes, among others, controlled temperature surfaces of IVF workstations, microscope heated stages and slide warmers.[37,109] All this equipment must be calibrated and tested regularly to ensure that the correct temperature is indicated.[36] The method and cost of testing and calibration differ. Equipment with digital calibration settings can usually be checked and calibrated by laboratory personnel.[36] However, large surface areas, such as IVF workstations, must be tested at multiple positions and the calibration of these areas may have to be performed by a qualified technician in order to ensure uniformity.[37,100] Calibration and checking of equipment cannot be performed merely by measuring the heated surface. A simulation of the work done with the equipment must be performed and the temperature of water/culture media used should be measured.[36,100] After calibration, it is important to take note of the surface temperature, as it may well be slightly above 37°C. This

temperature should be targeted when daily quality control (QC) checks are performed.[100] There have been many reports on the differences in temperature of various incubators, even of the same make and model, or variations in temperature at certain positions within a single incubator.[14,36,98,101,110] It is of the utmost importance to use incubators that are not only reliable, stable and calibrated, but also to identify hot or cold spots in the incubator.[100,101]

The mode of temperature regulation and the needs of the laboratory must always be given attention when a new embryo culture incubator is introduced. The direct heat transfer used in bench-top incubators ensures more stable temperature control and faster return to the desired temperature after opening than front-loaded box-type incubators.[100,110] Kelly *et al.* report internal temperature stability of within 0.1°C after having tested a bench-top incubator, although only 1.1°C stability after having tested a box-type incubator.[110] The times for temperature recovery of a culture dish in a bench-top and front-loaded incubator, after a five second opening of the incubator door, are reported as being less than five minutes and approximately thirty minutes, respectively.[98,110] On the other hand, the water jacket surrounding front-loaded box-type incubators can maintain temperature at the required level for much longer in the event of a power failure,[37,100] i.e. a water-jacketed incubator that maintains temperature with a loss of only 1°C per hour after losing power has an air- jacketed counterpart which lose 3°C per hour.[37] This may be an advantage should the electricity supply to a laboratory be unreliable, when compared to a bench-top incubator that will cool down to ambient temperature within an hour of losing power.[36,37]

2.3.2.2. Acidity of culture media

As with temperature control in the IVF laboratory, the acidity, or pH value, of culture media is of importance to embryo development and is a parameter that can easily be manipulated to the desired levels.[72] The pH of culture media affects the intra-cellular pH (pHi) of gametes and embryos within the culture media.[36,72]

As the pHi regulates multiple metabolic functions, such as gluconeogenesis, protein synthesis, membrane transport, cell division and differentiation,[14,69,111] a slight change in pHi can dramatically impact embryo metabolism.[37] Since pHi cannot be

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measured practically, most ART laboratories use the measurement of culture media pH or carbon dioxide (CO_2) as an indicator of optimal culture conditions.[14] It is known that the pHi of embryos is approximately 7.1-7.2.[72] However, due to acidification through intra-cellular metabolic processes a slightly higher culture media pH should be maintained.[72] The use of commercially available human IVF culture media is frequently recommended at pH 7.2-7.4.[37,72] The relationship between hydrogen (H^+) concentration and pH is described by the Henderson-Hasselbach equation ($pH = pK_a + \log_{10}[H^+]$).[71] From this equation it can be calculated that as the pH scale is logarithmic, a 0.2 change in pH is in effect a >60% change in H^+ concentration, therefore pH fluctuations in the laboratory should be kept as minimal as possible.[72]

Culture media used during IVF is manufactured with a bicarbonate-salt pH buffer that deprotonates in the culture media into bicarbonate (HCO_3^-) and the associated positive ion (dependent on the culture media manufacturer).[112-114] The CO_2 in air surrounding the culture media i.e. gas composition as used with the specific incubator, will dissolve in the culture media to a certain extent (dependent on the partial pressure of CO_2).[36,71,115] The CO_2 will combine with water in the culture media to form carbonic acid (H_2CO_3) and then dissociate into HCO_3^- and H^+ ions.[14,15] The amount of HCO_3^- already present in the culture media will regulate the dissociation of H_2CO_3.[15,115] An increase in CO_2 and the effectual increase in HCO_3^- will bind to H^+, resulting in an increase in H_2CO_3 concentration and a decrease in H^+ concentration. As pH is a measurement of acidity by H^+, the decrease in H^+ concentration means a decrease in acidity, i.e. an increase in pH value. This shows that by manipulating the CO_2 composition of the gas used in the incubator, the pH of culture media can be regulated.[69]

Most front-loaded box-type incubators are connected to pure CO_2 and an infra-red or thermo-conductivity CO_2 sensor measures the level of CO_2 in the incubator.[37,100] Infra-red CO_2 sensors are more accurate and faster than thermo-conductivity sensors, resulting in a faster CO_2 recovery rate after the incubator was opened,[15,37,116] but are sensitive to moisture and more expensive than thermo-conductivity sensors. As the growing embryo will not be exposed to oxygen (O_2) levels higher than approximately 8% *in vivo*, embryo culture in a lower oxygen (5%)

level is used increasingly in ART, especially when culturing to the blastocyst stage.[37,116] Most bench-top incubators maintain the correct gas mixture by the constant injection of a pre-mixed tri-gas mixture consisting of the desired CO_2 percentage, 5% O_2 with nitrogen (N_2) making up the remainder of the mixture. This provides a steady supply of the correct gas mixture, although it is dependent on a reliable source of gas cylinders that have the accurately mixed gas mixture and are supplied with a certificate of analysis.[36] The amount of CO_2 that will dissolve in culture media is dependent on the partial pressure of CO_2 in the complete gas mixture.[14,71] Since there is a change in atmospheric pressure with a variation in altitude, there will also be a change in CO_2 partial pressure. This implies that when altitude is changed, a change in CO_2 concentration is needed to ensure that the same amount of CO_2 dissolves in culture media, thereby providing the same pH.[14,115] Figure 2.5 shows that as altitude increases, a higher percentage of CO_2 needs to be used to make up the complete gas mixture and to attain the same pH value in culture media.[14]
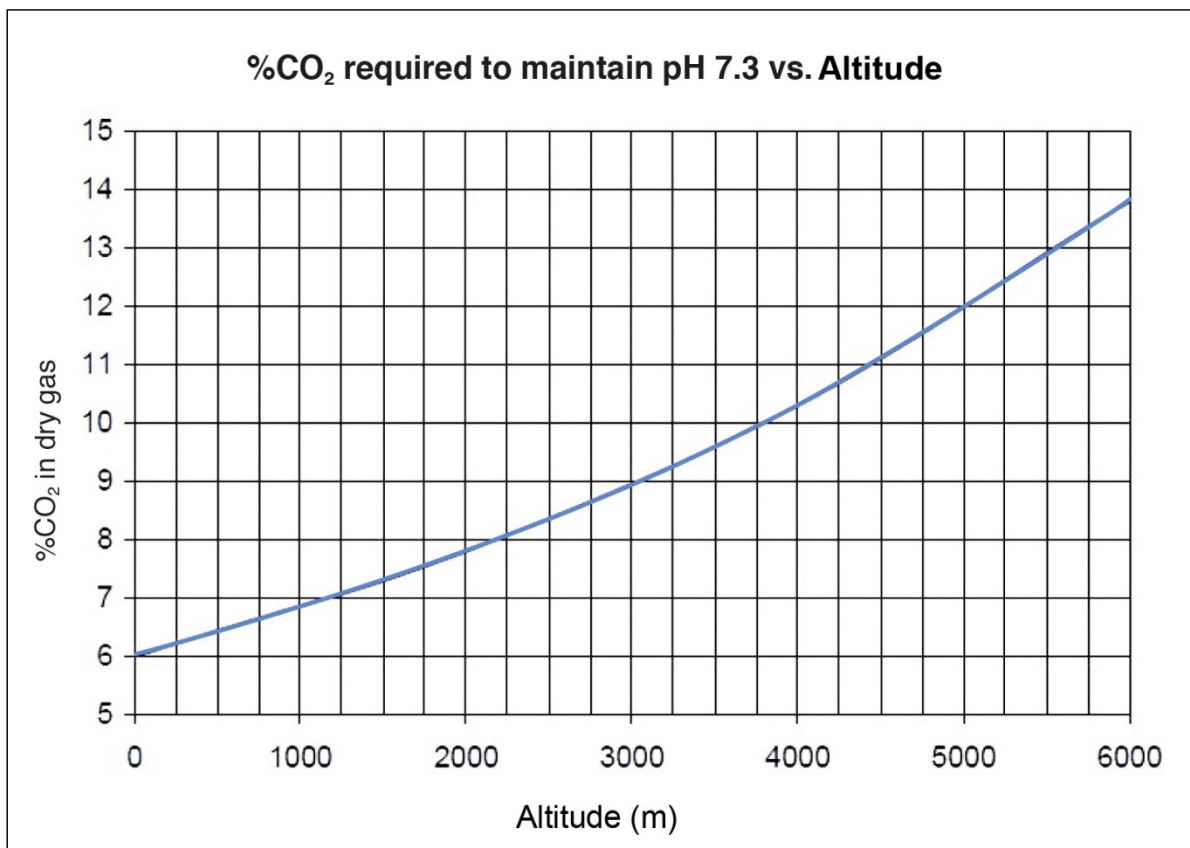
Figure 2.5: Relationship of altitude (meters above sea level) to percentage of carbon dioxide required to maintain culture media pH of 7.3 (adapted).[14]

One disadvantage of the pre-mixed gas system is the rigidity of the gas mixture, since the only way to change the gas composition used is to commission a new cylinder of gas.[37,69] An alternative to pre-mixed gas is available on larger bench-top incubators and some front-loaded incubators, where pure CO_2 and N_2 are connected to the incubator and mixed in a mixing chamber before being injected into the incubator. The N_2 is used to reduce the amount of O_2 in the gas mixture, and the CO_2 and O_2 levels can be set with a real-time response from the incubator.[37] Care must be taken that CO_2 and O_2 levels are regularly confirmed by external measurements when gasses are mixed within the incubator.[37,69] Alternative methods of gas supply for the culture of embryos have been suggested. The *de novo* production of CO_2 in a closed system reported by Swain provides the laboratory with sterile CO_2, without the impurities found in gas cylinders.[117] Another suggestion, which has been in use for years during bovine embryo culture, is the use of filtered expired air.[118] In this instance, embryo culture takes place in a closed environment and air from a person's lungs is exhaled through a filter into the closed system. As exhaled air has less O_2 (approximately 16%) and more CO_2 (approximately 4%) than ambient air, this is able to support embryo development.[118] These alternative methods of CO_2 supply have the potential to support more cost-effective methods of embryo culture.

2.3.2.3. Air quality and reactive oxygen species

The environment in which an embryo is cultured plays a significant role in the embryo's development. The culture media surrounding the embryo and the laboratory's atmosphere cumulatively affects the eventual quality of the embryo.[14,36,100] It has been proved that the following laboratory factors, among others, affect embryo development: air quality, whether filtered and whether it contains volatile organic compounds (VOCs); light intensity and ambient room temperature; disposables used during embryo culture; the culture media oil overlay; and the handling of the culture media.[14,15]

Air quality and the presence of VOCs in an IVF laboratory can be detrimental to embryo development and subsequent clinical pregnancy rates.[14,119] Although air quality can be measured by a particulate counter, it is reported that even a particulate count of less than 0.1 parts per billion can have VOCs such as toluene in significantly

higher numbers.[120] The presence of any particulate in the air poses a threat to the oocytes and embryos.[14] The safest option is to have a high efficiency particulate air (HEPA) or ultra-low particulate air (ULPA) filter system in-line with the air supply to the laboratory.[14] Further safeguards include an air-pressure difference of 10-15 pascals between the IVF laboratory and adjacent rooms[36] and having an activated carbon filter to collect VOCs not removed by particulate filtering and potassium permanganate filters to oxidize compounds such as alcohols and ketones not trapped by the carbon.[14,121] Buildings can produce VOCs for years after completion[36,119] and since the equipment in operation in the laboratory also generates VOCs,[121] air in the laboratory should be filtered continuously. A mobile air circulator with a HEPA filter, such as a CODA® filter could be used to clean the air present in the laboratory.[121] In-line HEPA filters should be employed to filter gas flowing into the incubators, as this gas that bypasses the laboratory's air filtration system will be in direct contact with the culture dishes.[37]

In many countries specific air quality standards are required in ART laboratories.[122] The Brazilian Cells and Germinative Tissues Directive stipulates air quality where cells are exposed to the environment should be at least equivalent to the International Organization for Standardization (ISO) cleanroom standard ISO 14644-1 class 5.[122] The European Union Directive 2006/86/EC states that Good Manufacturing Procedures (GMP) grade A air quality is required where cells are exposed to the environment, with a background environment of grade D or better (GMP grading progress from A to D in regards to air quality).[36,123] The ISO 14644-1 class 5 and GMP grade A standards are equivalent to each other and the US Federal Standard 209E class 100, which allows a maximum of 100 particles of 0.5 micron or larger per cubic foot of air.[36,124] Conversely, ISO 14644-1 class 8, GMP grade D and US Federal Standard 209E class 100 000 is considered air with a maximum of 100 000 particles of 0.5 micron or larger within one cubic foot volume.[36,124]

Working in a certified IVF workstation with a HEPA/ULPA filter, or even in a closed workstation that is HEPA/ULPA and VOC filtered and sometimes even ultra-violet treated, could assist in reducing the number of compounds with which a developing embryo comes into contact.[37] The last line of defence is the oil overlay, which forms a physical barrier that traps any particles that may end up in the culture dish and acts

as a sink to catch and hold minute levels of chemical or volatile compounds.[36,121] Not only does the oil overlay protect the developing embryos from VOCs, it also protects the media from evaporation and buffer against sudden changes in temperature and gas composition.[36]

Apart from physical contaminants, free radicals in culture media can also affect the development of embryos negatively.[125] Free radicals and reactive oxygen species (ROS) are by-products of cellular aerobic metabolism.[126,127] On the one hand, ROS is needed for certain steps in development, such as the capacitation and acrosome reaction of sperm,[35,54] and may even be implicated in the process of oocyte maturation.[127] Furthermore, high levels of ROS have been linked to increased sperm DNA fragmentation, lower oocyte fertilization rates, decreased embryo development resulting in embryos with uneven divisions and increased fragmentation, as well as decreased clinical pregnancy rates.[35,128,129] The manipulation of gametes and *in vitro* culture of embryos has been shown to produce increased levels of ROS due to exposure to light, unbalanced metabolic substrate concentrations, high oxygen levels, possibly xenobiotics amongst other factors.[128]

Antioxidant defence mechanisms are found in seminal plasma as well as in external and internal defence mechanisms in the *in vivo* oviduct system.[125,128] During *in vitro* culture of embryos however, the seminal plasma and external antioxidant defence mechanisms are removed.[35,128] The embryo's intra-cellular defence, which relies mainly on antioxidant enzymes,[127] remains to protect the developing embryo while a supplementary, artificial external defence is created by the addition of antioxidants to embryo culture media.[128] The presence of ROS should not be eliminated, because there is consensus that a minimal amount of ROS in culture media is advantageous.[125,127,128] The unnecessary build-up of ROS by reducing the number of laboratory procedures resulting in ROS production to a minimum, i.e. exposure to light, oxygen and increased metabolic by-products should be promoted to avoid oxidative stress.[128] The equilibrium between ROS and antioxidants is complex with the choice of antioxidant to add to culture media being difficult to ascertain.[112,125,128]

2.4. Operational expansion in assisted reproductive technology

Since the beginning of ART, there have been on-going investigations to improve embryo culture and the eventual clinical pregnancy rates. As the field of assisted reproduction expanded, more scientists added their efforts, all with different ideas and approaches to the problem, culminating in a wide variety of possible changes to the ART laboratory.[76]

One of the most exciting new developments in embryo culture incubation is the variety of time-lapse systems that have become commercially available.[112,130,131] Through these incubation systems, the developing embryos can undergo continual, real-time evaluation with no disturbance to culture conditions.[131,132] Apart from this exposure reduction of environmental stressors to the developing embryo, the time-lapse systems also provide vast amounts of information on the morphokinetic parameters during embryo development.[112,132]

Alongside the improvements in embryo culture and subsequent selection, embryo assessments can extend to genetic testing.[133] The practice of biopsy and pre-implantation genetic evaluation has gained momentum over the past few years[133-135] and a variety of biopsy techniques are available.[133-136] With the advancement of genetic testing and the increased survival rates of cryopreserved embryos after vitrification, embryo and specifically trophectoderm biopsy is proving to be an effective tool in the IVF laboratory.[132,133,136] Through the application of vitrification, the survival rate of cryopreserved oocytes and embryos have dramatically increased.[137] Due to this improvement and the known detrimental effect of ovarian stimulation on endometrial receptivity, more and more ART laboratories are considering freezing all embryos and transferring thawed embryos in a natural cycle.[137,138]

Apart from the recent developments and enhancement of basic practices in ART, many innovative techniques are being investigated to potentially improve embryo culture. Some of these include invasive procedures such as the biopsy of blastocoelic fluid to perform whole genome analysis,[139] the replacement of nuclear material between oocytes to prevent the inheritance of mitochondrial diseases,[140] three-dimensional *in vitro* ovarian follicle culture[134] and the use of precursor cells to

rejuvenate or mature oocytes.[76,141] Non-invasive developments include the design of morphokinetic algorithms to evaluate embryo development through time-lapse imaging,[142] micro-vibration of culture dishes,[143] measurement of metabolites and nutrient utilization in culture media[76,134] as well as the application of microfluidics in ART.[86]

Technology is opening a world of new information to embryologists, and it is easy to imagine a futuristic ART laboratory where a micro-vibrating microchip designed to use microfluidics is used in conjunction with time-lapse imaging to culture embryos in a perfect environment. Metabolomic and morphokinetic analysis can be used to identify embryos that should be biopsied for genetic testing, prior to vitrification and transfer in a natural cycle. One could even go as far as to theorise how high the potential clinical pregnancy rate of such a laboratory could be. However, new technology always comes with development costs, which may make this futuristic laboratory a very nice dream that stays in the shop window, because it is too expensive. A balance between the development of new equipment and effective use of current technology is cardinal. Combined application of a more physiological approach, basic embryology principles and advanced selection methods could be the key to facilitating accessible ART.[144,145]

2.5. Assisted reproduction in developing countries

Classification of developing countries

"Developing countries" is a term loosely used to describe countries with a low human development index (HDI), comprising a complex combination of economic indicators, literacy levels and life expectancy, and a less developed industrial base.[146] The UN classifies countries with moderate to low HDI as developing.[146,147] Previously, the World Bank's distinction between developing and developed countries was based on each country's Gross National Income (GNI).[148] In 2016, the World Bank chose to refrain from using these terms as the diversity of countries previously pooled together under "developing countries" is too great.[148] The World Bank still provides GNI information on countries, allocated to four categories (GNI): Low (≤$1045), Lower-middle ($1046 – 4125), Upper-middle ($4126 – 12735) and High (≥$12736) income economies (figure 2.6).[148] In this text, countries with Low and Lower-middle income economies will be regarded as "developing countries".
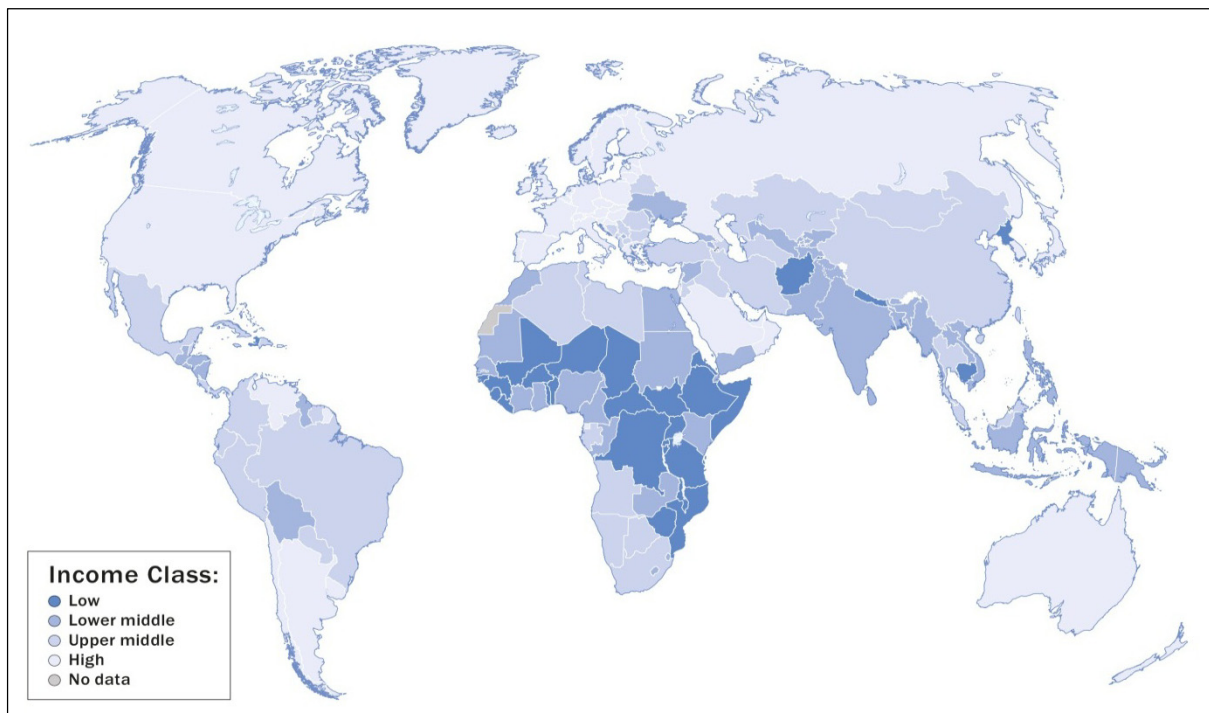
Figure 2.6: World map with countries colour-coded by income class according to gross national income (adapted).[148]

In 2016, the World Bank sorted 218 economies into the economy classes mentioned.[148] This number included all the World Bank member countries (n=189), as well as all the other economies with a population of >30 000, irrespective of political independence (figure 2.6, table 2.1).[148]

Table 2.1: World economies categorised per geographic area and gross national income, areas with high low and lower-middle income economies highlighted.[148]

Geographic area	Low Income	Lower-middle Income	Upper-middle Income	High Income	Total
Europe and Central Asia	0	7	14	37	58
East Asia and Pacific	1	15	8	14	38
South Asia	2	5	1	0	8
North America	0	0	0	3	3
Latin America and The Caribbean	1	5	20	16	42
Middle East and North Africa	0	7	6	8	21
Sub-Saharan Africa	27	13	7	1	48
Total	31	52	56	79	218

Out of the 218 economies evaluated, 31 were rated as Low-Income Economies and 52 as Lower-Middle Income Economies (table 2.1). The countries were also placed in seven geographic areas, concluding that most Low-Income Countries (87%) were in the Sub-Saharan Africa region, while Lower-Middle Income Economies were located in the East Asia and Pacific (28.8%), Sub-Saharan Africa (25%), Europe and Central Asia (13.5%), Middle East and North Africa (13.5%), South Asia (9.6%), and Latin America and The Caribbean (9.6%) regions.[148]

Infertility in developing countries

Most people in the world share the hope and expectation to have children, but childlessness is especially burdensome for lower-income populations from developing countries, where assisted reproductive services are limited and expensive.[8,21,31,149] In developed countries, the desire to have children stems from the motive for personal happiness and fulfilment, whereas in developing countries children are often seen as a religious and societal expectation, fulfilling a need to continue the family line, provide security in old age or secure rights of property and inheritance.[27,150]

Even though male factor infertility is implicated in approximately 50% of infertility cases in developing countries, this is rarely acknowledged and the female is often held accountable for the inability to procreate.[27,146] Infertility can be a stigma when a woman's societal status is largely defined by motherhood, and parenthood is a cultural expectation.[27-29] In many developing countries the inability to fall pregnant can lead to abandonment, isolation and psychological or even domestic abuse of women.[29,146,151,152] The stigma of infertility may be universal in developing countries, but how it is expressed is varied.[29,151] Physical violence can often be a consequence of a woman's inability to fall pregnant.[24] In sub-Saharan Africa, an infertile couple is more likely to adopt a child. However, in South Asia and the Middle East strong religious and cultural principles forbid this practice.[29] Some religions in China consider infertility as reprisal for past sins by either the person or his or her ancestors.[29,150] In Mexico and Brazil legislation limits ART procedures to married couples or those in stable relationships.[150] Although embryo cryopreservation is allowed in these countries, embryo donation is not an option.[150]

Infertility can be divided into two groups: Primary infertility carries the aetiology of anatomic, genetic, hormonal and immunological problems and is responsible for approximately 5% of infertility cases in the world, irrespective of the developmental state of the country. Secondary infertility includes preventable causes, mostly infection related, and the incidence differs significantly throughout the world.[24,29] Approximately 85% of infertile women are diagnosed with an infection-related cause of infertility in Africa[24] with post-abortion, -partum and sexually transmitted infections often being the cause.[27-29,153] Sexually transmitted diseases are responsible for more than 70% of cases of pelvic infection in sub-Saharan Africa, mainly *Chlamydia sp.* and *N. gonorrhoea*.[24] Focus on prevention of these infections, rather than on the treatment of infertility is one of the reasons that infertility care in Africa is all but absent.[27,28] Although prevention of sexually transmitted infections is important, pregnancy can be accomplished with ART practices, since couples with known blood-borne infections can use barrier contraceptives to prevent co-infection, while still having the opportunity to have biological children.[24,28,149,151]

Accessibility of ART in developing countries

Although the desperate need exists, ART is either unavailable or inaccessible to most of the population in the majority of developing countries.[24] A disparity is evident between access to health-care facilities in urban and rural areas of developing countries.[24] ART services are available in the private sector in some developing countries' capital cities. In the Middle East, Asia, India and Latin America more ART centres are found than in Africa, but procedures are very expensive, therefore only the affluent portion of the population can afford ART services.[24,27,29,150] In developing nations, high population growth rate is often provided as a reason for not addressing infertility needs with major health problems such as infectious diseases, malnutrition and maternal mortality taking centre stage (figure 2.7).[24,26,33,151]

At the beginning of the 21st century, interest in infertility and ART in developing countries increased significantly. At a meeting of the World Health Organisation (WHO) on Medical, Ethical and Social Aspects of Assisted Reproduction it was recommended that "Infertility should be recognised as a Public Health issue worldwide, including in developing countries" and added "Research is needed on innovative, low-cost ART procedures that provide safe, effective, acceptable and

affordable treatment for infertility".[29,152] In December 2006, the executive committee of the European Society of Human Reproduction and Embryology (ESHRE) established a special task force dedicated to infertility issues in developing countries. In 2007 the task force launched an action plan to promote affordable infertility diagnosis and treatment in developing countries, known as the Arusha project.[150]
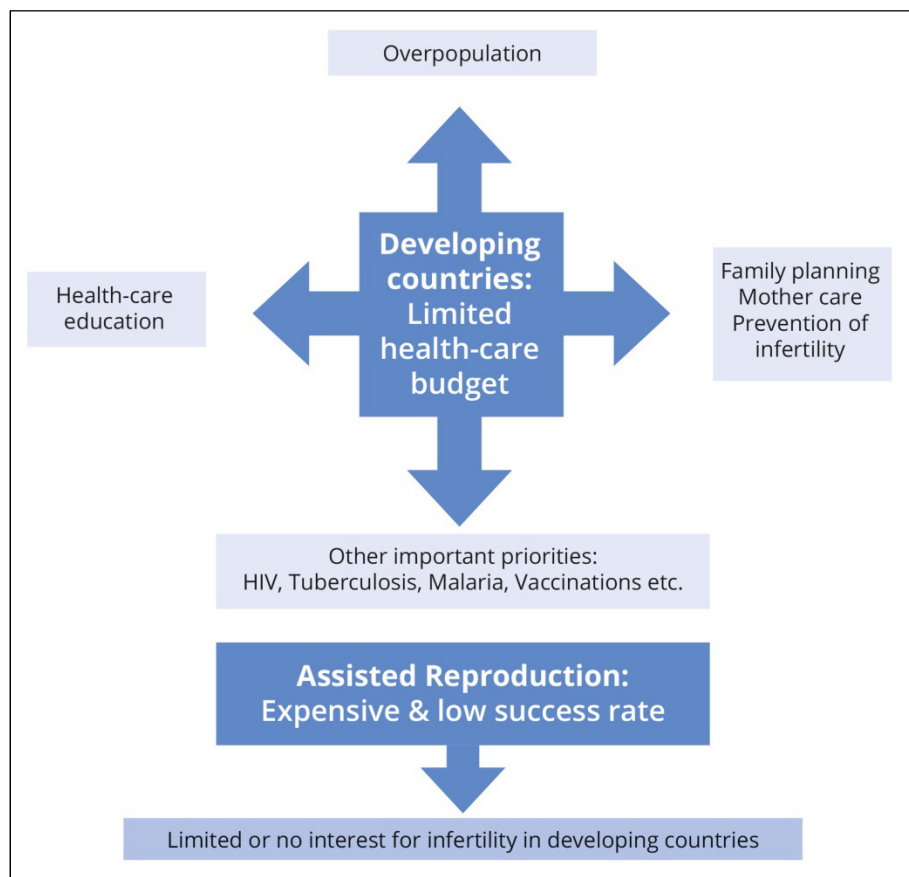
Figure 2.7: Factors impacting interest in assisted reproduction in developing countries (adapted).[151]

Some international groups focusing on low-cost, accessible ART have been founded. These include, but are not limited to, The Walking Egg (tWE) foundation, a non-profit organization collaborating with ESHRE and the UN's WHO; Family Health International, also a non-profit organisation managing research and public health needs; the Joyce Fertility Support Centre Uganda, a patient network focused on opening communications regarding infertility in Uganda and also Africa; The Population Council that conducts biomedical, public health and social science research; The Low Cost IVF Foundation, a non-profit organisation that encourages and supports low-cost ART options, as well as the United States-based charity

Friends of Low-Cost IVF.[27,28,150] All the role players agree that to address the problem effectively, both preventative health-care and ART should be made more accessible by increasing awareness of infertility and the number of ART facilities, as well as by reducing the cost of ART in developing countries.[150]

Setting up ART centres in a developing country

The challenge to provide accessible and affordable ART is formidable, as the setup and recurring costs of an ART clinic can be high.[146] The indirect costs to the health system arising from complications such as ovarian hyperstimulation and multiple pregnancies create a 'non-priority' status for the establishment of ART programmes in resource-poor countries.[24,146] Due to limited facilities, expertise and resources, comprehensive, cost-effective planning is required before starting a new ART facility in a developing country, as services that may be regarded as a given in developed countries are often not available in developing countries.[28,154-156]

One major drawback in developing countries is poor infrastructure.[28,155,157] The electricity supply may be infrequent with sudden power cuts and fluctuating voltages, with certain remote areas having no electricity.[157,158] A power generator or at least uninterrupted power supply is required for ART laboratories in developing countries, depending on the infrastructure and procedures that need to be performed at the laboratory.[36,158] Deliveries of disposable items, pre-mixed gas bottles, culture media and medication may be unreliable and cold chain maintenance cannot be assumed, with the majority, if not all, disposables and equipment having to be imported from developed countries.[7,156,158] Therefore, sufficient stock available to carry over when deliveries are delayed is essential.[155,157] Trained and experienced service engineers are not always on hand in developing countries.[158] Should a problem arise with equipment or general maintenance needs to be performed, a trained service engineer from abroad will have to be consulted. These delays may have implications for patient services or the operation of the equipment.[154,155,158,159]

Human resources in developing countries

Infertility treatment is a specialized field that requires trained personnel who are capable of providing a quality service to patients.[7,156] This is even more important in developing countries as unforeseen problems are more likely to occur and

experienced personnel are better equipped to handle these difficult situations. Unfortunately, very few accredited training facilities are available in developing countries.[155,156,158] Currently many ART facilities in developing countries are being operated by health-care professionals from developed countries who travel to the facility for short time periods.[7,156] Patients are batched for *locum* professionals to assist inexperienced local health-care workers and local personnel continue with diagnostic evaluations once ART experts depart.[155,156] Batching of patients ensures that the stock of disposables, culture media and medication are adequate.[155] Training of local health-care professionals in all relevant aspects of infertility is essential and training programmes run by experts from high-income countries help to develop local expertise.[7, 27,155] Local health-care workers are culturally sensitive, which patients appreciate because they perceive this as a safe treatment environment.[27]

Although the provision of ART to developing countries is fraught with complications, it is by no means impossible. Selected ART procedures, that can be performed within the infrastructure, resources and facilities available, can be offered to patients. Inexperienced staff can be trained in simple techniques and IVF culture systems can be modified to make these simpler and more robust.

2.6. Affordable and accessible assisted reproductive technology

With the high demand for infertility treatment that is not being met neither globally nor in developing countries,[28,33] there is an urgent need to increase accessibility to ART.[11,160] As the treatment of involuntary childlessness is expensive, a reduction in this cost will increase accessibility to these services.[27,28,30] The financial implications of setting up ART facilities are strongly aligned to the patients' level of affordability of treatment.[11] Should there be no assistance from the government, health schemes or donors, the cost of a private facility will eventually be borne by the patients themsleves.[11,30]

Comprehensive infertility care should focus not solely on ART, but on prevention and education as well.[7,161] Government-sanctioned education on reproductive health will provide long-term benefits by increasing awareness and reducing secondary infertility.[151,162] When the causes and treatment of childlessness are better

understood, public awareness will be raised, stigmatization will be reduced and preventative behaviour will follow.[161,163,164] Couples that are already suffering from infertility will still need the assistance of ART.[164] Accessibility can be realised only through an increase in the number of facilities and a decrease in the cost to patients.[160]

To increase the number of ART facilities in a specific country is a complex exercise. Communication should begin with government which has to take the initiative, by including basic fertility treatment in all current family planning programmes.[24,154,162] Local and international stake-holders, such as those companies supplying equipment, medication and disposables should be approached to discuss investment in a future market through subsidised prices and funding of training facilities.[146,153-155] Setting up new ART facilities in developing countries does not necessarily mean having fully functional, high-technology laboratories and treatment centres in place everywhere.[26,153] Different levels of treatment could be managed by providing basic diagnostic services, such as evaluation of tubal patency, semen analyses and follicular tracking at existing local clinics and medical facilities.[26,27,153] Less invasive treatment of unexplained infertility, using IUI and ovulation induction in a natural menstrual cycle which requires simple infrastructure, could be available at multiple health-care centres, while advanced, tertiary-level ART services can be confined to specialized facilities at hospitals and care centres in larger towns or cities.[26,27,146,165]

Construction of local facilities and infrastructure should be considered, coupled with sound financial planning.[146,155] With the associated costs of new technologies and improved culture systems, a "back-to-basic" approach in embryo culture could be adopted.[76,144,149] According to Huyser and Boyd, laboratory costs can constitute up to 35-48% of ART fees payable by patients.[6] A more cost-effective laboratory design will significantly increase the accessibility of ART to lower-income patients. Development of effective, low-cost instruments for the prevention, evaluation and treatment of infertility in low-resource countries is imperative.[24] Although curtailing these costs would reduce the cost of ART and facilitate accessibility, the quality of care provided to the patients should not be compromised.[153,160,164]

The search for alternative laboratory strategies provides an opportunity for innovative design. Some of the apparatus that has been suggested to lower the cost of ART include a submarine incubation system,[118,149] intra-vaginal or –uterine oocyte fertilization and embryo culture,[23, 153,166] as well as culturing in a closed system of glass tubes.[18] The submarine incubation system involves placement of culture dishes in a bag that has been filled with the correct gas mixture, sealed and placed in a circulating heated water bath at 37°C for the duration of culture.[118,153] The intra-vaginal culture could be performed in either a sealed vial filled with culture media[167] or a semi-permeable device called INVOcell[TM] that allows equilibrium between vaginal partial CO_2 and the culture media.[168] Culture of embryos in the uterus involves the use of a micro-perforated silicone elastomer tubing in an *in-utero* culture system (IUCS).[166] Oocytes are injected via ICSI after TVOA and placed inside the IUCS, which is placed in the patient's uterus via a standard embryo transfer catheter. The micro-perforations of the tubing, that allow the exchange of nutrients and specific complex elements in the uterine fluid, are too small for the embryo to escape.[166] An alternative laboratory strategy is culturing in a closed system of glass tubes, incorporating the *de novo* production of CO_2 by chemical reaction.[18] This closed system, the simplified tWE IVF culture system, is the focus of this study and will be described fully in the next section.

2.7. The Walking Egg

2.7.1. Beginnings of the foundation

The Walking Egg foundation (tWE, Genk, Belgium) is a non-profit organisation that focuses on childlessness in developing countries.[17,169] The foundation has adopted a multidisciplinary and global approach for raising awareness by: reviewing socio-cultural, ethical and economic facets of childlessness in developing countries; increasing accessibility to infertility diagnosis and treatment; and assisting other organizations and societies to reach the goal of "global access to infertility care".[169] The foundation had its origin in part from the meeting of minds of two of the foundation members, Prof Willem Ombelet and Mr Koen Van Mechellen in 1997.[16,169]

Ombelet, a gynaecologist specializing in the treatment of infertility in couples is the Head of the Department of Obstetrics and Gynaecology of the Ziekenhuis Oost Limburg Hospitals in Genk, Belgium and co-ordinator of the ESHRE Special Task

Force on Developing countries and infertility.[16,152] Van Mechelen is a Belgian artist whose passion is to bring people of different cultures together and has often worked alongside scientists to bring his art to life.[16] Van Mechelen is actively involved in fund-raising for tWE. One way in which he does this is by selling coloured lithos of his art work "The Walking Egg" (figure 2.8) for the approximate cost of a single IVF treatment in developing countries and donating all the proceeds to the foundation[170].

Figure 2.8: Coloured litho of "The Walking Egg" by Koen Van Mechelen.[170]

From the association of Ombelet and Van Mechelen after the "Andrology in the Nineties" meeting in 1997, the first "walking egg", a glass egg with the legs of a chicken was created.[16] Their collaboration did not end there and three years later the first issue of "The Walking Egg" magazine, a scientific magazine that also focused on art and philosophy, was published. The gap between science and art was bridged and six issues of the magazine followed.[16] A scientific-artistic project followed in December 2007 in Arusha, Tanzania, coinciding with the meeting of the ESHRE special task force that is dedicated to addressing infertility in developing countries.[169] The Arusha Project was born and in March 2010 tWE foundation was founded by Mr Koen Van Mechelen, Prof Willem Ombelet, Dr Annie Vereecken, who specializes in gynaecological pathology and non-invasive prenatal testing, and Dr Rudi Campo, President of the European Society for Gynaecological Endoscopy, to achieve the goals envisaged in the Arusha Project.[16,169]

The Walking Egg foundation's strategies are multi-faceted (figure 2.9), initiating advocacy and networking to raise awareness of the fertility needs in developing countries.[160,171] This process will include discussions with politicians, health-care providers and the scientific community, as well as reaching out to the 'man in the street' through the media and news agencies.[17,172] The other two elements of the foundation's plan are research and service delivery. The research outputs proposed by tWE are investigations on political, ethical, religious, socio-cultural and other aspects of infertility management as well as projects to develop and streamline one-step diagnostic procedures, to identify infertility needs and to do feasibility studies on low-cost ovarian stimulation and simplified IVF protocols.[17,160,169,172]
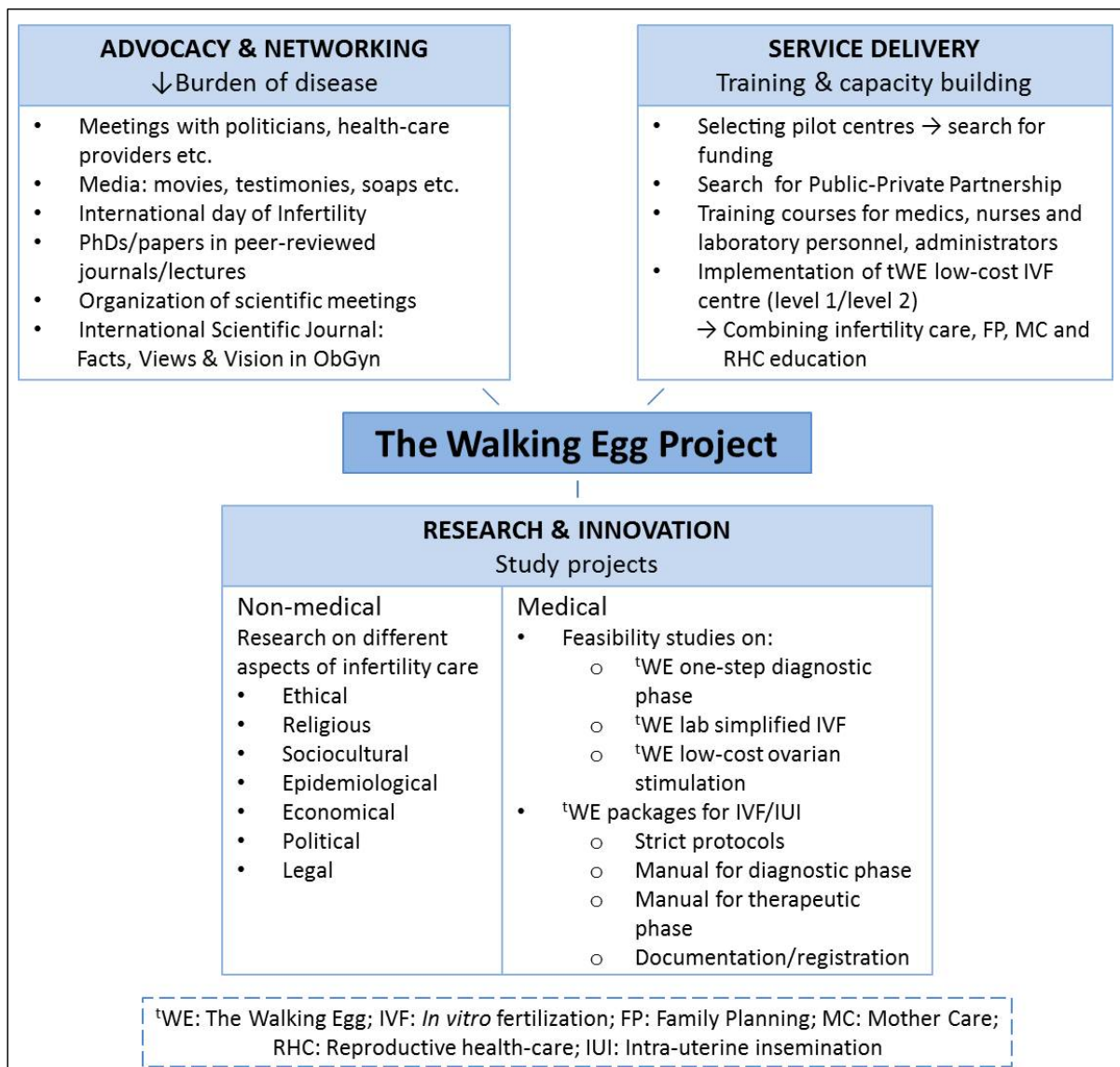
Figure 2.9: Operational plan of The Walking Egg foundation to increase awareness and implement ART services in developing countries (adapted).[160]

Lastly, to ensure service delivery in developing countries, the foundation will focus on the selection of possible sites for pilot-centres, procure public-private funding to facilitate these centres, initiate training courses for all levels of health-care workers involved in ART, and eventually put these into practice at level 1 and 2 low-cost tWE IVF centres.[160,163,171] Level 1 centres should be accessible to the general public and provide diagnostic infertility and basic ART services such as IUI, while level 2 centres located in centralised areas will provide level 1 services, along with more advanced ART, for instance IVF.[17,163] Both types of centres will provide family planning, reproductive health-care and mother-care services.[160] Although the inclusion of ICSI is not part of the initial roll-out, it may later be included at a level 3 centre.[17,163]

Some of the research conducted through the tWE foundation has already cumulated in workable solutions, especially the development and initiation of the simplified tWE IVF culture system.[18] The simplified culture system is designed to provide a basic, optimal environment for human embryo culture without the use of complex high-tech incubation equipment, medical gasses and infrastructure typical of IVF laboratories in high resource settings.[18]

2.7.2. Technical detail

The simplified tWE IVF culture system was developed by Prof Jonathan van Blerkom, a multi-award-winning scientist in the Department of Molecular, Cellular and Developmental Biology at the University of Colorado and IVF Laboratory Director at Colorado Reproductive Endocrinology in Denver (United States of America), whose research in the field of molecular and cellular aspects of early mammalian development is well known.[16,18] The design of the simplified culture system combines a simplistic chemical reaction and affordable, robust equipment to facilitate embryo culture.[18,173] The estimated cost when using the simplified tWE IVF culture system runs to approximately 10-15% of the cost of conventional IVF.[173] During embryo culture in the simplified system, culture media is kept in glass tubes which remain closed for the duration of culture, so that detrimental agents cannot enter the culturing environment (figure 2.10).[18] The culture media is maintained at the required temperature by encasing the glass tubes in a warming block (figure 2.10A & B). These warming blocks can be heated and need to be kept at a constant 37°C using any compatible heat regulating device.[18]
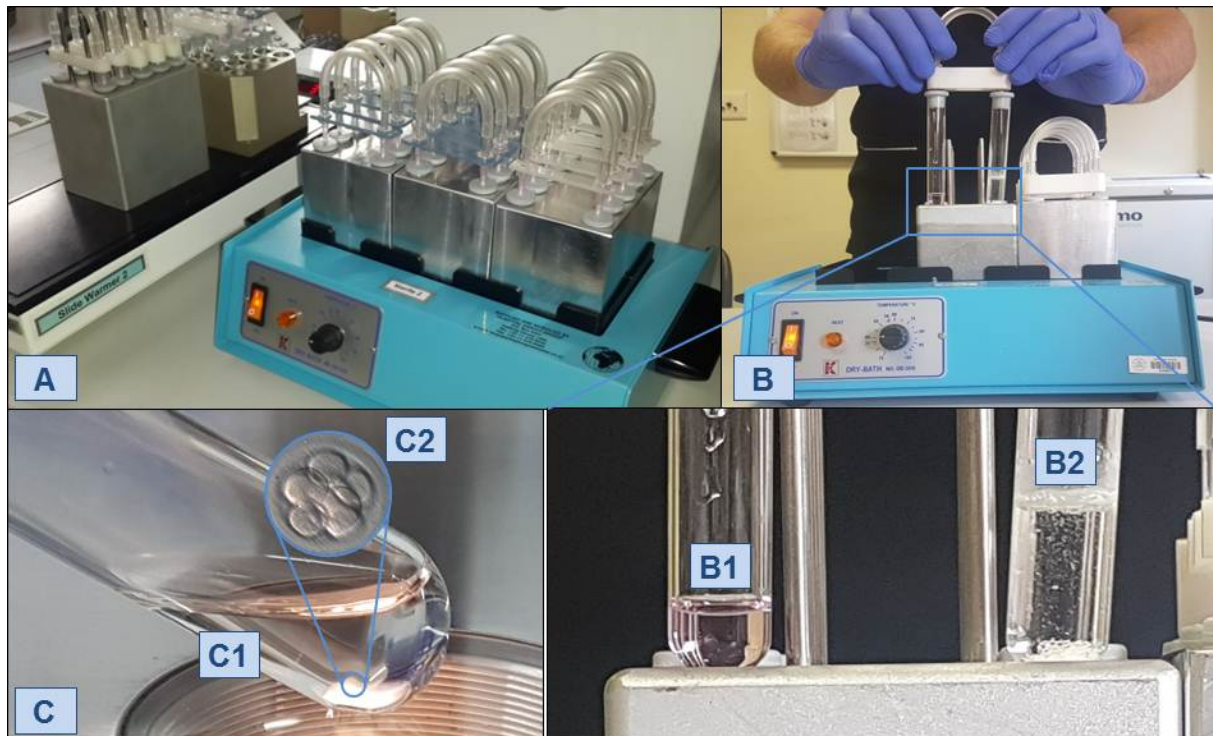
Figure 2.10: A: Gas equilibration of glass tubes with the simplified Walking Egg *in vitro* culture system. B: Gassing and culture tubes, connected by needles and tubing, B1: culture media with pink colour (pH>8.0) and B2: gassing tube with CO_2 being produced, seen as bubbles. C: Glass culture tube with C1: clear peach coloured culture media (pH ~7.3) and C2: an embryo in culture as seen with a stereoscopic microscope through the glass wall of the culture tube (simulated).

The simplified tWE IVF culture system utilises the principle of CO_2 being produced *de novo* in a glass tube, by the reaction of citric acid and sodium bicarbonate ($C_6H_8O_7$ + $3NaHCO_3 \rightarrow 3H_2O + 3CO_2 + Na_3C_6H_5O_7$), rather than using expensive medical grade gasses (figure 2.10B).[18,117] Gas generation and equilibration takes place before the introduction of cumulus-oocyte-complexes or sperm to the culture media. The tubes are closed prior to gas equilibration, kept stoppered during culture and all samples are injected through stoppers into the glass tubes using sterile needles, resulting in a closed CO_2 rich environment.[18,117] No media or cells are removed from the culture tubes prior to embryo transfer and embryo evaluations are performed through the glass tube, while remaining stoppered, by visualization of the embryo in culture via a stereoscopic microscope (figure 2.10C). The culture tubes containing the fertilized oocyte, along with the remaining cumulus and sperm cells, are kept in pre-heated warming blocks for up to three days, after which embryos are transferred into the female patient's uterus by conventional embryo transfer catheter.[18,174]

The laboratory setup for the simplified tWE IVF culture system consists of instruments used for semen preparation and equipment for the embryo culture system.[175] The andrology requirements can be as simple as a few pipettes and a microscope if semen samples are being processed by the swim-up technique, or with the addition of a centrifuge, density gradient centrifugation can be performed.[108] Due to the high prevalence of infectious semen samples in developing countries,[24] the application of density gradient centrifugation with a mechanical device, such as the ProInsert[TM] (PI15-5, Nidacon, Mölndal, Sweden), in the centrifugation tube can be used as an added risk reduction method to decontaminate semen samples.[157,176] The equipment associated with the simplified tWE IVF culture system are a heat regulation instrument to maintain temperature during embryo culture, a stereoscopic microscope used during oocyte aspiration, embryo evaluation and transfer and a filtered air environment where culture tubes can be prepared before being stoppered.[175] The filtered air environment can be created in a laminar flow cabinet with HEPA filter, or with a modified "humidicrib" chamber.[175]

With the simplified tWE IVF culture system, "room air without any filtration" (ISO 14644-1 class 9 standard)[15,124] would suffice, since closed-off culture tubes are used for culture.[36,175] A modified "humidicrib" chamber, such as a Cell-Tek microscope chamber (Cell-Tek 3000, Tek-Event Pty Ltd, Sydney, Australia) can be used during culture tube preparation, oocyte aspiration and embryo transfer.[177] Since this IVF chamber is HEPA filtered, the air quality inside the chamber is at an appropriate standard when oocytes and embryos are exposed to the environment (during oocyte aspiration and embryo transfer)[15] and when culture tubes are being prepared.[175] Additionally, the temperature regulation mechanism and stereoscopic microscope with which the IVF chamber is equipped provides an ideal micro-environment, where embryo evaluations can be performed without the risk of temperature fluctuation when culture tubes are removed from the warming blocks.[177]

2.7.3. Successes achieved

In the scientific community, tWE foundation has had multiple successes. Ownership of Facts, Views and Vision in Obstetrics and Gynaecology was obtained by tWE in June 2013.[178] Previously this was the international scientific journal of the Flemish Society of Obstetrics and Gynaecology (VVOG), with the first issue published in June

2009.[179]   Facts, Views and Visions remained the official scientific journal for the VVOG and in September 2013, the announcement was made that the journal would also be the official scientific journal for the International Society for Mild Approaches in Assisted Reproduction.[180]

When the simplified tWE IVF culture system was presented to the world for the first time at the annual ESHRE congress held in London in 2013, the reaction was overwhelming.   Aside from being a major point for discussion in the scientific community, newspapers, magazines and other news agencies also embraced the idea of an IVF system that cost less than 200 euro.[19]   Within hours the news spread from the United Kingdom[181] as far as the United States of America in the west[182] and Thailand in the east.[183]   In 2013, Prof van Blerkom was presented with the American College of Embryology's "Embryologist of the year 2013" award for his work in ART and, specifically, for the development of the simplified tWE IVF culture system.[19]

In 2014, the first babies born from the simplified tWE IVF culture system were reported by Van Blerkom *et al*.[18] and those born after cryopreservation and subsequent thawed embryo transfer were reported by Ombelet *et al*.[174]   The scientific paper by Van Blerkom *et al*., in which the first tWE babies were cited and the culture system described[18], was awarded the Robert G. Edwards Prize Paper Award for the best paper published in Reproductive BioMedicine Online in 2014.[177]   The first tWE laboratory in a developing country was established at the Pentecost Fertility Centre, Accra Ghana in 2015 by the Association of Childless Couples of Ghana (ACCOG) in collaboration with tWE foundation[7,19] and the first pregnancy from this facility was achieved by the end of 2016, with an expected birth date in August 2017.[184]

Apart from scientific breakthroughs, tWE foundation has also been raising awareness of infertility in developing countries and engaged in a search for collaborators to open new tWE laboratories. The foundation has assisted the ACCOG in the planning and running of the first two National Congresses on Infertility/Childlessness in Ghana in March 2014 and April 2015, respectively.[19,185]   In November 2014 tWE foundation, in collaboration with the University of Hasselt and the Flemish Interuniversity Council and University Development Co-operation, organized a conference on reproductive health entitled: "Global access to infertility care: The Walking Egg project", which was

hosted by the University of Stellenbosch, South Africa.[19]   In 2016 a two-day workshop was co-hosted by the Footsteps to Fertility and tWE foundations in Nairobi, Kenya, where students from the University of Amsterdam and the Technical University of Nairobi gave feedback on their investigations into the consequences of infertility in Nairobi, Kenya.[19]

Numerous visitors from across the globe (Argentina, Bolivia, Burundi, Colombia, Cyrus, Egypt, the Gambia, Ghana, Kenya, South Africa and Vietnam) visited tWE in Genk, Belgium from 2014 to the present to initiate collaborations with the foundation for either setting up the simplified culture system or becoming involved in improving ART accessibility in developing countries, with some attending training sessions by tWE team on the operation of tWE laboratory.[19]   Delegates from the foundation travelled to China and Kenya in 2016 to meet with role players to discuss possible future partnerships.[19]   In April 2015, with financial support from the Belgian Developmental Co-operation, a five-part TV series was produced on the start-up of the Pentecost Fertility Centre in Accra, Ghana, as well as a later compilation that focused on comments from the African team who worked with tWE team.[19,186]   The Walking Egg also received "The Best of What's New Award" from Popular Science Magazine in the health category in 2014 and won a silver medal in the category "Women's Health & Wellbeing" at the Edison Awards in 2016.[19]

2.8. Conclusion

It is imperative that any new techniques and technologies be thoroughly investigated to ensure optimal use and safety.[76]   To this end, some features of the simplified tWE IVF culture system were investigated at a tertiary public sector institution in South Africa. Performing the investigation at this location provided an independent platform for academics in a developing country, where the challenges of providing ART services are experienced first-hand.   The public sector institution has a technologically advanced ART laboratory that allows direct comparison between established, conventional ART laboratory procedures and the simplified tWE IVF culture system. Parameters evaluated were those deemed fundamental for optimal embryo culture, as well as showcasing the simplified culture system's reliability and stability when compared to conventional embryo culture.

Chapter 3

Materials and methods

Chapter 3 Materials and methods

3.1. Section 1: Quality control of the simplified culture system

Human embryo culture is dependent on a stable culture environment. The simplified Walking Egg (tWE) *in vitro* fertilization (IVF) culture system utilizes a simple design to regulate culture media temperature and pH (figure 3.1). These two parameters were investigated to ascertain whether the embryo culture environment mimics conventional assisted reproductive technology (ART) conditions reliably.
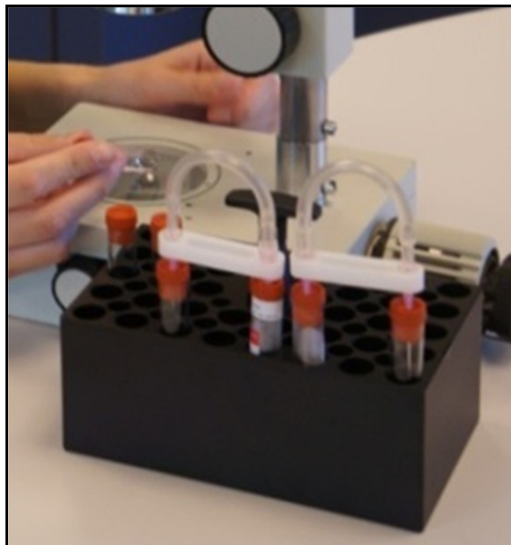
Figure 3.1: The simplified Walking Egg *in vitro* fertilization culture system.[177]

3.1.1. Temperature regulation

The first quality control (QC) checkpoint of the culture system was constant temperature control. Warming blocks used were heated to keep culture media in the glass tubes at a constant temperature of 37°C.[18] A single aluminium warming block, designed to enclose fully the standard tWE glass tubes, was supplied by tWE foundation to be used for the duration of the experiment. Three additional blocks were manufactured from aluminium, by artisans employed at the Steve Biko Academic Hospital, to the same dimensions as the one supplied (figure 3.2) and these blocks were used arbitrarily during the entire project. These warming blocks facilitated heat transfer rapidly by even heat distribution throughout the block with the customized fit around the glass tubes (TWE-1889-1114, The Walking Egg foundation, Genk, Belgium) ensuring minimal heat loss. A constant temperature is maintained when the culture media within the tubes remains at a stable temperature as was explored in the subsequent experimentation.
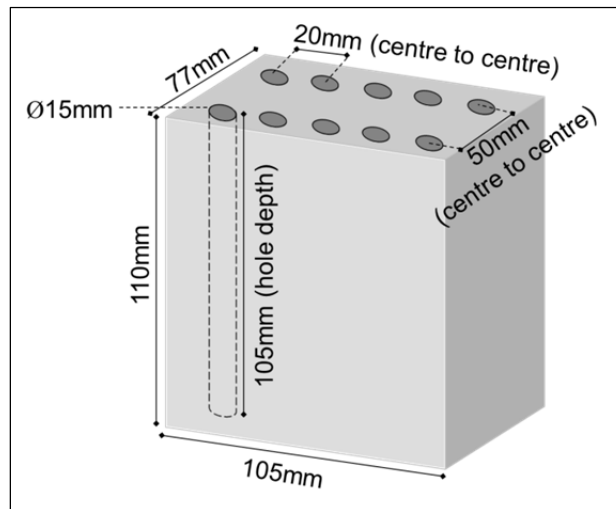
Figure 3.2: Dimensions of aluminium warming blocks used.

As the Global® Total® for Fertilization (LifeGlobal® Group, Guilford, CT, USA) culture media was the culture media of choice for the simplified tWE IVF culture system at the time investigation began,[175] it was used throughout the current experimentation period. This family of culture media is also the standard embryo culture media employed at the Reproductive Biology Laboratory (RBL), Steve Biko Academic Hospital, Pretoria, South Africa during IVF, providing a readily available supply.

During the course of investigation, different methods and devices in an ART laboratory that could be used to warm the blocks to 37°C were tested. As part of the maintenance schedule followed in the laboratory, all equipment are assessed yearly, with calibration if needed. When an instrument is actively in use in the laboratory, daily QC checks of appropriate parameters are performed to confirm successful operation and detect drifts timeously. All the instruments tested were operated without any additional interventions. Devices were grouped according to the manner in which each warming block could be heated. These include:

Enclosing the warming block: a **bench top warming oven** (Model 10-140E Incubator, Quincy Lab Inc., Chicago, Illinois) and a **water-jacketed embryo culture incubator** (no CO_2 connected) (Forma Series II Water Jacketed CO_2 incubator, Thermo Fisher Scientific Inc., Waltham, Massachusetts);

Partially enclosing the warming block: a **dry-bath style block heater** (DB-006, K-Systems Kivex Biotec A/S, Birkerod, Denmark) and **water bath** with circulation of heated water (CPM 200 Water bath, Laboratory Marketing Services CC, Maraisburg, South Africa); and

Single surface heating (warming block heated from bottom): a **digital slide warmer** (SW85, Adamas Instrumenten B.V., Rhenen, Netherlands) and **IVF workstation with heated surface** (Mobile IVF workstation L13, K-Systems Kivex Biotec A/S, Birkerod, Denmark).

Two placement positions were selected per device to determine the internal variation in temperature (figure 3.3). The warming oven and IVF workstation had blocks placed on the left- and right-hand side of each device, the incubator on the top left and bottom right shelves, the water bath and slide warmer at the front and back of the device, with blocks placed on the side and in the middle of the dry-bath.
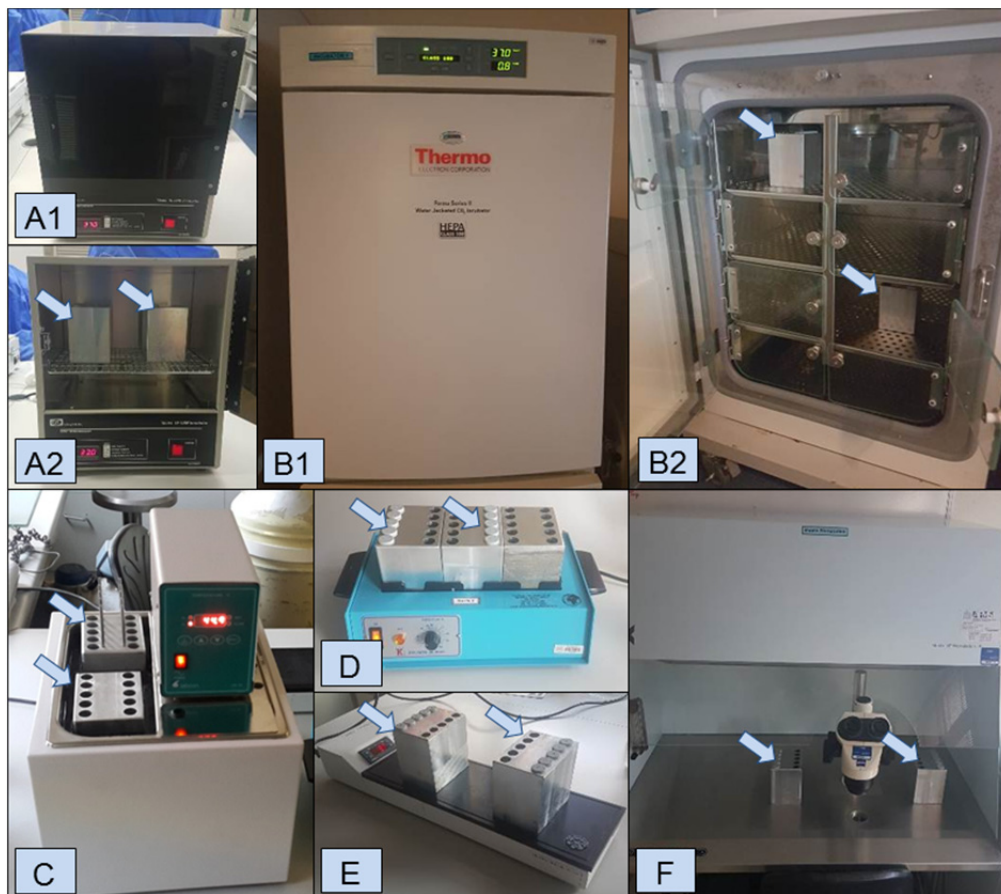
Figure 3.3: Heating devices used for temperature regulation and placement of warming blocks (indicated by arrows): A1&2: warming oven, B1&2: embryo culture incubator, C: water bath, D: dry-bath, E: slide warmer and F: IVF workstation.

For every temperature regulating instrument evaluated (n=6 devices), two warming blocks, housing five glass tubes with culture media each, were used to assess intra-variation of temperature. Temperature readings (figure 3.4) were performed at set

times i.e. early morning: 7h00 - 8h00, midday: 11h30 - 12h30 and afternoon: 14h30 - 15h30 over a four day period until a total of ten sets of temperatures per warming block had been collected (T4: n=100 culture media temperature measurements per instrument tested). With every repeat of temperature measurements, the warming block surface (T3, both warming blocks, n=20), heat regulating instrument (T2, two fixed positions, n=20) and ambient air (T1, single measurement, n=10) temperatures were recorded (n=900 total temperature measurements, figures 3.4 & 3.5).
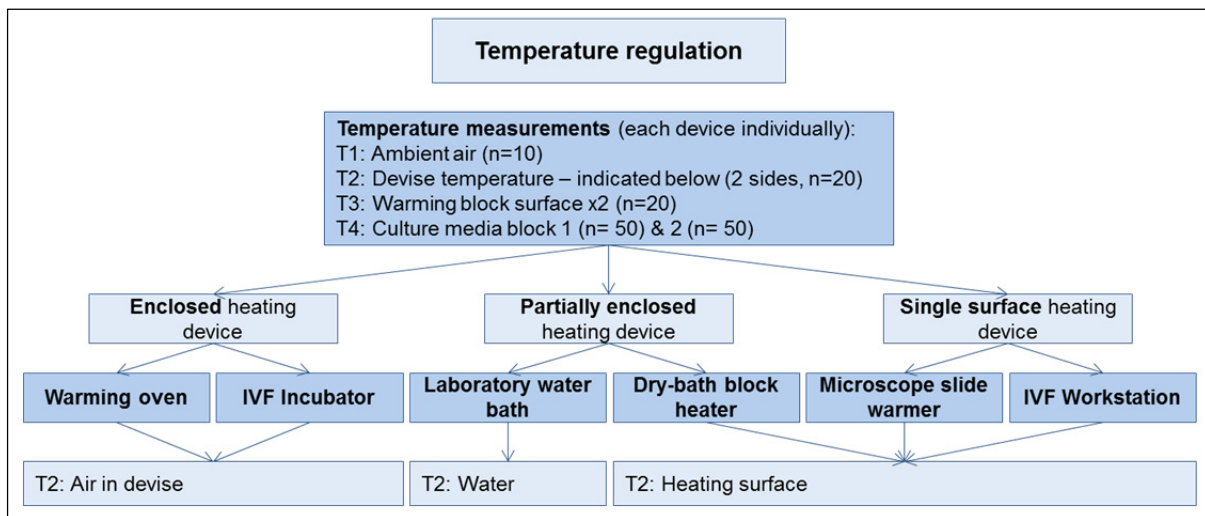
Figure 3.4: Flow diagram comparing heating devices and temperature readings measured for each device (n=900 total temperature measurements).
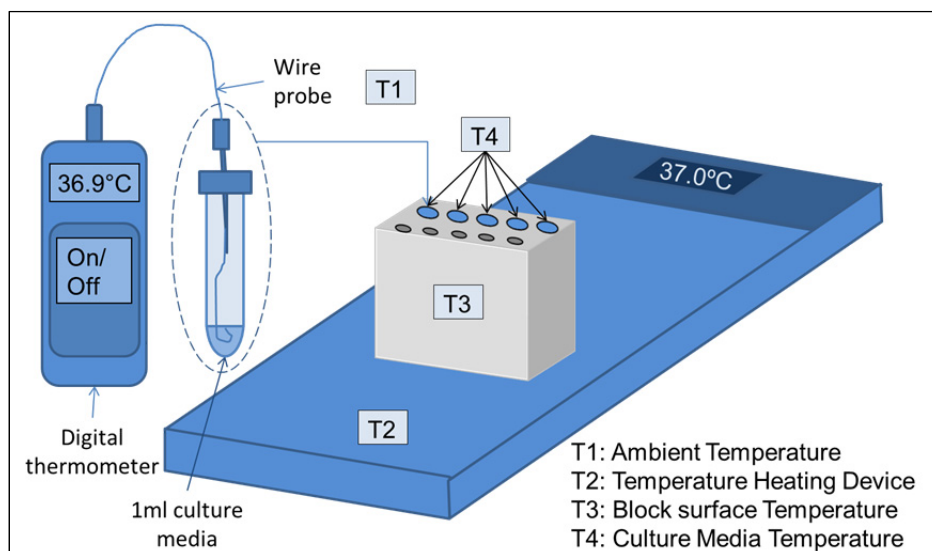
Figure 3.5: Illustrative display of different temperature measurement positions.

In order to perform the temperature measurements, one millilitre (1 ml) of culture media was pipetted into the glass tubes described using a calibrated 100 – 1000 µl

variable volume pipette (Finnpipette® F2, Thermo Fisher Scientific Inc., Waltham, Massachusetts) and sterile pipette tips (Finntip™ 1000, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The tubes were closed off with stoppers (TSB 2010034, The Walking Egg foundation, Genk, Belgium). The temperature of the culture media was measured by passing a wire probe (GTF 300, GHM Messtechnik GmbH Standort Greisinger, Regenstauf, Germany) through a 15 gauge needle (L.Y.S.A. HDN1515, Xiantao Rayxin Medical Products Co. Ltd, Xiantao, China) that was inserted through the stopper (figure 3.6A).
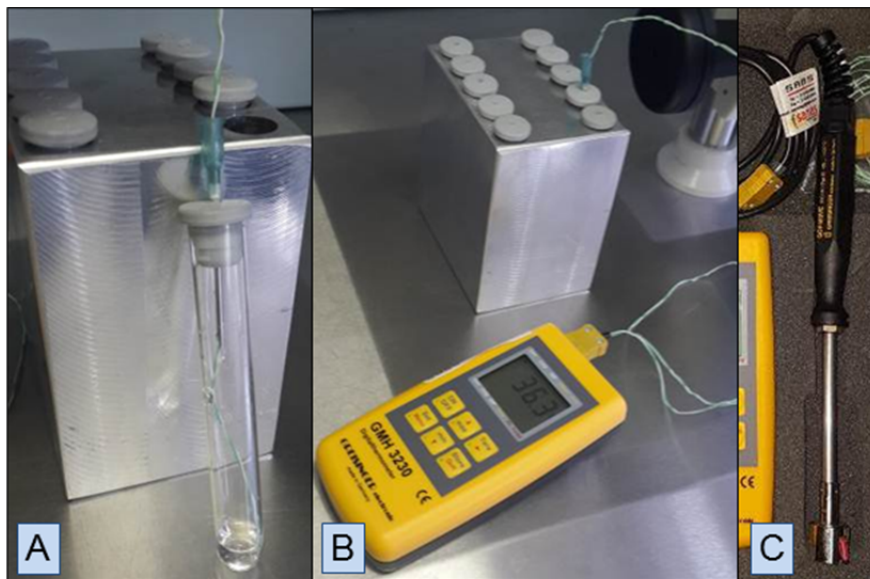
Figure 3.6: A: The placement of a wire probe for the *in situ* measurement of culture media temperature, B: the wire probe connected to a digital thermometer and C: an additional temperature probe to measure surface temperatures.

The wire probe was connected to an electronic thermometer (figure 3.6B) accurate to one decimal point (GMH G3230 Digitalthermometer, GHM Messtechnik GmbH Standort Greisinger, Regenstauf, Germany) calibrated by the South Africa Bureau of Standards at the onset of the project). The tubes were kept *in situ* in the warming block while temperatures were determined, ensuring accurate measurements while simulating actual culture conditions. All liquid and air temperatures were measured using the wire probe described, while surface temperatures were measured with the aid of a surface measuring probe (figure 3.6C, GOF400VE, GHM Messtechnik GmbH Standort Greisinger, Regenstauf, Germany) connected to the digital thermometer.

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3.1.2. Culture media pH

3.1.2.1. Regulation of pH by citric acid volumes

In the simplified tWE IVF culture system, glass tubes with 1 ml culture media each (*culture tube*) are connected via connection tubing (E87 10cm, The Walking Egg foundation, Genk, Belgium) and needles (305196 BD PrecisionGlide[TM] 18G, Becton, Dickinson and Company, Franklin Lakes, New Jersey) (figure 3.7A) to parallel placed glass tubes (*gassing tube*), containing a sodium bicarbonate (SB) pellet (Wolfs Sodium Bicarbonate Tablets, The Walking Egg foundation, Genk, Belgium) and a set volume (3 ml)[175] of citric acid (CA; CAS030, The Walking Egg foundation, Genk, Belgium; undisclosed concentration as determined and supplied by the manufacturer) (*connected culture and gassing tube referred to in text as a set of tubes*).   The culture media (Global® Total® for Fertilization) utilized for the experiments, has a recommended pH range of 7.20 – 7.40 (LifeGlobal® Quality Control Specifications; Gruber and Klein 2011) for embryo culture. An average pH of 7.30 ±0.05 was aimed for during the study.[187]

Figure 3.7: A: Connection of culture media and gassing tubes via tubing and needles and B: sets of connected tubes during gas equilibration.

During the period of experimentation, the CA volume in gassing tubes were adjusted from 1.2 to 3.0 in 0.2 ml increments and injected into each gassing tube containing SB using a 5 ml syringe (Quadroject® DIN13098, Matraplast Ltd, Budpest, Hungary) and needle (305196 BD PrecisionGlide[TM] 18G). The gassing tubes were connected to culture tubes containing 1ml Global® Total® for Fertilization culture media and the sets of tubes were kept in a warming block at 37°C for 18 hours (Figure 3.6B).

As the total volume of fluid within the gassing tubes is and connection tubing needed to remain constant,[175] any change in CA volume was compensated for by the addition of water. The amount of water added to CA was calculated to have a final volume of 3 ml in the gassing tube.[175] Ten sets of tubes were prepared with each volume of CA (10 sets x 10 volumes, n=100).

After the 18 hour incubation period, the needles connecting culture tubes to gassing tubes were removed. Hereafter the stoppered, gas equilibrated culture tubes were kept in the warming blocks, at 37°C[18] and 200 µl of the culture media was sampled using 1 ml syringes (1 ml/cc tuberculin syringe, Isidigi Medical Supplies, Centurion, South Africa) and 22 gauge needles (22 G x 1¼" Hypodermic needle, Isidigi Medical Supplies, Centurion, South Africa). Immediately after each sample was aspirated, the needle was removed from the syringe and the syringe capped with parafilm (LPPG7016-05, Lasec SA, Johannesburg, South Africa) to prevent exposure of the culture media to ambient air. The capped syringes were then transported in a portable warming oven at 37°C (G95E Portable incubator, K-Systems Kivex Biotec A/S, Birkerod, Denmark), to a calibrated blood gas analyser (Radiometer ABL 800 Flex, Radiometer Inc., Brea, California), where pH was measured. The data gathered were used to determine the volume of CA needed to obtain a pH of 7.30 ±0.05 in the culture media.

During the production of CO_2 in the connected glass tubes, the internal air pressure of the system increased and the pressure build-up caused some of the stoppers to be partially expelled from the tubes (figure 3.8A), resulting in a loss of CO_2 and an associated pH increase in the culture media. The issue was addressed by wrapping the stoppers and top 0.5cm of each tube in several layers of parafilm. Although this solution reduced the frequency of occurrence, a few stoppers were still pushed out, causing the parafilm to tear (figure 3.8B). This problem has been solved by tWE foundation by the introduction of sleeves (Versilic® Silcone Folding Skirt Stoppers 407015-20, The Walking Egg foundation, Genk, Belgium) to be placed over the stoppers. These sleeves fit over the stopper and top of the glass tube, securing the connection between the two (figure 3.8C).
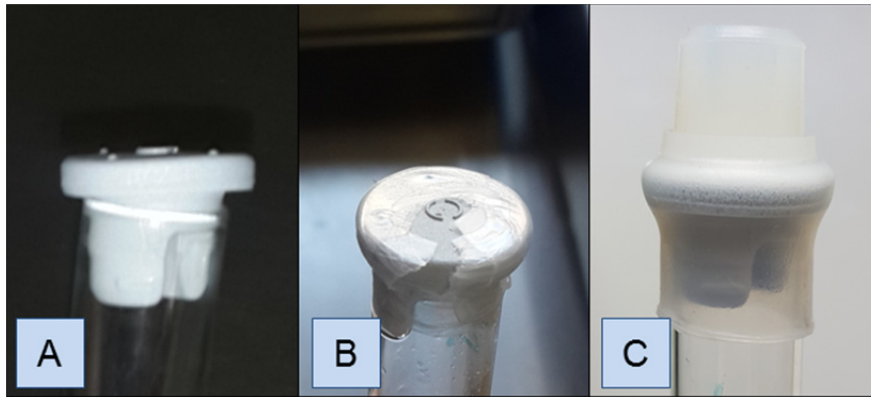
Figure 3.8: A glass culture tube with A: stopper pushed out; B: with torn parafilm and stopper pushed out; and C: with a sleeve to keep tube stopper in place.

3.1.2.2. *Temperature during gas equilibration*

The protocol for operating the simplified tWE IVF culture system prescribes that gas equilibration (through CO_2 production by SB and CA, described previously) should occur at 37°C.[18] Gas generation and equilibration at 37°C were compared to two lower temperatures (15°C and 25°C) over an extended time period (30 hours).

Gassing tubes (n=120) were prepared, each with a CA (1.7 ml) and water (1.3 ml) mixture (total 3 ml) and connected to a culture tube containing 1ml Global® Total® for Fertilization culture media. As a control, the pH of culture media in each culture tube were measured in 32 sets of tubes that were heated to 37°C during gas equilibration for time periods of 16 and 18 hours (indicating when CO_2 equilibration occurred) as well as 24 and 30 hours (demonstrating culture media pH stability over time). Sets of glass tubes (n=40) were left on a benchtop during gas equilibration in a room with air-conditioning that maintained the room temperature at 15°C, with an additional 48 sets of glass tubes left in a room with the temperature set at 25°C. Gas equilibration was stopped in the two test groups with the removal of the needles after 16 (25°C group only), 18, 20, 22, 24 and 30 hours of experimentation (n=8 per temperature/incubation time combination). The stoppered, gassed culture media tubes were then placed in a heated warming block at 37°C for one hour to heat the culture media to 37°C before pH could be determined. The pH values of all culture media samples were measured using a calibrated blood gas analyser, as described.

3.1.2.3. *Impact of altitude on citric acid volume required*

The effect of altitude on pH regulation in the culture tubes was evaluated by performing identical experiments at different locations in South Africa (at altitudes of 30, 350, 925, 1326 and 1627 meters above sea level, figure 3.9). Test sites were selected by altitude and ease of access and altitude was confirmed on location via global positioning system (Garmin Nuvi1300, Garmin Southern Africa (Pty) Ltd, Johannesburg, South Africa). At each location, ninety sets of tubes (n=450) were divided into groups of nine with increasing volumes of CA (1.2 – 2.2 in 0.2 ml increments) being added to the gassing tubes.
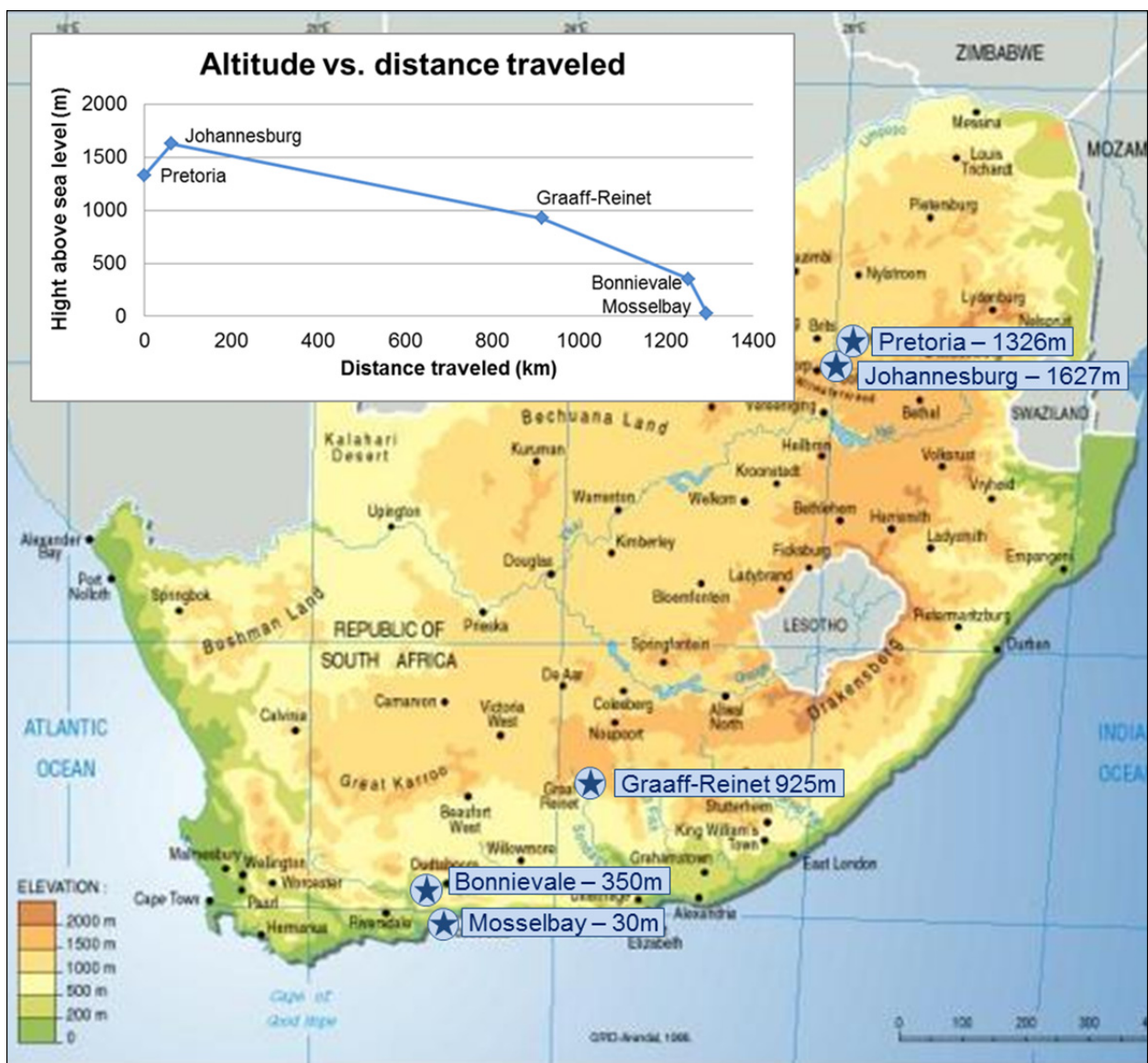
Figure 3.9: Relief map of South Africa[188] with indicators at sites where experiments were performed at various altitudes. Insert shows the altitude of each location vs. distance travelled from the reference laboratory.

All tubes were kept in warming blocks heated to 37°C using a slide warmer (SW85, Adamas Instrumenten, Rhenen, Netherlands) for 18 hours after the CA injection. To facilitate the mobility of repeated sampling at different locations, a portable pH meter (ICRI5209, Crison, Barcelona, Spain) accurate to two decimal points was used to measure culture media pH subsequent to equilibration. The pH meter was connected to a glass, double-junction, potassium chloride-filled pH probe (5029, Crison, Barcelona, Spain) to manufacturer's specifications (figure 3.10).

Figure 3.10: Portable pH meter and potassium chloride-filled glass probe.

3.1.2.4. *Influence of water to citric acid volume ratio*

The first protocols developed for the simplified tWE IVF culture system specified a 3 ml CA volume to obtain optimal culture media pH. As the culture system evolved and alternate glass tubes were introduced, the CA volume needed to facilitate culture media pH decreased. To maintain the uniformity of the culture system, a 3 ml volume of fluid was retained as part of the gas equilibration protocol, and changes in CA volume were compensated for by the addition of water.[175] This adjustment was evaluated by a comparison of gassing tubes with and without water added to the CA.

Sets of tubes (n=60) were divided into three groups of twenty, with each group injected with 1.4, 1.7 or 2.0 ml of CA. Half the gassing tubes in each group had water added to the CA up to a total volume of 3 ml (control) and the other half had CA with no water added (test). All tubes were incubated at 37°C for 18 hours, after which equilibration was stopped and the pH of the culture media was measured with a blood gas analyser, as described.

3.2. Section 2: Verification of insemination protocol

On completion of the QC assessment of the simplified tWE IVF culture system, an investigation into the insemination protocol of the system was undertaken. The simplified culture system employs a low number of motile sperm ($\leq 10 \times 10^3$) during the insemination of oocytes.[18,175] The current study set out to investigate the optimal number of sperm, using a bio-assay. Furthermore, the culture media and sperm used during the experiments were examined to assess the effect of high volumes of culture media with low numbers of sperm during insemination, on reactive oxygen species (ROS) generation and sperm deoxyribonucleic acid (DNA) integrity.

3.2.1. Insemination counts

The hemi-zona binding assay (HZA) is a bio-test employed to simulate sperm-zonae binding during IVF.[97] Couples seeking an assisted reproduction procedure at the RBL, Steve Biko Academic Hospital, Pretoria, South Africa were given an information leaflet and requested to give consent for the use of non-fertilized oocytes and/or spermatozoa following the ART procedure (*Addendum 7.2. Information leaflet and informed consent for non-clinical research*). Semen, processed according to standard RBL protocol (*Addendum 7.3.1 SOP F1.16.2 Sperm processing for therapeutic procedures*) and non-viable unfertilized oocytes remaining from attempted ART cycles at the RBL were used for the HZA.

3.2.1.1. Determining minimal sperm insemination numbers

The HZA bio-test was performed according to the RBL protocol and as described by Franken *et al.*[97] See Addendum 7.3.2 (*SOP F2.7.1 Hemi-zona Assay*) for a detailed description of the method. Oocytes (n=104) were bisected using a micro-blade (BD Micro-Sharp[TM], Beckton, Dickinson and Company, Franklin Lakes, NJ, USA) manipulated with a micromanipulator (Transferman NK2, Eppendorf, Hamburg, Germany) and the two halves of the zona pellucida were allocated to either the test or control group as illustrated in figure 3.11. The two hemi-zonae were then placed in separate 50 µl micro-drops of pre-gassed culture media (Global[®] Total[®] for Fertilization) drops covered by mineral oil (FertiCult[TM] - Mineral Oil, FertiPro NV, Beernem, Belgium) and washed spermatozoa were introduced to each of the micro-drops of culture media.
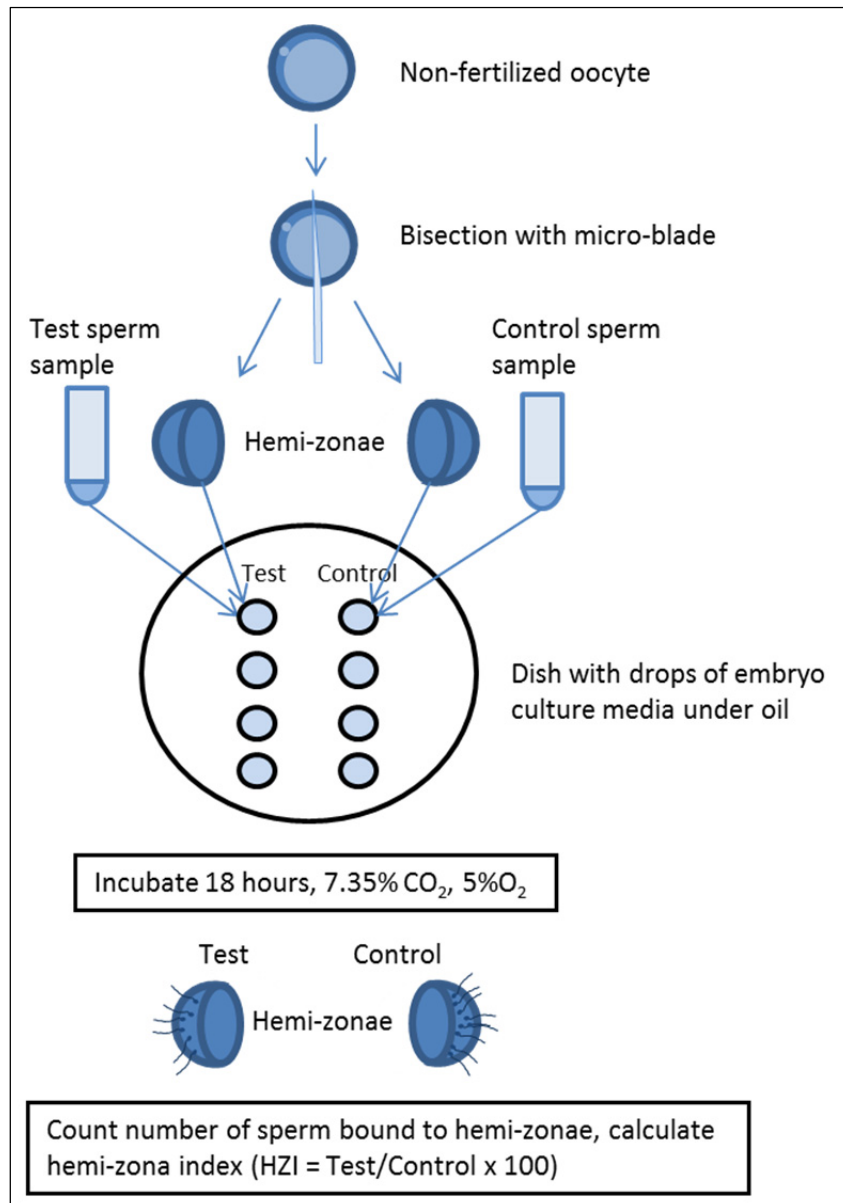
Figure 3.11: Schematic representation of the hemi-zona binding assay.

Insemination of the control group's hemi-zonae (n=104) was performed with 50×10^3 motile spermatozoa according to RBL protocol. The test group's hemi-zonae (n=104) were inseminated with lower numbers of spermatozoa, ranging from 0.5×10^3 to 20×10^3 motile spermatozoa (figure 3.12). The hemi-zonae and sperm were incubated for 18 hours in a humidified conventional embryo culture incubator (7.35% CO_2, 5% O_2, 37°C; K-Minc[TM], Cook Medical, Bloomington, Indiana). The time of incubation was selected to simulate the amount of time oocytes in the simplified culture system would be exposed to sperm before the first pre-zygote evaluation is performed.

After incubation, the bisected zonae were removed from the culture media and the number of spermatozoa bound to each hemi-zona counted in a double-blinded fashion by two evaluators at 400 times magnification (Axiovert 200; Zeiss, Oberkochen, Germany).

In order to standardize the assay and eliminate zona with diminished binding, hemi-zonae from the control group with fewer than 50 spermatozoa bound were considered non-optimal. The test-runs from both the control and test cohorts for these oocytes were discarded. The minimum sperm concentration, which would provide sufficient sperm binding, was determined from the results obtained.
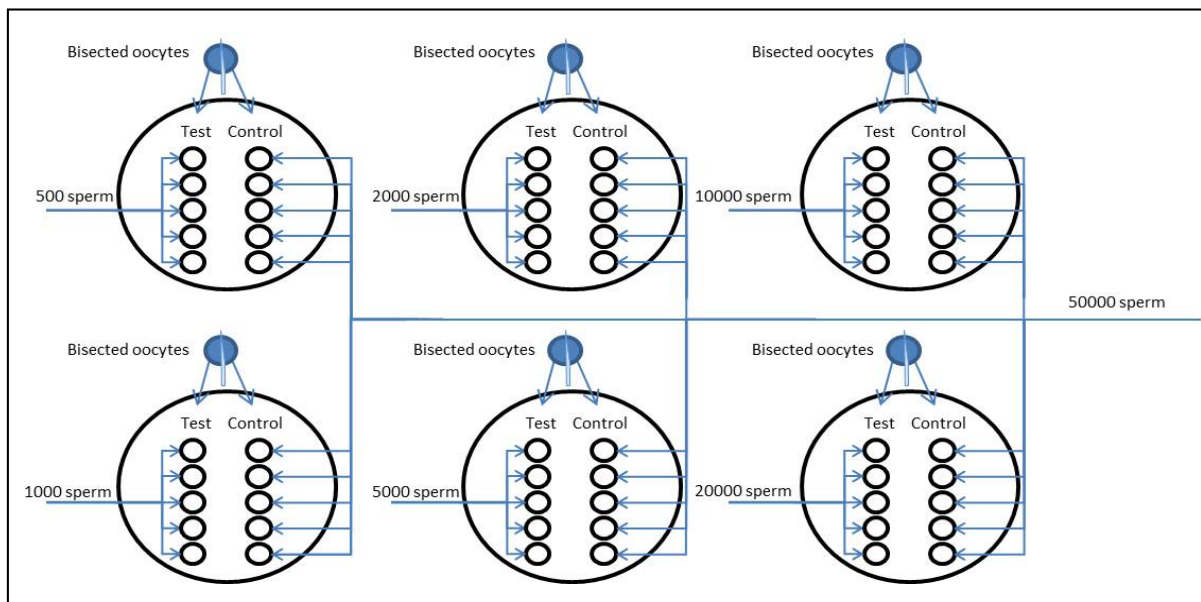
Figure 3.12: The test and control group used in the hemi-zona binding assay illustrating the increasing motile sperm concentrations within the test cohort.

3.2.1.2. Conventional culture vs. the simplified tWE IVF culture system

The minimal number of spermatozoa to inseminate with was determined by employing the simplified tWE IVF culture system and comparing this culture system with conventional micro-droplet culture, using the HZA bio-test. Non-viable, unfertilized oocytes were bisected (n=19) with each hemi-zona either placed in a 200 µl culture drop (control) (Global$^®$ Total$^®$ for Fertilization) or a tWE culture tube (test) containing 1 ml gassed culture media (Global$^®$ Total$^®$ for Fertilization), as is performed in the simplified culture system (figure 3.13). Both the test and control samples were inseminated with 5×10^3 motile spermatozoa (resulting in 5×10^3 and

25x10^3 motile spermatozoa per millilitre respectively). The samples were incubated for 18 hours in a conventional embryo culture incubator (control) and laboratory warm bath, set at 37°C (test). For each group, the hemi-zonae were removed from the culture media and the number of spermatozoa bound was counted.
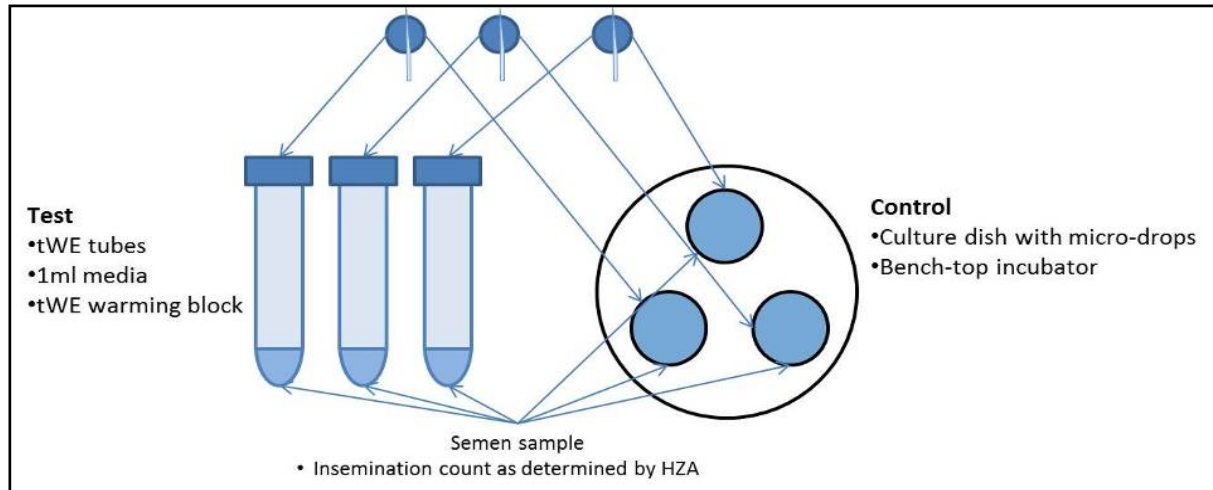
Figure 3.13: Representation of the hemi-zona binding assay (HZA): a comparison of conventional vs. the simplified Walking Egg (tWE) in vitro fertilization culture system.

As the prescribed insemination method of the simplified tWE IVF culture system indicates sperm to be added drop-wise to the culture tubes, using a needle and syringe,[18,175] the method had to verified before the experiment started. Using water, with trypan blue (15250061 0.4% Trypan Blue Solution, Thermo Fisher Scientific Inc., Waltham, Massachusetts) added for better visualization, drops of increasing volume (5 – 100 µl) was pipetted onto a petri dish (150360 90mm diameter Nunc^{TM} IVF Petri Dish, Thermo Fisher Scientific Inc., Waltham, Massachusetts) using a calibrated variable volume pipette (Finnpipette$^®$ F2). Alongside these drops, additional drops were made by expelling either a single or two drops of the coloured water from a 1 ml pipette with an 18 or 26 gauge needle (305196 18 G & 30511 26 G BD PrecisionGlide^{TM}) attached (figure 3.14). By visual comparison, it was determined that a single drop of fluid expelled from an 18 gauge needle is equivalent to approximately 25 µl in volume. Sperm samples used with the experiment was diluted to an appropriate concentration, as to have the 5 x 10^3 motile sperm in 25 µl. Sperm insemination was hence performed by expelling a single drop of the diluted sperm sample into tWE culture tube, using a 1 ml syringe and 18 gauge needle.
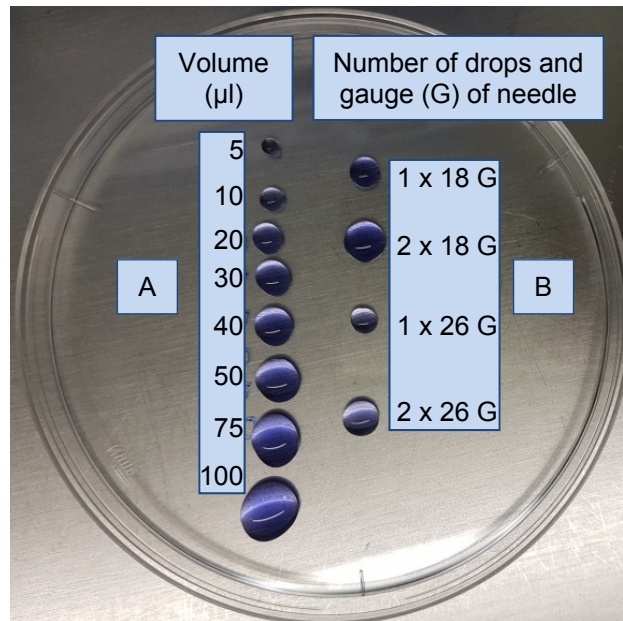
Figure 3.14: Determining droplet volume for use during the simplified Walking Egg *in vitro* insemination. A: Defined, increasing volumes and B: a single or two drops expelled from a syringe, using two needle gauges.

During experimentation difficulty was experienced with the retrieval of hemi-zonae from the culture tubes. Locating the hemi-zonae within the micro-droplets is already challenging as the zonae are transparent glycol-protein structures.[92] This proved to be near impossible when attempting to retrieve the hemi-zonae in the large volume of tWE tubes. After multiple attempts to perform the experiment, only three of the nineteen hemi-zonae incubated could be retrieved. The experimental design was then adapted to improve visualization of the hemi-zonae by replacing the tWE tubes with 4-well dishes. Non-viable, unfertilized oocytes were bisected (n=14) and the hemi-zonae were placed in either a 200 µl culture media drop (control) or a 1 ml culture media volume in a 4-well culture dish (179830 Nunc^{TM} 4-well dish, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The culture media containing hemi-zonae were inseminated with 5×10^3 motile spermatozoa and left to incubate for 18 hours in a conventional embryo culture incubator. The hemi-zonae were easily located and all hemi-zonae were retrieved after the test period.

3.2.1.3. Visualization of sperm-zona binding

To observe clearly and count the number of spermatozoa bound to the hemi-zonae accurately, staining with ethidium homodimer (EThD) was performed[189] and stained hemi-zonae were examined using a confocal laser-scanning microscopy system

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(LSM 510 Meta confocal, Zeiss, Oberkochen, Germany) mounted on an inverted microscope (Axiovert 200, Zeiss, Oberkochen, Germany) equipped with a 40X oil immersion objective and argon laser with 488nm excitation at the Laboratory for Microscopy and Microanalysis, University of Pretoria (figure 3.15).
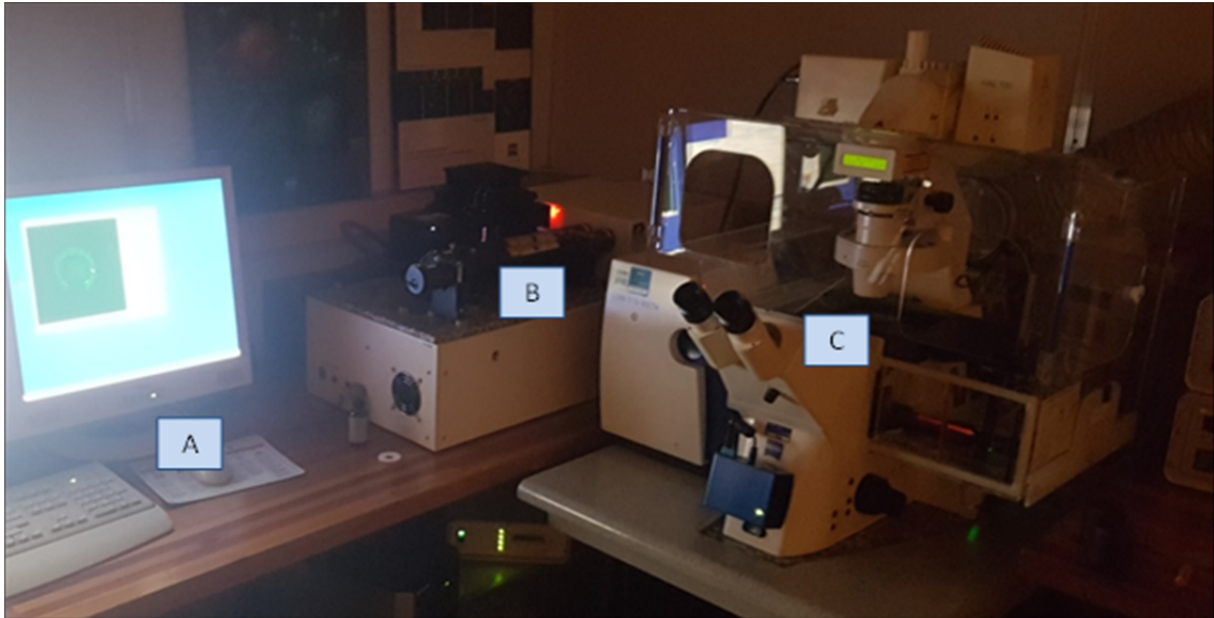
Figure 3.15: Confocal microscopy system with A: a computer with image processing software and B: a laser light module connected to the C: Zeiss Axiovert 200 inverted microscope with motorized stage and protective encasement.

The hemi-zonae were incubated for one hour at room temperature in phosphate buffered saline (PBS; P4417, Sigma-Aldrich Pty Ltd, Johannesburg, South Africa) containing 0.02 M glycine. Following incubation, the hemi-zonae were washed in PBS and placed in 4 µM EThD (EthD-2; LIFE Technologies, Johannesburg, South Africa) for 30 minutes (in the dark at room temperature). Subsequently, the hemi-zonae were washed three times in PBS.

Glass coverslips (22 x 22 mm microscope cover glass, Lasec SA, Johannesburg, South Africa) were prepared in advance by placing 1 µl droplets of clear nail polish on each corner and leaving it to dry. Droplets of antifade suspension (Prolong Diamond Antifade 5; LIFE Technologies, Johannesburg, South Africa) were added in the centre of each coverslip and the stained hemi-zonae were transferred to the antifade suspension. A microscope slide (76 x 26 x 1 mm microscope slide with ground edge, Lasec SA, Johannesburg, South Africa) was then settled onto each coverslip, after which the space between the coverslip and slide was filled with more

antifade suspension and the edges sealed with nail polish. The samples were briefly viewed under an inverted microscope (Stemi 2000, Zeiss, Oberkochen, Germany) to identify where the hemi-zonae were located on the slides. Placements of the hemi-zonae were indicated by marking the bottom of each slide with a permanent marker and the samples were kept in a dark box until examination.

3.2.2. Sperm deoxyribonucleic acid packaging

Sperm DNA integrity has been shown to have a positive correlation with ART results.[190] The increased culture media volume used with the simplified tWE IVF culture system, combined with a low insemination dose, may have a positive effect on sperm DNA packaging. Toluidine blue is an indirect sperm DNA integrity test that indicates lighter and darker staining of sperm, one with highly condensed chromatin packaging and the other with less densely packed chromatin.[191]

After the comparison between the two culture methods using the HZA had been done as described in section 3.2.1.2 (p. 55), the individual DNA packaging of spermatozoa remaining in the culture media was assessed. Due to low volumes and numbers of sperm (200 µl culture media and 5×10^3 motile sperm per sample), samples (n=36) were pooled (three samples pooled together to obtain n=12 pooled samples) before sperm and culture media were separated by centrifugation (Centrifuge 5417R, Eppendorf, Hamburg, Germany) at 500g for 10min.

After centrifugation of the pooled samples, the pellets containing spermatozoa were removed, smeared onto a glass microscope slide and left to air dry. The slides were stained with toluidine blue according to standard RBL protocol (*Addendum 7.3.3 SOP F1.6.1 Toluidine blue*). The samples were exposed to a 1:1 mixture of 96% ethanol and acetone for 30 minutes to fix the spermatozoa to the slide and allowed to air dry. Fixed slides were then stained with 0.1% nigrosine. Once the nigrosine background stain had dried, slides were placed in 0.1M HCl for 15 minutes to hydrolyse the spermatozoa and then rinsed twice with distilled water. Slides were finally submerged in a 0.05% toluidine blue solution for 15 minutes, removed and left to air dry.[86]

The stained spermatozoa were evaluated using a light microscope (Axioskop 40, Zeiss, Germany) and 40x-phase contrast objective. A total of 200 spermatozoa per sample were counted, distinguishing lightly stained (normal DNA packaging) from darker stained sperm (abnormal DNA packaging or DNA fragmentation present, Figure 3.16)[190] and the percentage of spermatozoa with DNA fragmentation was calculated.
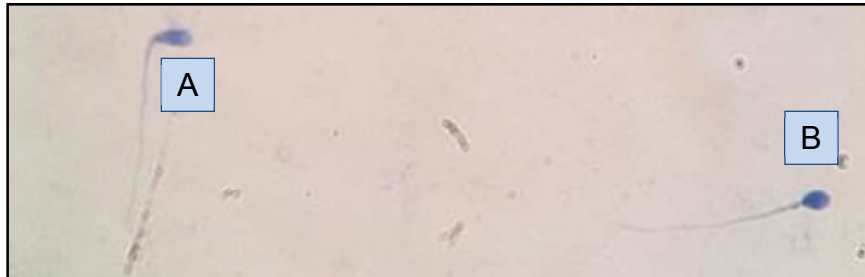
Figure 3.16: Spermatozoa stained with Toluidine blue showing A: normal DNA integrity with light stain and B: fragmented/abnormal DNA by dark stain.

3.2.3. Reactive oxygen species generation in culture media

Reactive oxygen species that are natural by-products of cellular metabolism,[127] are present in semen, as well as being produced during semen processing.[35] The presence of ROS is necessary to ensure normal physiological function of spermatozoa, oocytes and pre-implantation embryos.[35] However, increased ROS can lead to oxidative stress and associated harmful effects.[35,127] The fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; $C_{24}H_{16}Cl_2O_7$; D6883,Sigma-Aldrich Pty Ltd, Johannesburg, South Africa) reacts to both free ROS and reactive nitrogen species, and is oxidized to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF).[192,193] The measured fluorescent intensity of DCF is proportional to the total free radical i.e. ROS content of a sample.[192] Subsequent to the experiment described in section 3.2.2, the supernatant after the removal of spermatozoa, was processed and stored for subsequent ROS evaluation.

In addition, for evaluation of the contribution of cumulus cells on ROS generation during incubation, tWE culture tubes (n=6) with 1 ml culture media and conventional IVF insemination dishes with 200 µl culture media drops (n=6) were prepared and gassed. Excess, discarded cumulus cells, removed during a standard oocyte aspiration were then cut into approximately 10 x 10 x 5 mm sections and each piece

placed with either 5 x 10^3 motile spermatozoa in a pre-equilibrated culture tubes or 50 x 10^3 motile spermatozoa in the conventional IVF insemination droplets. The culture tubes were kept in a warming block at 37°C for the duration of the experiment and the insemination dishes kept in a conventional embryo culture incubator (7.35% CO_2, 5% O_2, 37°C).

The experiment's setup simulated the cumulus-oocyte-complex insemination and subsequent continuous culture of embryos in the simplified tWE IVF culture system and during conventional IVF respectively. At the start of culture, 50 µl culture media was removed from each culture tube and insemination drop. The culture tubes and insemination dishes were then incubated for 18 hours, after which another 50 µl sample of culture media was removed. During conventional IVF, fertilized oocytes would be removed from the insemination after 18 hours of culture. Therefore, the insemination dishes were not cultured longer than 18 hours and no more sampling from these dishes was performed. The culture tubes simulating the simplified tWE IVF culture system were cultured to 114 hours after insemination, with 50 µl culture media samples being removed from each culture tube at 66 and 114 hours after insemination. Culture media samples were removed from the culture tubes using a needle and syringe, without opening the tubes. All samples were centrifuged at 1x10^4 G for 5 minutes to eliminate any insoluble particles and the supernatant removed for storage in 2 ml Cryovials^{TM} at -196°C to batch samples for evaluation.

An H_2O_2 standard control was prepared on the day of experimentation by diluting a starting concentration of 30 µM H_2O_2 (H1009, Sigma-Aldrich Pty Ltd, Johannesburg, South Africa) in Global® Total® for Fertilization stepwise by removing 500 µl of the current concentration and adding 1000 µl Global® Total® for Fertilization. The process was repeated until concentrations of 30, 10, 3.333, 1.111, 0.370, 0.123, 0.041 and 0.014 µM H_2O_2 were obtained. Test samples were thawed to room temperature just prior to the readings being performed.

A working solution of 10 µM DCF-DA in PBS was freshly prepared before experimenting and 50 µl DCF-DA, along with 50 µl volume of either sample (n=6 repeats per test sample) or H_2O_2 concentration (n=3 repeats per concentration) was pipetted into separate wells of a white bottomed 96-well plate (Nunc, Thermo

Scientific, Roskilde, Denmark) to have a final volume of 100 µl in each well. Negative controls of Global® Total® for Fertilization, water and 10 µM DCF-DA (n=2 repeats each) were also prepared. The 96-well plate was placed in a multimode plate reader (Biotek Synergy 2, Biotek Instruments, Winooski, Vermont) (figure 3.17). Excitation was performed at 485nm, and fluorescence emission at 590nm was detected.

Figure 3.17: Biotek Synergy 2 multimode plate reader used for fluorescence detection to measure the presence of reactive oxygen species.

3.3. Statistical analysis

3.3.1. Section 1: Culture media temperature and pH assessments

Section 1 detailed an explorative study into the effect of changing environments on culture media temperature and pH. Due to the nature of the experiments, descriptive statistics were utilized to determine standards and interpret data.

3.3.1.1. Evaluation of temperature control devices

Six temperature control devices were assessed using four parameters. A data summary for the parameters was done by instrument through reporting of mean and standard deviations. Within each of the devices, two sides were compared at the

0.05 level of significance in respect of three parameters (device, warming block and culture media temperature) using a student two-sample t-test when equal variance was assumed. Pearson's product-moment correlation coefficient was used to assess the correlation between culture media temperature and each of the other three parameters.

3.3.1.2. *Manipulation of culture media pH*

(i) The optimal volume of CA to provide culture media pH of 7.30 ±0.05 (37°C, 18 hours equilibration) was sought and CA volumes ranging from 1.2 – 3.0 ml were evaluated. Citric acid volumes were compared in respect of pH in a one-way analysis of variance. Multiplicity in pair-wise testing between CA volumes was evaluated using the Bonferroni statistical adjustment.[194] The linear relationship between pH and CA volume was graphically displayed together with mean pH at each CA volume.

(ii) An assessment of pH levels at three temperatures at six time points were performed by a two-way analysis of variance that also included the interaction between temperature and time, which was of primary interest. The linear relationship between pH and time for each temperature was displayed graphically.

(iii) The relationships between culture media pH, CA volume and altitude were investigated. A two-way analysis of variance was employed to consider culture media pH for six CA volumes at five different altitudes, with inclusion of the interaction between pH value and altitude. A graphic representation was used to display the linear relationship between pH and CA volume at each of the altitudes.

(iv) Culture media pH levels were assessed for three CA volumes with or without the addition of water in a two-way analysis of variance. The linear relationship between pH and CA volume for both groups was charted.

3.3.2. *Section 2: Hemi-zona assay and subsequent analyses*

(i)   A one-way analysis of variance with Bonferroni adjustment was employed to compare sperm-zonae binding numbers in six tests and one control group after HZA with decreasing sperm numbers. Descriptive statistics were used to determine 99% confidence intervals for insemination groups of interest in respect of sperm-zonae binding.

(ii)   The derived minimum insemination number was used to compare two culture methods by HZA. A paired t-test was performed to compare the association between sperm-zona binding in the test and control groups. Testing was done at the 0.05 level of significance.

(iii)   Assessments of sperm and culture media remaining after the completion of the previous experiment were used to evaluate sperm DNA fragmentation and culture media ROS generation. Wilcoxon's signed-rank test compared test vs. control groups in both cases at a 0.05 level of significance.[195]

Chapter 4

Results

Chapter 4 Results

4.1. Section 1: Quality control of the simplified culture system

The culture of embryos relies on three essential aspects: (1) providing the growing embryo with the necessary nutrients through suitable culture media; (2) regulation of culture media temperature and pH; and (3) reducing detrimental influences from affecting the growing embryo.[14,55,187] Investigations into vital parameters involved in embryo culture with the simplified Walking Egg (tWE) *in vitro* fertilization (IVF) culture system were undertaken. The use of different instruments to heat culture media to 37°C, the control of culture media pH under various conditions and the sperm insemination protocol of the simplified tWE IVF culture system were investigated. Results obtained were processed using statistical analyses and results for graphic displays are shown in Addendum 7.1.

4.1.1. Temperature regulation

During conventional embryo culture, the temperature of culture media is regulated to 37°C by a purpose-built, micro-processor-controlled IVF incubator.[14] For the simplified tWE IVF culture system any type of heating apparatus to regulate the warming blocks and to maintain the culture media temperature can be used, as long as the temperature is kept constant at 37°C.[18] The capacity of six different heating instruments to provide a stable temperature of 36.6-37.0°C during embryo culture was explored. The equipment tested comprised a selection found in standard IVF laboratories. Two instruments that enclose (warming oven and incubator), two that partially enclose (water bath and dry-bath) and two that touch only the surface (slide warmer and IVF workstation) of the warming block were tested (figures 3.3 p. 45 & 3.4, p. 46).

During the experiment the room temperature (T1: ambient) and three parameters per instrument were measured (T2: device, T3: warming block and T4: culture media temperature) and each test was repeated ten times to indicate their ability to regulate temperature when set at 37°C. Two sides of each instrument (figure 3.3, p. 45) were measured each time to determine intra-device variation. The mean and standard deviation (SD) values of temperatures measured over time are depicted in table 4.1.

Table 4.1: Temperature comparison (mean ±SD) of six heating devices (grouped A: enclosed, B: partially enclosed and C: single surface heating), indicated by the measurement of ambient (T1, n=60), device (T2, n=120), warming block (T3, n=120) and culture media (T4, n=600) temperatures (n_{Total}=900).

	Side 1	*Side 2*	*Difference (p-value)*
Warming oven	**(A) Enclosed heating**		
T1: Ambient	25.4°C ±1.95	N/A	N/A
T2: Device	36.7°C ±0.30	36.7°C ±0.30	0.000 (1.000)
T3: Block	36.6°C ±0.48	36.0°C ±0.61	0.660 (0.015)
T4: Media	37.2°C ±0.28	36.2°C ±0.38	0.982 (0.000)
Incubator			
T1: Ambient	26.4°C ±1.67	N/A	N/A
T2: Device	36.7°C ±0.10	36.6°C ±0.11	0.167 (0.536)
T3: Block	36.6°C ±0.09	36.6°C ±0.10	0.011 (0.960)
T4: Media	36.9°C ±0.09	36.8°C ±0.08	0.084 (0.715)
Water bath	**(B) Partially enclosed heating**		
T1: Ambient	25.3°C ±0.76	N/A	N/A
T2: Device	37.4°C ±0.05	37.4°C ±0.05	0.000 (1.000)
T3: Block	36.8°C ±0.15	36.8°C ±0.16	0.011 (0.886)
T4: Media	37.4°C ±0.04	37.3°C ±0.06	0.028 (0.215)
Dry-bath			
T1: Ambient	24.6°C ±1.41	N/A	N/A
T2: Device	36.8°C ±0.16	36.8°C ±0.16	0.000 (1.000)
T3: Block	36.2°C ±0.15	36.2°C ±0.19	0.030 (0.820)
T4: Media	36.7°C ±0.08	36.7°C ±0.11	0.024 (0.765)
Slide warmer	**(C) Single surface heating**		
T1: Ambient	24.7°C ±0.67	N/A	N/A
T2: Device	36.7°C ±0.58	35.8°C ±0.53	0.800 (0.011)
T3: Block	36.1°C ±0.18	35.6°C ±0.16	0.420 (0.000)
T4: Media	36.7°C ±0.09	36.2°C ±0.16	0.454 (0.000)
IVF w-station			
T1: Ambient	26.8°C ±0.68	N/A	N/A
T2: Device	37.1°C ±0.20	37.3°C ±0.16	0.189 (0.045)
T3: Block	36.3°C ±0.39	36.3°C ±0.35	0.022 (0.905)
T4: Media	36.8°C ±0.18	36.8°C ±0.20	0.054 (0.667)

Although all the equipment were set at 37°C, when considering culture media temperature, the results indicated a drift of approximately -0.3°C in the dry-bath and slide warmer and approximately 0.2 and 0.4°C in the warming oven and water bath respectively.

The temperature regulation outside of the desired range did not exclude any of the equipment from this investigation, however the inherent inconsistency exhibited between instruments, as is also reported by Walker *et al.*,[101] warrants the need for accurate calibration of heat regulating equipment. If, for some reason, an instrument could not be calibrated, the change in temperature should be compensated for by adaptation of the temperature set point, with a regular quality control schedule to confirm that no drift in temperature is occurring.

Intra-device variation

The difference in temperature between side 1 and 2 of each heating instrument (figure 3.3, p. 45) was calculated and compared with a two-sided *t*-test (equal variance) to determine whether the difference was significant ($p < 0.05$). Figure 4.1 illustrates these results (mean) in all groups (see Addendum 7.1.1 for data set).
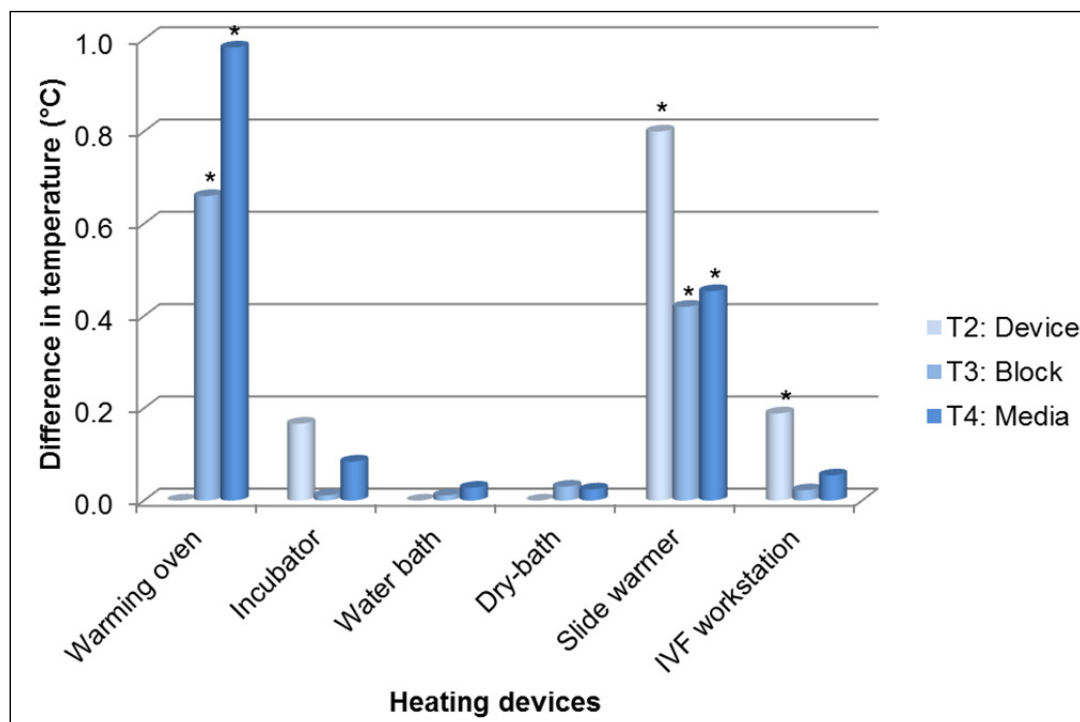
Figure 4.1: Temperature variation between two sides of each heating device (* statistical significance $p < 0.05$, n=140 per device).

The warming oven and slide warmer showed a much wider variation between sides than would be ideally suited to embryo culture. The cause of this variation is the placement of the heating element within the instrument i.e. in the warming oven the heating element is found on the left hand-side, resulting in slightly higher temperatures on that side. Should a device with a high intra-variation be used with the simplified tWE IVF culture system, calibration and positioning of warming blocks in a specific area in the instrument would be advisable. Although a wide variation was noted in the warming oven in the warming block and culture media temperatures, no variation was observed in the device temperature. Due to the design of the instrument, the temperature probe could be passed only through a single hole at the top of the oven. The two positions measured by alternating the angle of the wire probe were very close, hence the limited proximity of the two positions could have accounted for the lack of variation in the results.

Temperature stability over time

Conventional embryo culture incubators are able to maintain their temperature at 37°C with an approximate 0.2°C fluctuation,[14] therefor the similar ability capacity of each of the tested instruments was a point of interest. Due to the known effects of heat-shock proteins on embryo development,[104] temperature variation was deemed preferable below, rather than above, 37°C. Therefore, 36.6-37.0°C was prescribed as the preferred physiological range for embryo culture. For each instrument ten repeats of five temperature measurements was used to determine the stability of temperatures over four days. The mean temperature and fluctuation (95% confidence-interval (CI)) of each instrument are listed in table 4.2.

Table 4.2: Culture media temperature and fluctuations for each of the six instruments evaluated (n=300).

Instrument	Mean	Fluctuation (95%CI)
Warming oven	37.2°C	±0.41°C
Incubator	36.9°C	±0.11°C
Water bath	37.4°C	±0.06°C
Dry-bath	36.7°C	±0.09°C
Slide warmer	36.7°C	±0.12°C
IVF workstation	36.8°C	±0.21°C

Figure 4.2 shows the mean culture media temperature values of the repeats per heating device (see Addendum 7.1.1 for data set).
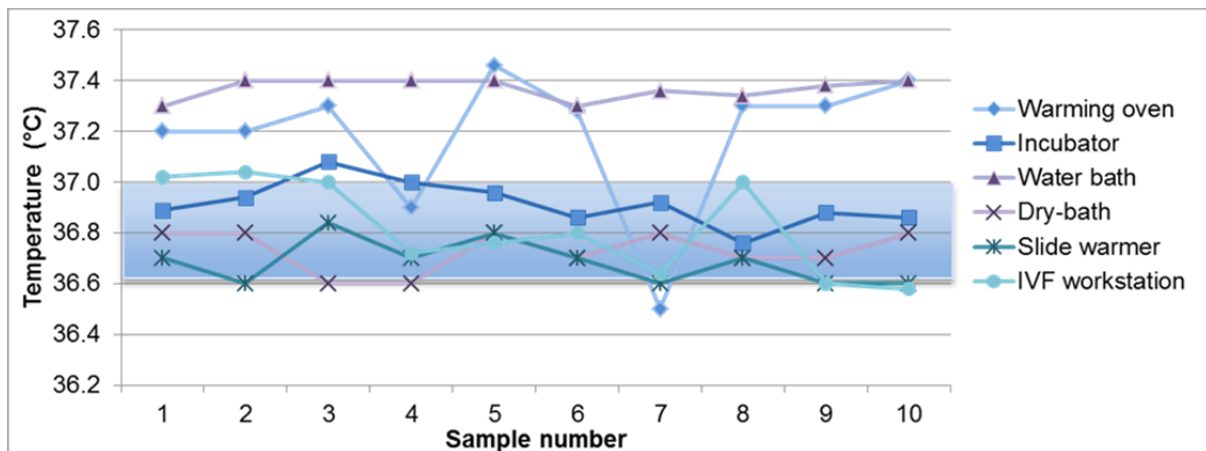
Figure 4.2: The mean culture media temperature per repeat for each device, with the acceptable ±0.2°C range (36.6 – 37.0°C) indicated in blue (n=300).

According to the data, the warming oven did not show sufficiently stable temperatures that could be used during embryo culture. Furthermore, the IVF workstation presented a temperature fluctuation of 0.21°C. All the other instruments tested (incubator, water bath, dry-bath and slide warmer) displayed temperature fluctuations of <0.2°C.

Correlations: culture media vs. ambient, device and warming block temperatures
The correlation of culture media temperature vs. ambient, device and warming block surface temperature was determined and each coefficient of variance (CV) (p-value) is shown in table 4.3. Absolute CV of ≥0.7 indicated a strong correlation and 0.7>|CV|>0.5 a moderate correlation between culture media and the other parameters measured.

The warming block temperature correlated with culture media temperature in all apparatus, except the IVF-workstation. The warming oven and incubator showed the strongest correlation, while the dry-bath and slide warmer indicating a strong correlation when CV values were rounded to 0.7. The water bath's warming block temperatures correlated moderately with culture media temperature. Device temperature correlated with culture media temperature in the incubator (strong), as well as the water bath and dry-bath (moderate), while ambient temperature only

showed correlation to culture media temperature in results from the IVF workstation (moderate correlation, CV rounded to 0.5). All the results with moderate or strong correlations had p-values of <0.05.

Table 4.3: Pearson's correlation coefficient of variance values (p-value) for ambient, device and warming block temperatures, compared to culture media temperature (n=300, correlations are color-coded).

Device	T1: Ambient	T2: Device	T3: Block	
Warming oven	0.2530 (0.282)	0.0158 (0.947)	0.7400 (0.000)	
Incubator	-0.3289 (0.183)	0.7694 (0.000)	0.9504 (0.000)	
Water bath	0.2541 (0.309)	0.5824 (0.011)	-0.5386 (0.021)	*T4: Media*
Dry-bath	0.2817 (0.229)	0.0765 (0.749)	0.6832 (0.001)	
Slide warmer	-0.1209 (0.612)	0.5148 (0.020)	0.6987 (0.001)	
IVF w-station	-0.4799 (0.044)	-0.0702 (0.782)	0.0749 (0.768)	
All combined	0.1496 (0.112)	0.5392 (0.000)	0.7809 (0.000)	

Correlations: Strong correlation (|CV|≥0.7, p<0.05)
Moderate correlation (0.7>|CV|>0.5, p<0.05)

Negative correlations were identified between the warming block and culture media temperatures of the water bath, as well as between ambient and culture media temperatures of the IVF workstation. The negative correlation in the water bath can possibly be explained by the cooling effect of evaporating water that may have splashed onto the surface of the warming block while circulating, resulting in a biased result when the warming block surface temperature was measured. The IVF workstation's negative correlation is most likely due to the large surface area of the workstation and the constant flow of air over this surface.

4.1.2. Culture media pH

The regulation and stability of culture media pH are imperative to keep internal pH of the cells in culture at the desired level since a change in intracellular pH can affect embryo development and metabolism. [14,112] During human embryo culture, most commercially available culture media have a recommended pH range of 7.2 – 7.4[187] and during the experiments a culture media pH of 7.3 ±0.05 was strived for.

4.1.2.1. Regulation of pH by citric acid volumes

An analysis of variance showed a highly significant (p <0.0001) difference between the means of culture media pH values obtained from different citric acid (CA) volumes. A Bartlett's test for equal variance calculated no significant variance between groups (p=0.361) and equal variance was assumed. The linear relationship between culture media pH and CA volume can be seen in figure 4.3 (data set in Addendum 7.1.1). The CA volume range between 1.6 and 2.0 ml showed mean pH values approximately within the 7.3 ±0.05 range sought.
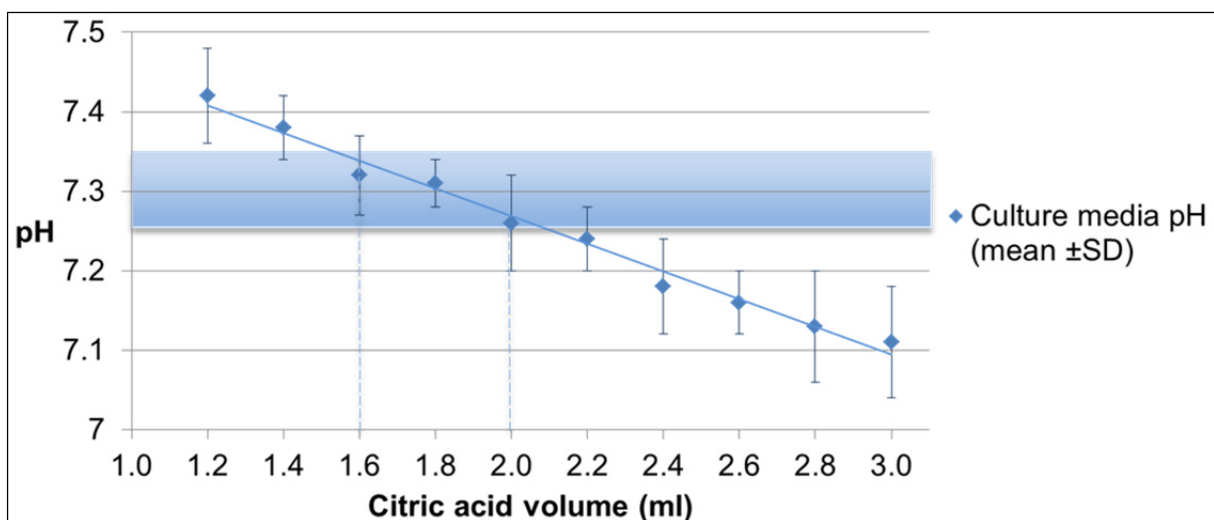
Figure 4.3: Linear relationship of culture media pH and increasing volumes of CA after gas equilibration (optimal pH range of 7.3 ±0.05 highlighted, n=100).

4.1.2.2. Temperature during gas equilibration

The simplified tWE IVF culture system follows a protocol of gas equilibration for 18 hours at 37°C.[18] Gas equilibration at 37°C vs. room temperature (15 and 25°C) was compared and culture media pH values measured (figure 4.4). According to Van Blerkom *et al.*, the culture media pH value will plateau out after gas equilibration at 37°C has been completed.[18] Measurements were therefore taken after 16, 18, 24 and 30 hours incubation at 37°C to show this plateau. Gas equilibration was performed at 16, 18, 20, 22, 24 and 30 hours of incubation for the test groups (15 and 25°C). The culture media pH was still high above the physiological pH range required at 15°C, after 18 hours of incubation, whereby the 16 hour time of incubation was not necessary. The mean (±95% CI) culture media pH values were used to display graphically the impact of temperature on gas equilibration in figure 4.4 (see Addendum 7.1.1 for data set).
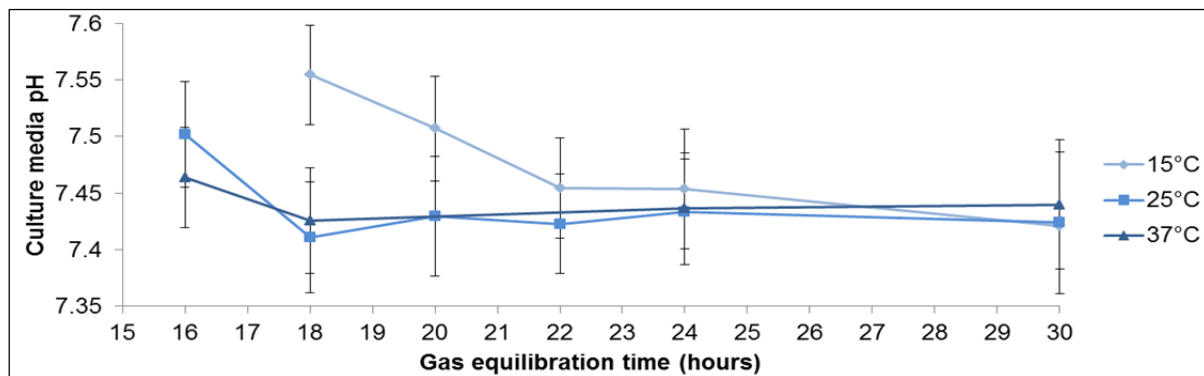
Figure 4.4: Culture media pH after gas production at various temperatures (15, 25 and 37°C) and increasing time intervals (16 - 30 hours) of equilibration (n = 120).

The experimental results clearly show a plateau in culture media pH levels with gas equilibration at 15 and 25°C, although the plateau occurs only after 22 hours of incubation at 15°C. With this in mind no heating equipment should be required during gas equilibration as long as sufficient time is allowed for gas equilibration when it is performed at room temperature.

4.1.2.3. *Impact of altitude on citric acid volume required*

It is well documented that the relationship between carbon dioxide (CO_2) levels required to effect a specific culture media pH are influenced by air pressure as well as altitude.[14,115,187] An increased amount of CO_2 is needed to ensure a particular pH of culture media at higher altitudes, as is explained by the Henderson-Hasselbach equation.[71] The simplified tWE IVF culture system is closed-off from the environment, with gas pressure in the system regulated by the production of CO_2 gas. No publications on the influence of various altitudes on the simplified system are cited. This unknown variable was therefore considered in the current investigation with increasing CA volumes (1.2 – 2.2 ml) utilized during gas equilibration at various locations with different altitudes i.e. to determine whether there would be an effect with a change in altitude (five locations, 30 – 1627 meters above sea level) on culture media pH within the system (figure 3.9, p. 51).

From the results obtained, no difference in mean culture media pH could be linked to a change in altitude between 30 and 1627 meters above sea level. This implies that the simplified tWE IVF culture system can be employed without any amendments to protocol at most if not at all altitudes. Predictive margins (95% CI) of culture media

pH values for all locations are plotted against CA volume in figure 4.5 (see Addendum 7.1.1 for data set). As seen in figure 4.5, the six measurements for each of the five locations followed the same trend, without any shift in pH when values from the same volume of CA and different altitudes are considered.
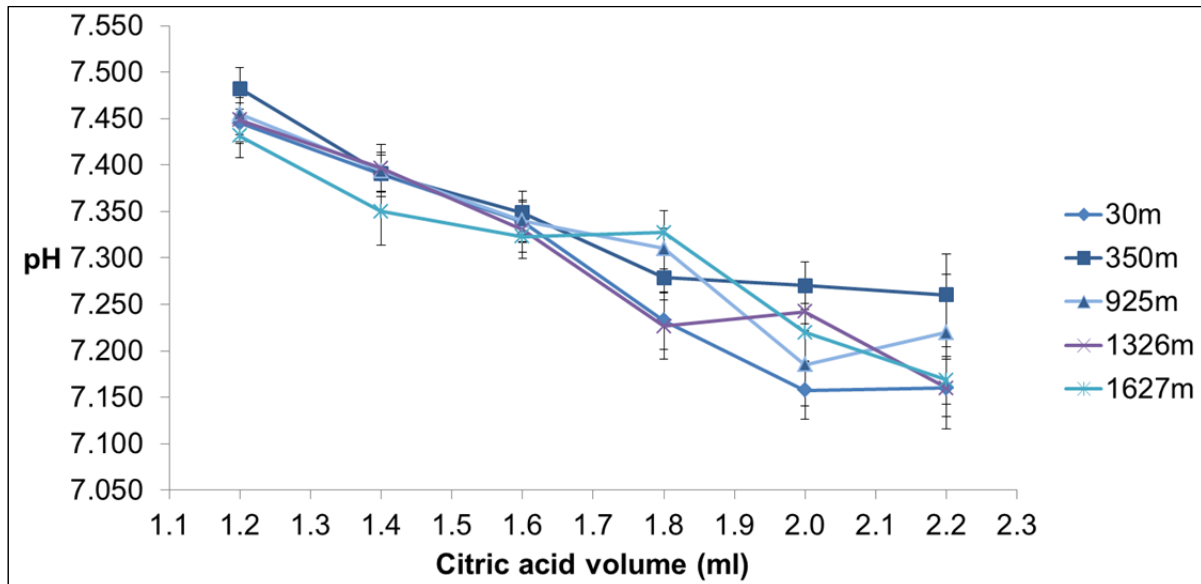
Figure 4.5: The effect of altitude on gas equilibration depicted by culture media pH values, after gas equilibration with increasing volumes of citric acid (n=450).

4.1.2.4. Influence of water to citric acid volume ratio

According to the simplified tWE IVF culture system protocol, a total volume of 3 ml is prescribed to be used during gas equilibration.[175] When the CA volume to be injected does not reach 3 ml, the CA must be diluted with sufficient water to obtain a 3 ml volume.[175] The necessity to dilute CA with water was investigated since this could pose a possible margin of error during preparation. Gas equilibration of sets of culture tubes with three different volumes of CA (1.4, 1.7 and 2.0 ml) were prepared, with and without the addition of water prior to gas equilibration. The mean culture media pH (±SD) after equilibration of each CA volume is depicted in figure 4.6 (data set in Addendum 7.1.1).

All the results indicated that the culture tubes from the test group (without water) measured lower culture media pH values than the tubes in control group (with water dilution). The results from the three CA volume groups (1.4, 1.7, 2.0 ml) showed a 0.155, 0.124 and 0.001 pH value difference respectively. The addition of water can

clearly be seen to have had an impact on gas equilibration, especially in tubes with lower CA volumes, where more water would have to be added to obtain the final 3 ml volume.
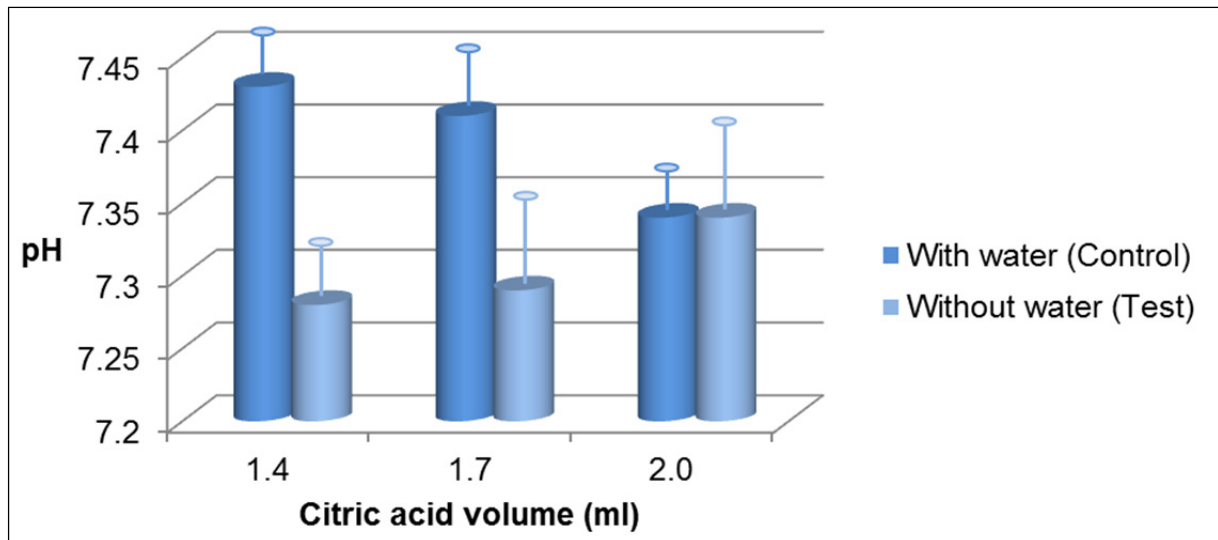
Figure 4.6: The effect of citric acid dilution with water (final volume of 3 ml) and without, on culture media pH after gas equilibration (n=60).

4.2. Section 2: Verification of insemination protocol

The simplified tWE IVF culture system follows an insemination protocol of ≤10 x 10^3 motile sperm per culture tube containing 1 ml culture media and one oocyte-cumulus-complex.[18,175]   During conventional IVF, higher numbers of sperm (~25 x 10^3 – 100 x 10^3 motile sperm) are inseminated in lower volumes (~50 – 250 µl) of culture media.[15,86]   An investigation into insemination with minimal sperm was performed with the use of a hemi-zona bio-test during the current study.

4.2.1. Insemination counts

4.2.1.1.   Determining minimal sperm insemination numbers

Binding of sperm to the zona pellucida, after insemination with various amounts of sperm, was compared by a modified hemi-zona binding assay (HZA).[97]   Semen samples from five donors were used and sperm parameters can be seen in figure 4.7 (see Addendum 7.1.1 for data set), indicating that all semen samples used had sperm parameter values above the lower reference limits as indicated by the 5th addition of the World Health Organization's Laboratory manual for the examination and processing of human semen.[108]
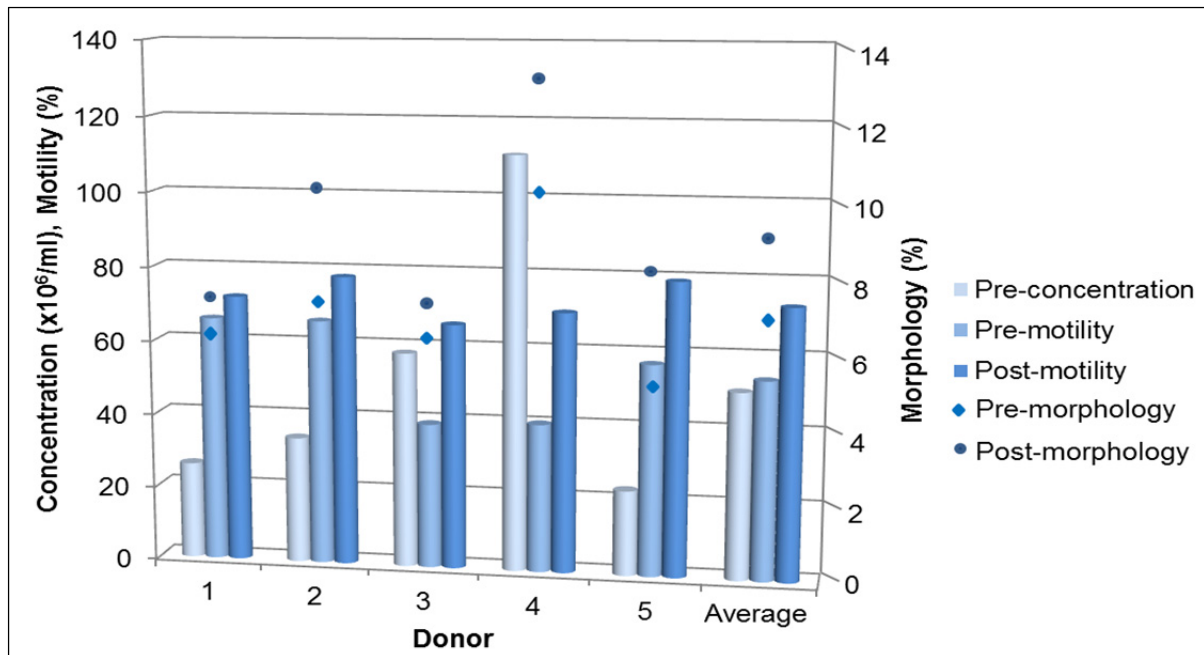
Figure 4.7: Individual and average sperm parameters (bar chart on primary axis: concentration and motility; X-Y scatter plot on secondary axis: morphology) of donors (n=5) used during the hemi-zona assay.

Sperm-zona binding was microscopically counted in a double-blinded fashion after decreasing numbers of motile sperm ($50 \times 10^3 - 0.5 \times 10^3$) were used for insemination in 50 µl culture media (18 hours incubation in a conventional IVF incubator). The number of sperm bound to the hemi-zonae (mean ±SD & 95% CI), per group of sperm insemination number, is listed in table 4.4.

Table 4.4: Mean binding (95% CI) of spermatozoa to hemi-zonae after insemination with decreasing numbers of sperm (n=104).

Sperm insemination number ($x10^3$/ml)	Mean no sperm bound	(95% CI)
0.5	13.948	(9.78 ; 18.12)
1	20.533	(16.21 ; 24.85)
2	35.314	(28.14 ; 42.49)
5	39.638	(32.06 ; 47.22)
10	62.824	(50.68 ; 74.97)
20	73.256	(61.92 ; 84.59)
50	119.720	(105.08 ; 134.36)

The binding of 20 sperm to a hemi-zona is considered as a cut-off value indicating that the zona is of acceptable quality for the bio-test.[196] Sufficient sperm-zona binding is also indicative of the fertilization potential of a sperm sample.[196] The results from the current HZA indicated >20 sperm binding from sperm insemination numbers of as few as 1×10^3 motile sperm. When considering the 95% CI, the 1×10^3 motile sperm insemination sample showed a lower limit of 16.21 sperm bound. However all the groups with $>2 \times 10^3$ motile sperm used for insemination displayed lower limits of >20 sperm bound per oocyte. Therefore a minimum cut-off value of 2×10^3 motile sperm for insemination during IVF is recommended, but only after all sperm parameters have been taken into account. Increasing the sperm insemination number to 5×10^3 motile sperm may be advisable when sperm with lower parameters are used.

4.2.1.2. Conventional culture vs. the simplified tWE IVF culture system

Subsequent to determining the minimum cut-off value to be used for sperm insemination, an evaluation of this low insemination with conventional culture vs. the simplified tWE IVF culture system was carried out. Sperm hemi-zonae binding in 200 µl and 1 ml culture media was compared after insemination with 5×10^3 motile sperm. The mean (±SD) sperm binding per hemi-zona numbered 22.36 ±5.06 (200 µl) and 12.79 ±7.75 (1 ml) for the two groups respectively (figure 4.8). The difference between the two groups was calculated as 42.8% with 95% CI of [31.71%; 82.58%].
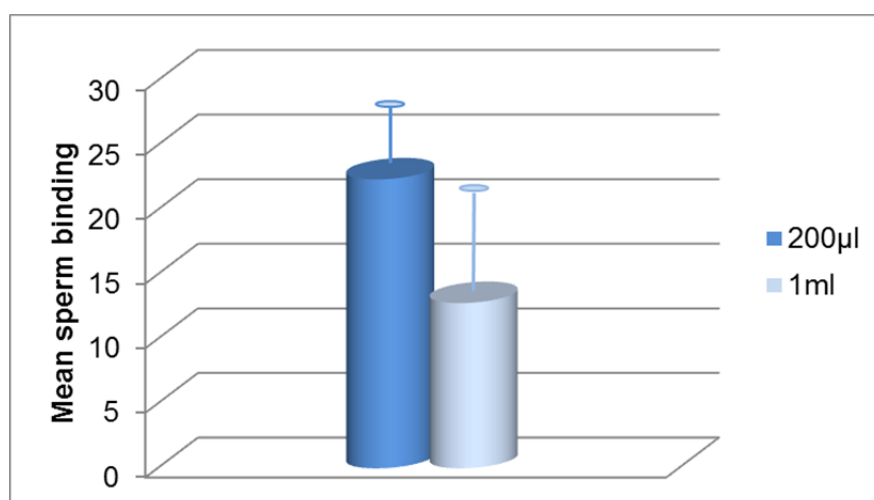
Figure 4.8: Mean sperm binding (±SD) to hemi-zonae in 200 µl vs. 1 ml culture media after insemination with 5×10^3 motile sperm (n=19).

4.2.1.3. Visualization of sperm-zona binding

Visualization of sperm bound to hemi-zonae was done using an inverted Zeiss Axiovert 200 microscope at 200x magnification with phase and PlasDIC modulation contrast. Hemi-zonae were stained with ethidium homodimer (EthD) and evaluated by a confocal laser-scanning microscopy system mounted on an inverted Zeiss Axiovert 200 microscope, equipped with a 40X oil immersion objective and argon laser with 488nm excitation. The visualization of a hemi-zona with sperm bound, as observed with the inverted microscope via various modulation contrast methods as well as with the confocal microscope is shown in figure 4.9.
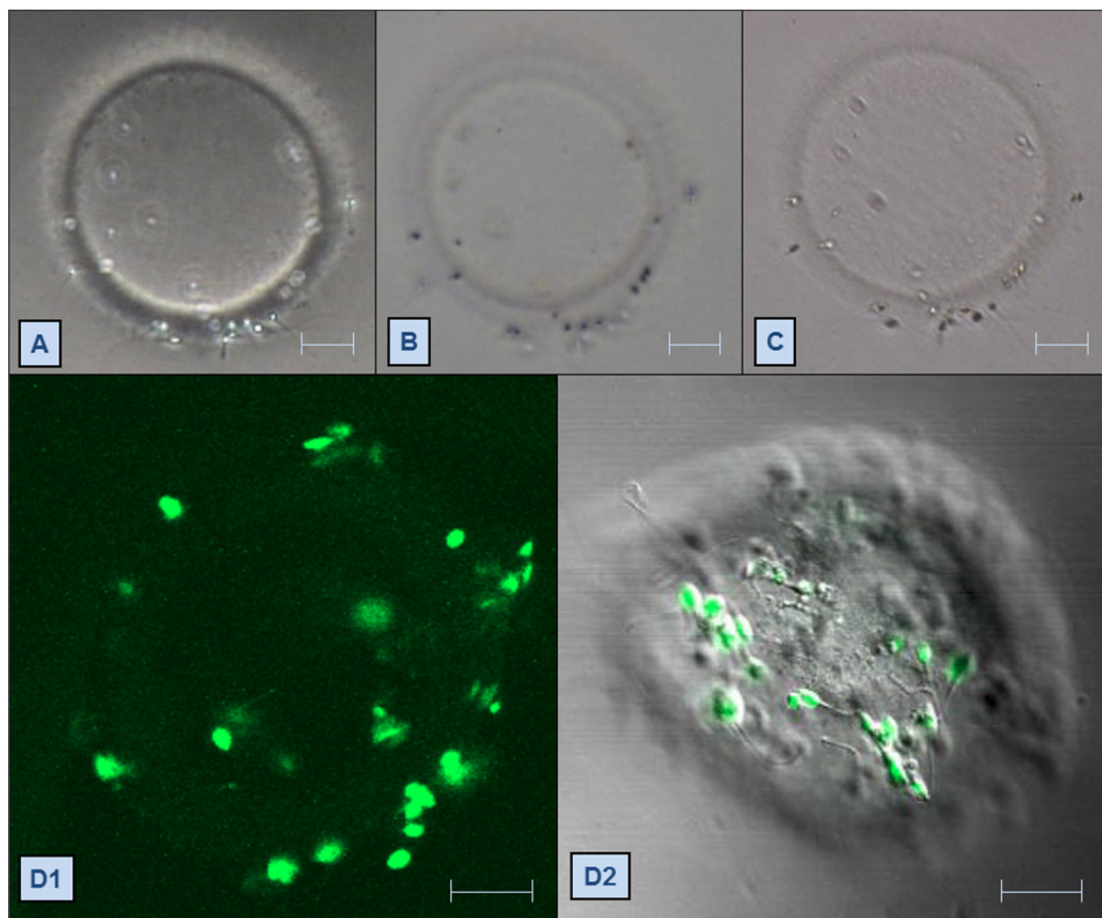
Figure 4.9: Micrographs of a hemi-zona with sperm bound taken at 200x magnification (scale-bar 20µm) with A: phase 1, B: phase 2, C: PlasDIC contrast, and D: confocal microscopy that shows D1: a single focal plane and D2: confocal microscopy with phase 2 contrast overlay.

All microscopy methods compared provided sufficient visualization to be able to count the sperm bound to the hemi-zonae. The evaluation of a hemi-zona with unstained sperm was performed by passing through different focal planes while

looking at the hemi-zonae and counting all bound sperm. Stacking of photos of the stained hemi-zonae by the confocal microscope imaging software and three dimensional rendering of these images facilitated the process of identifying sperm on different focal planes. Super-imposing fluorescent micrographs and phase contrast micrographs clearly displayed sperm cells, although this was of little assistance to count the number of sperm bound as a single focal plane had to be evaluated at a time. Staining hemi-zonae with EThD and the subsequent evaluation with confocal microscopy provided good imaging of sperm bound to hemi-zonae. Similarly, phase contrast microscopy displayed sufficient visualization, without the extra expense and time of staining, and meticulous treatment of fluorescent slides. Since the evaluation of hemi-zonae was not enhanced by staining with EthD and as the staining adds numerous additional steps to the procedure, with added cost implications, it is not advised to combine this procedure with the HZA.

4.2.2. *Sperm deoxyribonucleic acid packaging*

The selection of sperm with less deoxyribonucleic acid (DNA) fragmentation can improve ART results i.e. through a decrease in miscarriages.[190] Sperm remaining in culture after insemination in 200 µl and 1 ml culture media, as described in the previous experiment, were isolated and sperm DNA integrity was assessed. The mean (±SD) percentage of sperm with normal DNA packaging from the two culture media volumes were 78.8% ±2.71 and 79.2% ±4.02 (figure 4.10). No significant difference in DNA integrity was observed between the two test groups as a two-tailed *t*-test calculated a p-value of 0.901 between them. The results indicated that insemination in a larger volume of culture media does not impact on DNA packaging of the inseminating sperm sample.
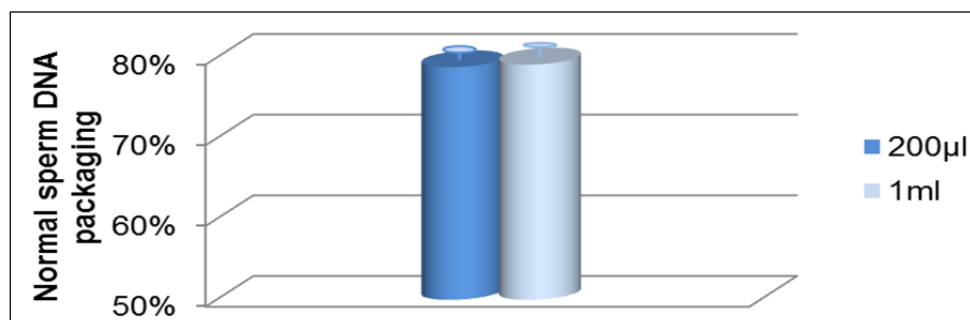
Figure 4.10: Mean (±SD) sperm DNA integrity after insemination in 200 µl and 1 ml culture media (n=12).

4.2.3. Reactive oxygen species generation in culture media

Reactive oxygen species (ROS) may have a negative influence on embryo development, particularly on cleavage and fragmentation rates of embryos in culture.[35] To measure the ROS in culture media, fluorescence generated by the conversion of non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) to fluorescent 2',7'-dichlorodihydrofluorescein (DCF) was assessed[192] using a Biotek Synergy 2 multimode plate reader and reported as relative fluorescence units (RFU). As a positive control, increasing concentrations of hydrogen peroxide (H_2O_2; 0.014 – 30 µM) was added to Global® Total® for Fertilization and treated with DCF-DA, after which DCF fluorescent emissions was measured. Negative controls included water and Global® Total® for Fertilization treated with DCF-DA as well as DCF-DA only containing wells. The amount of fluorescence measured in the respective controls can be found in tables 4.5 & 4.6.

Table 4.5: Fluorescence measured (RFU ±SD) in Global® Total® for Fertilization with different H_2O_2 concentrations (positive controls, n=24)

Positive controls		Fluorescence (RFU ±SD)
H_2O_2 Concentration (µM)	0.014	41228 ±841
	0.041	41511 ±652
	0.123	41137 ±963
	0.37	41062 ±625
	1.111	41177 ±191
	3.333	41183 ±409
	10	42338 ±191
	30	43997 ±493

Table 4.6: Fluorescence measured (RFU ±SD) in wells containing Global® Total® for Fertilization (culture media), water and DCF-DA (negative controls, n=10)

Negative controls	Fluorescence (RFU ±SD)
Culture media	39812 ±84
Water	37367 ±274
DCF-DA	38086 ±711

DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate

Generation of ROS in 200 µl and 1 ml culture media, after insemination with 5 x 10^3 sperm and 18 hour incubation, was determined by measurement of fluorescence after treatment with DCF-DA. The fluorescence in the two groups measured (mean RFU ±SD) 40 735 ±195 and 40 796 ±693, respectively, with no significance difference between them (p>0.05).

In addition to this investigation, the generation of ROS in culture media incubated in the simplified tWE IVF culture system and in a conventional IVF culture system was compared. The addition of 5 x 10^3 motile sperm and a 10 x 10 x 5 mm piece of cumulus cells to culture media in tWE culture tubes simulated continuous culture in the simplified tWE IVF culture system.[18] Opposite tWE culture tubes, the injection of 50 x 10^3 motile sperm in a 200 µl drop of culture media containing a similar sized piece of cumulus cells portrayed conventional IVF culture.[15,86] The culture tubes were incubated continuously for 114 hours (5 days) and samples of the culture media in the tubes were removed at 0, 18, 66 and 114 hours. The conventional IVF insemination dish was incubated for 18 hours and culture media samples were removed at 0 and 18 hours. The culture media samples were treated as described to measure the amount of ROS present. The mean (±SD) fluorescence values obtained from these samples are displayed in table 4.7. The differences in fluorescence observed between the different sample groups, however were not statistically significant (p>0.05).

Table 4.7: Mean fluorescence (RFU ±SD) measured in culture media samples, comparing the simplified Walking Egg (tWE) and conventional *in vitro* fertilization (IVF) culture systems.

Time (hours)	Fluorescence (RFU ±SD)	
	Conventional IVF	*Simplified tWE IVF*
0	39938 ±525	39960 ±815
18	40825 ±192	40315 ±716
66	N/A	40659 ±693
114	N/A	41182 ±857

Since the measurement of fluorescence provides a relative value, the fluorescence measured in Global® Total® for Fertilization (negative control) was considered as a baseline with no ROS generation. The mean RFU value measured in this control was subtracted from all the other measurements, providing calculated, comparable values. Figure 4.11 display the trend in these fluorescence values that can be seen over time for the two culture systems compared, superimposed over the positive control (increasing H_2O_2 concentrations) values obtained. ROS generation increases over time for both culture systems, but at a lower rate for the simplified tWE IVF culture system than for conventional IVF.
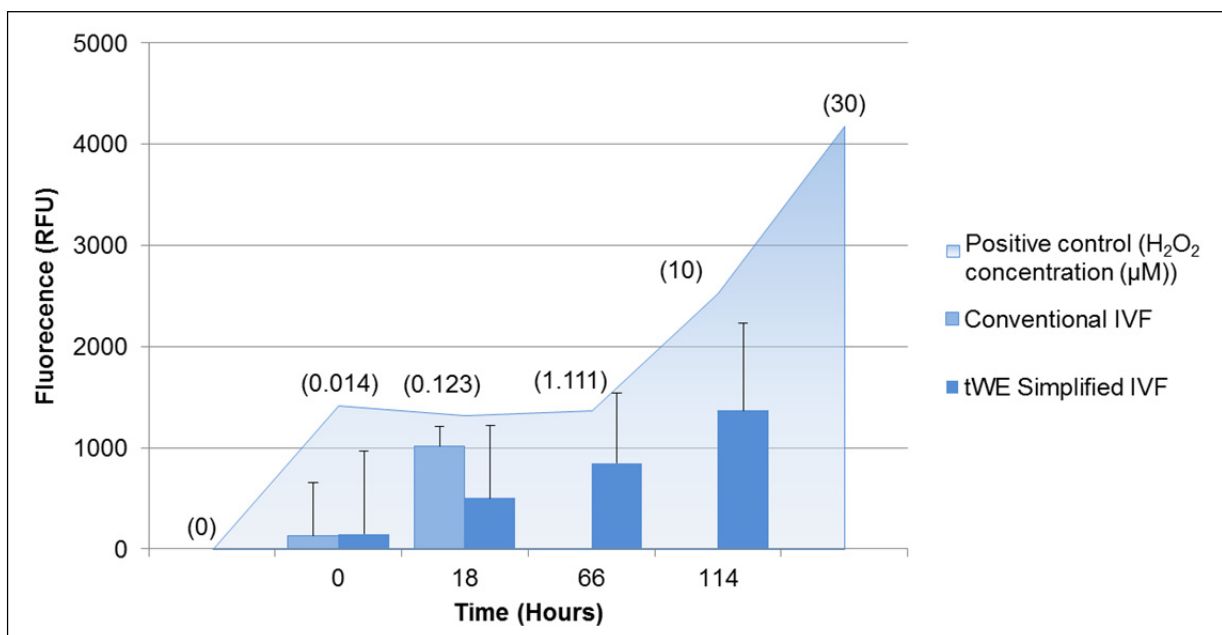
Figure 4.11: Calculated (measured value minus baseline) mean fluorescence (RFU ±SD) values of test samples (n=36) over time, comparing the simplified Walking Egg (tWE) and conventional *in vitro* fertilization (IVF) culture systems.

Chapter 5

Discussion and conclusion

5.1. Discussion

5.2. Recommendations and conclusion

Chapter 5 Discussion and conclusion

5.1. Discussion

5.1.1. Overview

The health-care budgets in developing countries are limited, with infertility not prioritized for funding.[17,27,149,197] The need for assisted reproductive treatment in developing countries was overlooked in the past, and in the main part this trend has continued.[17,26,29,156] As the availability of assisted reproductive services is scarce in low-resource countries, it can be found mostly in the private sector, with the accompanying inflated costs of treatment.[27,198] Government subsidies or insurance coverage for assisted reproductive treatment is almost non-existent in these regions, resulting in "out of pocket" payments by patients.[6,26,198] The financial implications can frequently be disproportionate to their financial means, with assisted reproduction costing over 40 times the monthly minimum wage in some developing countries.[28,197] These inflated expense often affects less afluent couples more. Dyer *et al.* report that 51% of the lowest income couples from a public sector setting in South Africa would occur catastrophic expenditure in their attempts to fall pregnant, whereas their higher income counterparts would be similarly affected in only 2% of couples.[197]

Personal financial instability due to attempts to conceive, especially when restricted funds available are spent on ineffectual infertility treatments such as traditional medicine, is a major problem in low-resource countries that needs attention.[26,174,198] To address the affordability of assisted reproductive technology (ART) in developing nations, basic ART procedures, such as minimal ovarian stimulation and intra-uterine insemination (IUI), can be employed to reduce the treatment cost.[26,33] However, due to complicated causes of infertility and the uniqueness of each patient, this is not always a viable option.[26] Combined with minimal stimulation, initiation of a low-cost *in vitro* fertilization (IVF) protocol will reduce patients' expenses.[29,152] One such low-cost IVF system has been developed by the Walking Egg (tWE) foundation.[160] This simplified culture system use basic IVF principles, which are combined with innovative design and practical applications, to circumvent not only the use of expensive equipment, but also to provide a robust system for use in developing countries.[18,173] The *de novo* production of carbon dioxide (CO_2) and closed culture employed by the system achieves stable culture media pH, with culture media

temperature being the only variable to monitor during embryo culture.[18,175] In the current research, this pioneering system has been investigated and related to conventional culture methods. As publications in this regards are sparse, results were compared to known standards and conclusions made from logical analyses of the findings.

5.1.2. Section 1: Quality control of the simplified tWE IVF culture system

5.1.2.1.   Temperature regulation

Ideally, media temperature should be maintained at 37°C during embryo culture.[104] However, temperature regulation stability via incubators or other temperature regulation devices can vary.[36,98,101] The smallest amount of temperature variance is preferred, as temperature fluctuations have detrimental influences on embryo development.[36] Temperature regulation of the simplified tWE IVF culture system was assessed, using six different heat regulation instruments (a bench-top warming oven, a front-loading water-jacket embryo culture incubator without CO_2 connected, a dry-bath style block heater, a water bath with circulation of heated water, a digital slide warmer and an IVF workstation with heated surface).

The equipment was selected to include instruments found in a standard IVF laboratory and the focus of the research was temperature stability over time and intra-device variation, as well as the correlation between culture media temperature and other parameters measured. Culture media temperature was considered to be a pivotal parameter. The target temperature for this parameter was 36.6-37.0°C[14,36,110] and was selected, instead of 36.8-37.2°C, because of the negative effect heat-shock proteins have on embryo development.[104]

Enclosed heating

The two instruments evaluated, that completely enclose the simplified tWE IVF culture system's warming blocks, were the bench-top warming oven (Model 10-140E Incubator, Quincy Lab) and front-loaded embryo culture incubator (Forma Series II Water Jacketed CO_2 incubator). Distinct differences were recorded in temperature regulation by the two devices. The incubator showed stable temperature regulation that remained consistent over time, with small variations between the two placement

positions. The warming oven had much greater variations in temperatures measured both over time and at the different placement positions.

The embryo culture incubator can be considered as a viable option to utilize with the simplified tWE IVF culture system as the instrument can maintain a stable temperature within the desired range and has enough space for multiple warming blocks. The warming oven is able to hold only two or three warming blocks. Temperature fluctuations over time and between warming blocks exclude this type of equipment for use with the culture system. When a warming oven of this type is available at the laboratory as part of the andrology section, it can be considered as a back-up instrument to temporarily house warming blocks should the dedicated warming device be out of commission.

Partially enclosed heating

The water bath (CPM 200 Water bath, Labcon) and dry-bath (DB-006, K-Systems), which partially enclose the simplified tWE IVF culture system warming blocks, both of which has displayed the ability to regulate culture media temperature with very small variability over time. These instruments also have the least intra-device variability of all the equipment tested. Both devices would maintain constant temperature when used with the simplified tWE IVF culture system, although either can accommodate at most three warming blocks.

When embryos cultured with the simplified system are evaluated, the block containing culture tubes for a specific patient is placed on a warm surface close to the microscope, with tubes being removed from the block one by one to perform the embryo evaluation.[18, 175] Should a water bath be used for embryo culture with the simplified tWE IVF culture system, the block will have to be dried every time it is removed from the water bath to avoid temperature fluctuation due to evaporation and to keep the laboratory dry. The water bath also has the additional disadvantage of warm circulating water being a possible source of contamination.

Single surface heating

The equipment that warm the warming blocks by conducting heat to the base of the warming blocks, are the IVF workstation (Mobile IVF workstation L13, K-Systems)

and the digital slide warmer (SW85, Adamas Instrumenten). Both instruments have shown the ability to maintain a steady temperature over time. The IVF workstation was marginally less stable than the slide warmer, with miniscule fluctuations outside of the stability range aimed for (±0.21 vs 0.2°C). The IVF workstation recorded a limited difference in culture media temperatures between two placement positions, compared to the slide warmer which indicated a large variation.

The IVF workstation is able to provide a sizeable area in which to place multiple warming blocks and can also maintain temperatures within a range at which embryo culture is possible. The ambient temperature did not correlate with the culture media temperature on any of the instruments assessed, apart from the IVF workstation. The assumption can be made that temperature stability is more difficult to maintain over such a large surface area. The design of the IVF workstation compensates for this weakness with the use of multiple heating elements.[37] Temperature mapping of a large surface area heat regulation device, such as the IVF workstation, is therefore imperative, since the different heating elements can cause hot- and cold-spots.[36] The slide warmer on the other hand, can consistently provide stable temperatures, although the limited space available is approximately the size of four warming blocks. When the slide warmer is temperature mapped to identify areas of varying temperature, space compatibility with the simplified tWE IVF culture system may be reduced.

Synopsis

Although all the equipment tested, except the warming oven, can be used with the simplified tWE IVF culture system (Table 5.1), the space constraints of the water bath, dry-bath and slide warmer may limit their use. The small number of warming blocks that can be used with these instruments may result in the need for multiple devices to handle multiple patient cases simultaneously.

No one group of equipment, i.e. enclosed, partially enclosed or single surface heating, showed superior results to any of the others. However, the design of different instruments within a group has proven to affect variability. The deduction can be made that a purpose-built instrument, such as an embryo culture incubator, would be more reliable in regard to its operation than a piece of equipment used

outside of its intended use. However, the cost implication of a purpose-built instrument vs. a generic instrument should be considered.

Table 5.1: Summation of devices discussed, indicating the ability to satisfy specific requirements as well as overall compatibility with the simplified Walking Egg *in vitro* fertilization culture system.

Device	Temperature range	Stability over time	Intra-devise variability	Space for warming blocks	Simplified tWE IVF culture
Warming oven	✘	✘	✘	✓	✘
Incubator	✓	✓	✓	✓	✓
Water bath	✓ⁱ	✓	✓	✓ⁱ	✓
Dry-bath	✓ⁱ	✓	✓	✓ⁱ	✓
Slide warmer	✓ⁱ	✓	✓ⁱ	✓ⁱ	✓ⁱ
IVF workstation	✓	✓ⁱ	✓	✓	✓

tWE: The Walking Egg, IVF: *In vitro* fertilization, ✓: Satisfactory, ✓ⁱ: Intermediate, ✘: Non-satisfactory

Practical considerations should take into account the effect of a power outage on embryo culture, which is not uncommon in many developing countries.[158] Water-jacketed IVF incubators have been known to maintain temperature when electricity supply is cut, and the volume of water found in a water bath would also act as a heat sink when switched off. A single surface heating instrument or dry-bath would most likely experience a temperature decrease at a faster rate in the same situation, indicating the incubator or water bath as more optimal solutions when the supply of current is unpredictable. However, when an uninterrupted power supply is used to facilitate short periods of power outages, a water- jacketed incubator may drain the battery supply more rapidly than an instrument consuming less energy. A water-jacketed IVF incubator is recommended as the ideal instrument for the simplified tWE IVF culture system when finances allow, while a dry-bath is a more affordable alternative that can be used in conjunction with an uninterrupted power supply.

5.1.2.2. Culture media pH

The intra-cellular pH (pHi) of gametes and embryos regulates various metabolic functions.[72] As the culture media pH is directly linked to pHi, the pH stability of culture media is exceptionally important.[14,37,72] The *de novo* production of carbon

dioxide (CO_2) by chemical reaction in a glass tube is employed by the simplified tWE IVF culture system to manipulate culture media pH.[173] After CO_2 equilibration and pH stabilization, the culture tubes are removed from the gas generator, but kept closed to maintain pH stability.[18] During experimentation with the simplified tWE IVF culture system, Global® Total® for Fertilization was used, which has a recommended pH range of 7.20 – 7.40.[13] An average pH of 7.30 ±0.05 was targeted during the research study.[173,187]

Citric acid volume

During experimentation, the range of CA volumes that could result in a pH of 7.3 ±0.05 was determined. The pH values of predetermined CA volumes indicated a linear decrease in pH as CA volume increased. From these results, a 1.8 ml CA volume could be identified to facilitate pH values of 7.3 ±0.05 when considering the variance in pH values between gassing tubes with the same volume of CA added.

Furthermore, the effect of a change in altitude on pH regulation in the simplified tWE IVF culture system was evaluated. A decrease in altitude is associated with an increase in atmospheric pressure, which in turn elevates CO_2 partial pressure and lowers culture media pH during *in vitro* culture of embryos.[14,115] The results obtained from testing the simplified tWE IVF culture system, however, recorded no differences in pH values at selected altitudes (ranging from 30 to 1627 meters above sea level) in South Africa. Since the simplified culture is performed in a closed system, the air pressure within the glass tubes increases as CO_2 is produced, as was observed through several stoppers being pushed out of the glass tubes. The use of the sleeves supplied by tWE foundation to secure the stoppers in the gassing and culture tubes is therefore considered obligatory. This increased pressure will presumably negate the difference in atmospheric pressure, resulting in the same partial pressure for CO_2 in glass tubes equilibrated at varying altitudes. Therefore, when equilibrating culture tubes for the simplified tWE IVF culture system at various altitudes, no compensation in CA volume is required.

Change of protocol

As part of the research conducted, the gas equilibration protocol of the simplified tWE IVF culture system was explored. Aspects considered were (i) the temperature at

which gas equilibration occurs and (ii) the need for water to be added to CA during gas equilibration. For the first aspect, it was shown that a decrease in temperature to 25°C did not affect the gas equilibration considerably and at 15°C the pattern of gas equilibration was observed to be similar to that at higher temperatures, merely occurring over a longer time period. This offers the possibility of performing gas equilibration at room temperature, rather than at 37°C, combined with an increase in equilibration time from 18 to 24 hours. By changing the protocol in this manner, space availability in heat regulating equipment can be allocated solely to warming blocks housing embryo culture tubes.

Results obtained, while the second aspect was being investigated, indicated a marked difference in culture media pH when water was omitted from the gassing tube. When utilizing the simplified tWE IVF culture system, adherence to the protocol in this respect is strongly recommended. The addition of water to the gassing tube may, however, introduce potential risks to the culture system, by the addition of an extra step in the protocol and by possible contamination if the water contains impurities. This concern is being addressed by tWE foundation. Since commencement of the current research, a comprehensive study initiated by tWE foundation has been launched and an official roll-out of the new protocol will follow once this has been completed. The new protocol will include the use of 1 ml CA with altered concentration that does not require dilution with water.[184]

5.1.3. Section 2: Verification of insemination protocol

Currently there is no universal protocol in practice for conventional IVF procedures. A generalised protocol, encompassing the fundamental actions performed during conventional IVF can however be assumed.[15,86,199] The simplified tWE IVF culture system, on the other hand, follows a protocol described by Klerkx *et al.* as well as Van Blerkom *et al.*[18,173] Upon comparison, some of the differences between the simplified culture system and conventional IVF can be identified:

i.   Oocyte insemination is performed with a lower number of sperm;

ii.   Culture media volume is higher;

iii.   Oocytes/embryos are exposed to culture media containing sperm and cumulus cells for a longer incubation time; and

iv.   The culture environment is closed off from the ambient environment.

These modifications to protocol were investigated during experimentation and the resulting effects recorded. Specific focus was placed on the number of spermatozoa to be used for insemination and the effect thereof on sperm deoxyribonucleic acid (DNA) fragmentation. The combination of fewer sperm in a larger volume of culture media, along with extended exposure to sperm and cumulus cells during closed culture was also considered by evaluating the generation of reactive oxygen species (ROS).

5.1.3.1. Number of sperm inseminated

The comparison of DNA fragmentation of sperm used for insemination with the simplified tWE IVF culture system and conventional IVF indicated no significant difference in the current study. Since the volume of culture media and the number of sperm used for insemination does not influence sperm DNA fragmentation, the reduced sperm for insemination protocol can be applied with the simplified tWE IVF culture system.

To determine the lowest number of spermatozoa that can be used for oocyte insemination, an altered hemi-zona assay (HZA) was employed.[97] With the application of a cut-off limit of 20 bound sperm per hemi-zona,[196] 2×10^3 motile sperm was identified as the lowest number to be used for insemination during IVF, irrespective of the culture system. However, after the volume of culture media had been increased in-line with the simplified tWE IVF culture system protocol, the corresponding reduction in sperm concentration resulted in a decline in the number of sperm bound to hemi-zonae. To compensate for this lower binding, the number of sperm to be inseminated could be increased. A minimum cut-off of $2 - 5 \times 10^3$ motile sperm for insemination in the simplified tWE IVF culture system is therefore proposed, depending on a holistic consideration of all sperm parameters.

5.1.3.2. Long exposure time and reactive oxygen species

The extended culture of embryos in media containing cumulus and sperm cells, as is performed in the simplified tWE IVF culture system, could present a potential vulnerability due to a build-up of ROS over time. The cells contained in the culture tube would continue cellular metabolism until programmed and non-programmed cell

death occurs, with the metabolic by- and cellular breakdown-products continuously generating ROS.[126] Therefore, with the extended culture as performed with the simplified tWE IVF culture system, a linear increase in ROS over time can be expected.

A comparison of the level of ROS generation between the simplified culture system and conventional IVF, however, displays a lower presence of ROS in the simplified culture system. The values obtained indicated no significant difference between any of the groups tested, although an observational evaluation of the geometric averages obtained does indicate a trend. The amount of ROS generated during conventional IVF in the first 18 hours of incubation was more than that produced in the simplified culture system over sixty-six hours (day 3 of culture). During extended culture for 114 hours (day 5 of culture) in the simplified culture system, the ROS generated did surpass that of conventional IVF, although by only approximately one third of the ROS measured from conventional IVF culture. It is hypothesised that the low numbers of sperm and high culture media volume of the simplified tWE IVF culture system counteract the increased culture time, thereby minimizing ROS exposure of embryos remaining in the culture media in which fertilization occurred.

5.2. Recommendations and conclusion

Based on the current research, the following recommendations towards the simplified tWE IVF culture system can be made:

- Any stable and reliable, calibrated warming device can be used for heat regulation during embryo culture, combined with a quality control protocol to monitor and correct temperature drifts.

- Important parameters (when considering heat regulation) are intra-device variability, temperature stability over time, space availability and power consumption.

- The protocol for gas equilibration as stipulated by tWE foundation should be adhered to in regards to volumes of CA and the addition of water.

- Gas equilibration can be performed at room temperature, however the equilibration time should be extended from 18 to 24 hours.

- Insemination of oocytes can be performed with $2 - 5 \times 10^3$ motile sperm, depending on all sperm parameters.

- Embryos can be cultured in the insemination tubes for at least three days without having to be concerned about ROS build-up in the culture tube.

- To ensure optimal use of this remarkably designed system in developing countries, adequate training of local health-care professionals is essential.

The simplified system could be considered as innovative due to the use of minimalistic numbers of sperm for fertilization and continuous culture, where embryos remain in the same culture media with the remaining sperm and cumulus cells.[18] Interestingly, this is mirrored by the historic remark by Craft in 1982. He reported that the removal of fertilized oocytes from the dish containing cumulus cells was deemed unnecessary.[200] He also stated that 10×10^3 sperm can affect fertilization and speculated that even less may be sufficient, presenting the debate that men with oligozoospermia could father a child through ART.[200] The same comment was made by Klerkx *et al.*[173] and Van Blerkom *et al.*,[18] regarding the use of the simplified tWE IVF culture system for men with moderate to severe male factor infertility in clinics where ICSI is not available. Therefore, the simplified tWE IVF culture system propels toward basic ART principles. Furthermore, the simplified tWE IVF culture system eliminates multiple quality control steps when compared to conventional IVF, reducing potential complications and risks, as is indicated in figure 5.1.

In a conventional IVF laboratory, expensive and sensitive equipment is used, with quality control being a necessity to ensure optimal operations.[15] The need for medical grade gas and the associated administrative work, to arrange for the correct gas mixtures and the quality control thereof, are replaced in the simplified tWE IVF culture system with a single glass tube producing CO_2.[18] An added benefit of the *de novo* production of CO_2 in a closed system is the absence of impurities found in ambient air and compressed gas tanks,[117] excluding the need of in-line gas filters and whole-room air filtration installations. The need for micro-processor-controlled IVF incubators is also eliminated, since the culture tube is stoppered and gas mixtures in the tube cannot be altered after gas equilibration.[18] Temperature regulation of the system relies on the heating of warming blocks, which can be obtained by using simpler heat regulation devices.[18, 173]
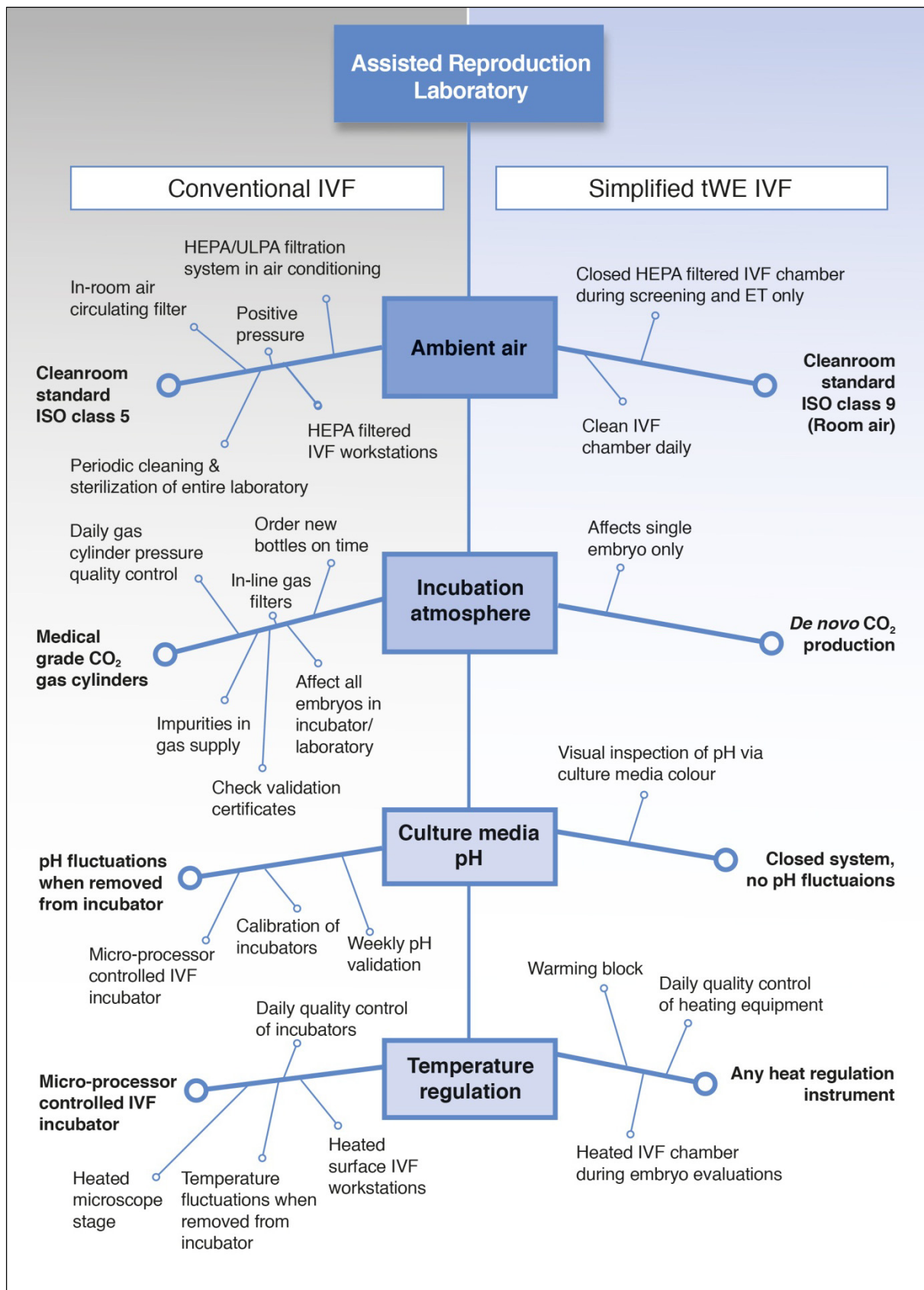
Figure 5.1: Summary of the quality control requirements of a conventional IVF laboratory vs. the simplified Walking Egg *in vitro* culture system (references to different actions in text).

The differences identified between conventional IVF and the simplified tWE IVF culture system provide ample evidence towards an alternative, more economical laboratory design. Considering the drive towards accessible and affordable infertility treatments,[8,17,31] the simplified tWE IVF culture system has the potential to be a cost-effective alternative to conventional IVF. Furthermore, combining this effective culture system with an oocyte aspiration during a natural or mild stimulation cycle, along with a single embryo transfer, contributes towards affordable ART, while also reducing the associated risks and costs due to multiple pregnancies.[17,26,27] Despite minimal stimulation and natural cycle IVF being associated with lower numbers of oocytes retrieved, improved egg quality and reduced aneuploidy rates have been reported, thereby improving the prognosis on selected patients.[27,201]

The design of the simplified tWE IVF culture system that was evaluated in the study has proved to be a reliable and robust addition to the field of ART. In February 2017, at the Best of ASRM and ESHRE meeting hosted in Paris, France it was reported that sixty babies has been born by January 2017 after the use of the simplified culture system.[177] This information as well as the first birth expected during August 2017 in Ghana[184] provide further proof that the simplified tWE IVF culture system can indeed be a tool to facilitate the accessibility of ART to developing countries. In developing countries, infertility care is probably one of the most misjudged and neglected health-care issues,[161] but as the old saying goes, "you have to eat an elephant one bite at a time". The Walking Egg foundation has certainly started "eating its elephant" and this study was conducted to reinforce and evaluate an innovative and already successful embryo culture method.

Chapter 6

References

Chapter 6 References

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Chapter 7

Addendums

7.1.   Statistical data

7.2.   Information leaflet and informed consent for non-clinical research

7.3.   Standard operative procedures

7.4.   Reporting of Results

Chapter 7 Addendums

7.1. Statistical data

7.1.1. Data sets for graphic representations

Table 7.1 (Figure 4.1): Difference in temperatures measured (n=900) at two placement positions (p-value)

Device	T1: Ambient	T2: Device	T3: Block
Warming oven	0.0000 (1.000)	0.6600 (0.015)	0.9820 (0.000)
Incubator	0.1667 (0.536)	0.0111 (0.960)	0.0844 (0.715)
Water bath	0.0000 (1.000)	0.0111 (0.886)	0.0278 (0.215)
Dry-bath	0.0000 (1.000)	0.0300 (0.820)	0.0240 (0.765)
Slide warmer	0.8000 (0.011)	0.4200 (0.000)	0.4540 (0.000)
IVF workstation	0.1889 (0.045)	0.0222 (0.905)	0.0544 (0.667)

Table 7.2 (Figure 4.2): The mean (±SD) culture media temperature per repeat for each device (n=300)

Mean (±SD)		1	2	3	4	5	6	7	8	9	10	Mean
Enclosed heating	Warming oven	37.2°C ±0.00	37.2°C ±0.07	37.3°C ±0.00	36.9°C ±0.00	37.5°C ±0.05	37.3°C ±0.04	36.5°C ±0.10	37.3°C ±0.00	37.3°C ±0.00	37.4°C ±0.00	37.2°C ±0.28
	Incubator	36.9°C ±0.05	36.9°C ±0.11	37.1°C ±0.04	37.0°C ±0.00	37.0°C ±0.05	36.9°C ±0.05	36.9°C ±0.04	36.8°C ±0.05	36.9°C ±0.04	36.9°C ±0.05	36.9°C ±0.09
Partially enclosed heating	Water bath	37.3°C ±0.00	37.4°C ±0.00	37.4°C ±0.00	37.4°C ±0.00	37.4°C ±0.00	37.3°C ±0.00	37.4°C ±0.05	37.3°C ±0.05	37.4°C ±0.04	37.4°C ±0.00	37.4°C ±0.04
	Dry-bath	36.8°C ±0.00	36.8°C ±0.00	36.6°C ±0.00	36.6°C ±0.00	36.8°C ±0.00	36.7°C ±0.00	36.8°C ±0.00	36.7°C ±0.00	36.7°C ±0.00	36.8°C ±0.00	36.7°C ±0.08
Single surface heating	Slide warmer	36.7°C ±0.00	36.6°C ±0.00	36.8°C ±0.05	36.7°C ±0.00	36.8°C ±0.00	36.7°C ±0.00	36.6°C ±0.00	36.7°C ±0.00	36.6°C ±0.00	36.6°C ±0.00	36.7°C ±0.09
	IVF w-station	37.0°C ±0.04	37.0°C ±0.05	37.0°C ±0.00	36.7°C ±0.04	36.8°C ±0.05	36.8°C ±0.07	36.6°C ±0.05	37.0°C ±0.00	36.6°C ±0.05	36.6°C ±0.33	36.8°C ±0.18

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Table 7.3 (Figure 4.3): Culture media pH (±SD) after gas equilibration with increasing volumes of CA (n=100)

Citric acid volume (ml)	Mean culture media pH ±SD
1.2	7.42 ±0.06
1.4	7.38 ±0.04
1.6	7.32 ±0.05
1.8	7.31 ±0.03
2	7.26 ±0.06
2.2	7.24 ±0.04
2.4	7.18 ±0.06
2.6	7.16 ±0.04
2.8	7.13 ±0.07
3	7.11 ±0.07

Table 7.4 (Figure 4.4): Culture media pH values (±95%CI) after gas equilibration at 15, 25 and 37°C (n=120).

Mean culture media pH ±95%CI		Time (hours) of gas equilibration					
		16	18	20	22	24	30
Temperature (°C)	15.0		±0.047	±0.044	±0.047	±0.044	±0.053
	25.0	±0.047	±0.049	±0.053	±0.044	±0.047	±0.063
	37.0	±0.044	±0.047			±0.049	±0.057

Table 7.5 (Figure 4.5): Culture media pH values after gas equilibration with increasing volumes of citric acid and at different altitudes (n=450).

Mean ±95%CI		Location (meters above sea level)				
		Mosselbay (30)	Bonnievale (350)	Graaff-Reinet (925)	Pretoria (1326)	Johannesburg (1627)
Citric acid volume (ml)	1.2	7.445 ±0.022	7.483 ±0.022	7.455 ±0.022	7.449 ±0.024	7.431 ±0.024
	1.4	7.390 ±0.024	7.391 ±0.020	7.394 ±0.028	7.397 ±0.025	7.350 ±0.036
	1.6	7.339 ±0.022	7.349 ±0.024	7.340 ±0.022	7.330 ±0.024	7.323 ±0.024
	1.8	7.233 ±0.031	7.279 ±0.024	7.310 ±0.022	7.227 ±0.036	7.327 ±0.024
	2.0	7.158 ±0.031	7.270 ±0.025	7.185 ±0.044	7.242 ±0.021	7.220 ±0.031
	2.2	7.160 ±0.044	7.260 ±0.044	7.220 ±0.062	7.160 ±0.031	7.168 ±0.025
	All	7.312 ±0.011	7.355 ±0.010	7.337 ±0.012	7.323 ±0.011	7.324 ±0.011

Table 7.6 (Figure 4.6): The effect of citric acid dilution with water (final volume of 3 ml) and without, on culture media pH after gas equilibration (n=60).

Mean ±95%CI		Water	w/o Water
Citric acid volume (ml)	1.4	7.43 ±0.03	7.28 ±0.04
	1.7	7.41 ±0.06	7.29 ±0.06
	2.0	7.34 ±0.03	7.34 ±0.06

Table 7.7 (Figure 4.7): Sperm parameters (concentration prior to semen processing and, progressive motility and morphology both pre- and post-semen processing) per semen donor and average of all donors (n=5) as used during the hemi-zona assay.

	Donor					
	1	2	3	4	5	Average
Pre-concentration ($x10^3$/ml)	26	34	58	111	23	50.40
Pre-motility (%)	66	66	39	40	57	53.60
Pre-morphology (%)	6	7	6	10	5	6.80
Post-concentration ($x10^3$/ml)	25	21	21	20	25	22.40
Post-motility (%)	72	78	66	70	79	73.00
Post-morphology (%)	7	10	7	13	8	9.00

Table 7.8 (Figure 4.11): Calculated (measured value minus baseline) mean fluorescence (RFU ±SD) values of test samples (n=36) over time, comparing the simplified Walking Egg (tWE) and conventional *in vitro* fertilization (IVF) culture systems.

Time (hours)	Fluorescence (RFU ±SD)	
	Conventional IVF	*Simplified tWE IVF*
0	126 ±525	148 ±815
18	1013 ±192	503 ±716
66	N/A	847 ±693
114	N/A	1370 ±857

7.1.2. Descriptive Statistics

Temperatures
Group 1 – Warming oven

```
-> ttest t1_amb if group == 1, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------
   Group |    Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------
       1 |     10      25.43    .6160898    1.948247    24.03631   26.82369
       2 |     10      25.43    .6160898    1.948247    24.03631   26.82369
---------+-------------------------------------------------------------
combined |     20      25.43    .4240221    1.896284    24.54251   26.31749
---------+-------------------------------------------------------------
    diff |                 0    .8712825                -1.830497   1.830497
-----------------------------------------------------------------------
    diff = mean(1) - mean(2)                               t =   0.0000
Ho: diff = 0                                degrees of freedom =       18
   Ha: diff < 0               Ha: diff != 0               Ha: diff > 0
 Pr(T < t) = 0.5000       Pr(|T| > |t|) = 1.0000       Pr(T > t) = 0.5000
-> table side if group == 1, c(N t2_device mean t2_device sd t2_device)
format(%9.3f)
---------------------------------------------------------
    side |   N(t2_dev~e)  mean(t2_dev~e)   sd(t2_dev~e)
---------+-----------------------------------------------
       1 |          10         36.680          0.305
       2 |          10         36.680          0.305
---------------------------------------------------------
-> ttest t2_device if group == 1, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------
   Group |    Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------
       1 |     10      36.68    .0963788    .3047765    36.46198   36.89802
       2 |     10      36.68    .0963788    .3047765    36.46198   36.89802
---------+-------------------------------------------------------------
combined |     20      36.68    .0663324    .2966476    36.54116   36.81883
---------+-------------------------------------------------------------
    diff |                 0    .1363002                -.2863561   .2863561
-----------------------------------------------------------------------
    diff = mean(1) - mean(2)                               t =   0.0000
Ho: diff = 0                                degrees of freedom =       18
   Ha: diff < 0               Ha: diff != 0               Ha: diff > 0
 Pr(T < t) = 0.5000       Pr(|T| > |t|) = 1.0000       Pr(T > t) = 0.5000
-> table side if group == 1, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
---------------------------------------------------------
    side |   N(t3_block)  mean(t3_block)   sd(t3_block)
---------+-----------------------------------------------
       1 |          10         36.620          0.476
       2 |          10         35.960          0.611
---------------------------------------------------------
-> ttest t3_block if group == 1, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------
   Group |    Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------
       1 |     10      36.62    .1504065    .4756272    36.27976   36.96024
       2 |     10      35.96    .1933334    .611374     35.52265   36.39735
---------+-------------------------------------------------------------
combined |     20      36.29    .1412165    .6315392    35.99443   36.58557
---------+-------------------------------------------------------------
    diff |           .6599998   .2449489                .1453814   1.174618
-----------------------------------------------------------------------
    diff = mean(1) - mean(2)                               t =   2.6944
Ho: diff = 0                                degrees of freedom =       18
   Ha: diff < 0               Ha: diff != 0               Ha: diff > 0
 Pr(T < t) = 0.9926       Pr(|T| > |t|) = 0.0148       Pr(T > t) = 0.0074
```

```
-> table side if group == 1, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
-----------------------------------------------------
    side |  N(t4_media)  mean(t4_media)   sd(t4_media)
---------+-------------------------------------------
       1 |          10          37.184          0.283
       2 |          10          36.202          0.377
-----------------------------------------------------
-> ttest t4_media if group == 1, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10      37.184    .089532    .2831251    36.98146    37.38654
       2 |      10      36.202    .1191992   .3769408    35.93235    36.47165
---------+--------------------------------------------------------------------
combined |      20      36.693    .1339856   .599202     36.41256    36.97344
---------+--------------------------------------------------------------------
    diff |              .982      .1490786              .6687975    1.295202
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                  t =   6.5871
Ho: diff = 0                                   degrees of freedom =       18
    Ha: diff < 0               Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 1.0000       Pr(|T| > |t|) = 0.0000        Pr(T > t) = 0.0000
```

Group 2 – Dry-bath

```
. for var t1_amb t2_device t3_block t4_media: table side if group == 2, c(N X mean
X sd X) format(%9.3f) \ ttest X if group == 2, by( side )
-> table side if group == 2, c(N t1_amb mean t1_amb sd t1_amb) format(%9.3f)
-----------------------------------------------------
    side |  N(t1_amb)   mean(t1_amb)    sd(t1_amb)
---------+-------------------------------------------
       1 |          10          24.580          1.407
       2 |          10          24.580          1.407
-----------------------------------------------------
-> ttest t1_amb if group == 2, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10      24.58     .4449221   1.406967    23.57352    25.58648
       2 |      10      24.58     .4449221   1.406967    23.57352    25.58648
---------+--------------------------------------------------------------------
combined |      20      24.58     .3062164   1.369441    23.93908    25.22092
---------+--------------------------------------------------------------------
    diff |              0         .6292148              -1.321931   1.321931
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                  t =   0.0000
Ho: diff = 0                                   degrees of freedom =       18
    Ha: diff < 0               Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5000       Pr(|T| > |t|) = 1.0000        Pr(T > t) = 0.5000
-> table side if group == 2, c(N t2_device mean t2_device sd t2_device)
-----------------------------------------------------
    side |  N(t2_dev~e)  mean(t2_dev~e)   sd(t2_dev~e)
---------+-------------------------------------------
       1 |          10          36.830          0.371
       2 |          10          36.830          0.371
-----------------------------------------------------
-> ttest t2_device if group == 2, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10      36.83     .1174264   .1613347    36.56436    37.09564
       2 |      10      36.83     .1174264   .1613347    36.56436    37.09564
---------+--------------------------------------------------------------------
combined |      20      36.83     .0808184   .1614307    36.66085    36.99916
---------+--------------------------------------------------------------------
    diff |              0         .1660659              -.3488916   .3488916
------------------------------------------------------------------------------
```

```
       diff = mean(1) - mean(2)                                         t =    0.0000
Ho: diff = 0                                     degrees of freedom =        18
    Ha: diff < 0                  Ha: diff != 0                   Ha: diff > 0
 Pr(T < t) = 0.5000         Pr(|T| > |t|) = 1.0000          Pr(T > t) = 0.5000
-> table side if group == 2, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
-------------------------------------------------------
     side |   N(t3_block)  mean(t3_block)    sd(t3_block)
----------+--------------------------------------------
        1 |           10          36.160           0.288
        2 |           10          36.190           0.292
-------------------------------------------------------
-> ttest t3_block if group == 2, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10       36.16   .0909212    .1515181     35.95432    36.36568
       2 |      10       36.19    .092436    .1923082      35.9809     36.3991
---------+--------------------------------------------------------------------
combined |      20      36.175   .0631934    .2826097     36.04273    36.30727
---------+--------------------------------------------------------------------
    diff |             -.0299999   .1296575                -.3024003    .2424004
------------------------------------------------------------------------------
       diff = mean(1) - mean(2)                                         t =   -0.2314
Ho: diff = 0                                     degrees of freedom =        18
    Ha: diff < 0                  Ha: diff != 0                   Ha: diff > 0
 Pr(T < t) = 0.4098         Pr(|T| > |t|) = 0.8196          Pr(T > t) = 0.5902
-> table side if group == 2, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
-------------------------------------------------------
     side |   N(t4_media)  mean(t4_media)    sd(t4_media)
----------+--------------------------------------------
        1 |           10          36.660           0.178
        2 |           10          36.684           0.177
-------------------------------------------------------
-> ttest t4_media if group == 2, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10       36.66   .0561743    .0776386     36.61499    36.70500
       2 |      10      36.684   .0558411    .1145851     36.61758    36.75042
---------+--------------------------------------------------------------------
combined |      20      36.672   .0386455    .1728279     36.59111    36.75289
---------+--------------------------------------------------------------------
    diff |             -.0239998   .0792072                -.1904079    .1424083
------------------------------------------------------------------------------
       diff = mean(1) - mean(2)                                         t =   -0.3030
Ho: diff = 0                                     degrees of freedom =        18
    Ha: diff < 0                  Ha: diff != 0                   Ha: diff > 0
 Pr(T < t) = 0.3827         Pr(|T| > |t|) = 0.7654          Pr(T > t) = 0.6173
```

Group 3 – Slide warmer

```
. for var t1_amb t2_device t3_block t4_media: table side if group == 3, c(N X mean
X sd X) format(%9.3f) \ ttest X if group == 3, by( side )

-> table side if group == 3, c(N t1_amb mean t1_amb sd t1_amb) format(%9.3f)

-------------------------------------------------------
     side |   N(t1_amb)  mean(t1_amb)    sd(t1_amb)
----------+--------------------------------------------
        1 |          10        24.650         0.669
        2 |          10        24.650         0.669
-------------------------------------------------------
```

```
-> ttest t1_amb if group == 3, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10      24.65    .2114764    .668747     24.17161    25.12839
       2 |      10      24.65    .2114764    .668747     24.17161    25.12839
---------+----------------------------------------------------------------
combined |      20      24.65    .145548     .6509106    24.34536    24.95464
---------+----------------------------------------------------------------
    diff |                  0    .2990728               -.6283286    .6283286
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                  t =   0.0000
Ho: diff = 0                                  degrees of freedom =       18
   Ha: diff < 0                  Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5000          Pr(|T| > |t|) = 1.0000        Pr(T > t) = 0.5000
-> table side if group == 3, c(N t2_device mean t2_device sd t2_device)
format(%9.3f)
----------------------------------------------------------
    side |  N(t2_dev~e)  mean(t2_dev~e)    sd(t2_dev~e)
---------+------------------------------------------------
       1 |         10        36.680           0.581
       2 |         10        35.880           0.673
----------------------------------------------------------
-> ttest t2_device if group == 3, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10      36.68    .1836664    .5808041    36.26452    37.09548
       2 |      10      35.88    .2128117    .6729698    35.39859    36.36141
---------+----------------------------------------------------------------
combined |      20      36.28    .1647326    .7367065    35.93521    36.62479
---------+----------------------------------------------------------------
    diff |           .7999996    .2811088                .2094119    1.390587
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                  t =   2.8459
Ho: diff = 0                                  degrees of freedom =       18
   Ha: diff < 0                  Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9946          Pr(|T| > |t|) = 0.0107        Pr(T > t) = 0.0054
-> table side if group == 3, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
----------------------------------------------------------
    side |  N(t3_block)  mean(t3_block)    sd(t3_block)
---------+------------------------------------------------
       1 |         10        36.050           0.178
       2 |         10        35.630           0.157
----------------------------------------------------------
-> ttest t3_block if group == 3, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs       Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10      36.05    .0562732    .1779514    35.9227     36.1773
       2 |      10      35.63    .0495534    .1567015    35.5179     35.7421
---------+----------------------------------------------------------------
combined |      20      35.84    .060437     .2702824    35.7135     35.9665
---------+----------------------------------------------------------------
    diff |           .4200001    .0749814                .26247      .5775301
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                  t =   5.6014
Ho: diff = 0                                  degrees of freedom =       18
   Ha: diff < 0                  Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 1.0000          Pr(|T| > |t|) = 0.0000        Pr(T > t) = 0.0000
```

```
-> table side if group == 3, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
----------------------------------------------------------
     side |   N(t4_media)  mean(t4_media)   sd(t4_media)
----------+-----------------------------------------------
        1 |          10         36.684          0.086
        2 |          10         36.230          0.164
----------------------------------------------------------
-> ttest t4_media if group == 3, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10      36.684    .0272929    .0863076    36.62226    36.74574
       2 |      10       36.23    .0517473    .1636393    36.11294    36.34706
---------+--------------------------------------------------------------------
combined |      20      36.457    .0593523    .2654317    36.33277    36.58123
---------+--------------------------------------------------------------------
    diff |              .4540009    .0585037                .3310891    .5769126
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   7.7602
Ho: diff = 0                                    degrees of freedom =       18
    Ha: diff < 0                  Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.0000
```

Group 4 – Water bath

```
. for var t1_amb t2_device t3_block t4_media: table side if group == 4, c(N X mean
X sd X) format(%9.3f) \ ttest X if group == 4, by( side )
-> table side if group == 4, c(N t1_amb mean t1_amb sd t1_amb) format(%9.3f)
----------------------------------------------------------
     side |   N(t1_amb)   mean(t1_amb)    sd(t1_amb)
----------+-----------------------------------------------
        1 |          10         25.256          0.763
        2 |          10         25.256          0.763
----------------------------------------------------------
-> ttest t1_amb if group == 4, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10    25.25556    .2544662    .7633987    24.66876    25.84236
       2 |      10    25.25556    .2544662    .7633987    24.66876    25.84236
---------+--------------------------------------------------------------------
combined |      20    25.25556    .1745624    .7406056    24.88726    25.62385
---------+--------------------------------------------------------------------
    diff |                     0    .3598696               -.7628895    .7628895
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.0000
Ho: diff = 0                                    degrees of freedom =       18
    Ha: diff < 0                  Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 0.5000         Pr(|T| > |t|) = 1.0000          Pr(T > t) = 0.5000
-> table side if group == 4, c(N t2_device mean t2_device sd t2_device)
format(%9.3f)
----------------------------------------------------------
     side |   N(t2_dev~e)  mean(t2_dev~e)   sd(t2_dev~e)
----------+-----------------------------------------------
        1 |          10         37.367          0.050
        2 |          10         37.367          0.050
----------------------------------------------------------
-> ttest t2_device if group == 4, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10    37.36667     .016667    .0500011    37.32823     37.4051
       2 |      10    37.36667     .016667    .0500011    37.32823     37.4051
---------+--------------------------------------------------------------------
combined |      20    37.36667    .0114335    .0485082    37.34254    37.39079
---------+--------------------------------------------------------------------
    diff |                     0    .0235708               -.0499678    .0499678
```

```
-----------------------------------------------------------------------------
     diff = mean(1) - mean(2)                                      t =   0.0000
 Ho: diff = 0                                  degrees of freedom =       18
     Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5000         Pr(|T| > |t|) = 1.0000         Pr(T > t) = 0.5000
-> table side if group == 4, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
---------------------------------------------------------
     side | N(t3_block)  mean(t3_block)   sd(t3_block)
----------+----------------------------------------------
        1 |          10          36.833          0.158
        2 |          10          36.822          0.164
---------------------------------------------------------
-> ttest t3_block if group == 4, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------------
       1 |      10    36.83333    .0527044    .1581131     36.7118    36.95487
       2 |      10    36.82222    .0547156    .1641467     36.69605     36.9484
---------+-------------------------------------------------------------------
combined |      20    36.82778    .0368758    .1564508     36.74998    36.90558
---------+-------------------------------------------------------------------
    diff |            .0111109    .0759707                -.1499397    .1721616
-----------------------------------------------------------------------------
     diff = mean(1) - mean(2)                                      t =   0.1463
 Ho: diff = 0                                  degrees of freedom =       18
     Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5572         Pr(|T| > |t|) = 0.8855         Pr(T > t) = 0.4428
-> table side if group == 4, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
---------------------------------------------------------
     side | N(t4_media)  mean(t4_media)   sd(t4_media)
----------+----------------------------------------------
        1 |          10          37.361          0.040
        2 |          10          37.333          0.051
---------------------------------------------------------
-> ttest t4_media if group == 4, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------------
       1 |      10    37.36111    .0131705    .0395116    37.33074    37.39148
       2 |      10    37.33333    .0169968    .0509903    37.29414    37.37253
---------+-------------------------------------------------------------------
combined |      20    37.34722    .0109607    .0465022     37.3241    37.37035
---------+-------------------------------------------------------------------
    diff |            .0277782    .0215024                -.0178049    .0733613
-----------------------------------------------------------------------------
     diff = mean(1) - mean(2)                                      t =   1.2919
 Ho: diff = 0                                  degrees of freedom =       18
     Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8926         Pr(|T| > |t|) = 0.2148         Pr(T > t) = 0.1074
. for var t1_amb t2_device t3_block t4_media: table side if group == 5, c(N X mean
X sd X) format(%9.3f) \ ttest X if group == 5, by( side )
-> table side if group == 5, c(N t1_amb mean t1_amb sd t1_amb) format(%9.3f)
---------------------------------------------------------
     side | N(t1_amb)  mean(t1_amb)   sd(t1_amb)
----------+----------------------------------------------
        1 |          10          26.811          0.677
        2 |          10          26.811          0.677
---------------------------------------------------------
```

Group 5 - Workstation

```
-> ttest t1_amb if group == 5, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------------
       1 |      10    26.81111    .2257361    .6772082    26.29056    27.33166
       2 |      10    26.81111    .2257361    .6772082    26.29056    27.33166
```

```
---------+-----------------------------------------------------------------------
combined |    20    26.81111   .1548537   .6569885     26.4844   27.13782
---------+-----------------------------------------------------------------------
    diff |                 0    .319239              -.6767565   .6767565
---------+-----------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.0000
Ho: diff = 0                                   degrees of freedom =       18
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5000        Pr(|T| > |t|) = 1.0000        Pr(T > t) = 0.5000
-> table side if group == 5, c(N t2_device mean t2_device sd t2_device)
format(%9.3f)
-----------------------------------------------------------
    side |    N(t2_dev~e)   mean(t2_dev~e)    sd(t2_dev~e)
---------+-------------------------------------------------
       1 |            10           37.056           0.201
       2 |            10           37.244           0.167
-----------------------------------------------------------
-> ttest t2_device if group == 5, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------------
       1 |      10    37.05555   .0668976   .2006928    36.90129   37.20982
       2 |      10    37.25555   .0555559   .1644478    37.11633   37.37256
---------+-------------------------------------------------------------------
combined |      20       37.15   .0479993   .2036438    37.04873   37.25127
---------+-------------------------------------------------------------------
    diff |            -.1888898   .0869583              -.3732333  -.0045464
-----------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =  -2.1722
Ho: diff = 0                                   degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0226        Pr(|T| > |t|) = 0.0452        Pr(T > t) = 0.9774

-> table side if group == 5, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
-----------------------------------------------------------
    side |    N(t3_block)   mean(t3_block)     sd(t3_block)
---------+-------------------------------------------------
       1 |            10           36.322           0.409
       2 |            10           36.300           0.367
-----------------------------------------------------------
-> ttest t3_block if group == 5, by( side )
Two-sample t test with equal variances
-----------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+-------------------------------------------------------------------
       1 |      10    36.32222    .136196   .3940858    36.00815   36.63629
       2 |      10        36.3   .1224746   .3544238    36.01757   36.58243
---------+-------------------------------------------------------------------
combined |      20    36.31111   .0888889   .3771237    36.12357   36.49865
---------+-------------------------------------------------------------------
    diff |             .0222236   .1831649              -.3660687   .4105159
-----------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.1213
Ho: diff = 0                                   degrees of freedom =       18
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5475        Pr(|T| > |t|) = 0.9049        Pr(T > t) = 0.4525
-> table side if group == 5, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
-----------------------------------------------------------
    side |    N(t4_media)   mean(t4_media)     sd(t4_media)
---------+-------------------------------------------------
       1 |            10           36.840           0.279
       2 |            10           36.786           0.247
-----------------------------------------------------------
```

```
-> ttest t4_media if group == 5, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10       36.84    .0929904    .1789713    36.73626    36.94374
       2 |      10    36.78556    .0823799    .2017397    36.66862    36.90250
---------+----------------------------------------------------------------
combined |      20    36.81278    .0606221    .2571979    36.68488    36.94068
---------+----------------------------------------------------------------
    diff |             .0544446    .1242323               -.2089161    .3178053
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.4382
Ho: diff = 0                                    degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                    Ha: diff > 0
 Pr(T < t) = 0.6665         Pr(|T| > |t|) = 0.6671         Pr(T > t) = 0.3335
. for var t1_amb t2_device t3_block t4_media: table side if group == 6, c(N X mean
X sd X) format(%9.3f) \ ttest X if group == 6, by( side )
-> table side if group == 6, c(N t1_amb mean t1_amb sd t1_amb) format(%9.3f)
-------------------------------------------------------
    side |    N(t1_amb)   mean(t1_amb)    sd(t1_amb)
---------+---------------------------------------------
       1 |         10         26.433        0.500
       2 |         10         26.433        0.500
-------------------------------------------------------
```

Group 6 – W/j-Incubator

```
-> ttest t1_amb if group == 6, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10    26.43333    .1666666    .4999999     26.049    26.81767
       2 |      10    26.43333    .1666666    .4999999     26.049    26.81767
---------+----------------------------------------------------------------
combined |      20    26.43333    .1143324    .4850712    26.19211    26.67455
---------+----------------------------------------------------------------
    diff |                    0    .2357022               -.4996664    .4996664
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.0000
Ho: diff = 0                                    degrees of freedom =       18
    Ha: diff < 0                 Ha: diff != 0                    Ha: diff > 0
 Pr(T < t) = 0.5000         Pr(|T| > |t|) = 1.0000         Pr(T > t) = 0.5000
-> table side if group == 6, c(N t2_device mean t2_device sd t2_device)
format(%9.3f)
-------------------------------------------------------
    side |   N(t2_dev~e)  mean(t2_dev~e)   sd(t2_dev~e)
---------+---------------------------------------------
       1 |         10         36.744        0.445
       2 |         10         36.578        0.653
-------------------------------------------------------
-> ttest t2_device if group == 6, by( side )
Two-sample t test with equal variances
--------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+----------------------------------------------------------------
       1 |      10    36.74444    .1482406    .1047219     36.4026    37.08629
       2 |      10    36.57778    .2178034    .1065101    36.07552    37.08003
---------+----------------------------------------------------------------
combined |      20    36.66111    .1293874    .5489444    36.38813    36.93409
---------+----------------------------------------------------------------
    diff |             .1666667    .2634646               -.3918533    .7251866
--------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                    t =   0.6326
Ho: diff = 0                                    degrees of freedom =       18
    Ha: diff < 0                 Ha: diff != 0                    Ha: diff > 0
 Pr(T < t) = 0.7320         Pr(|T| > |t|) = 0.5359         Pr(T > t) = 0.2680
```

```
-> table side if group == 6, c(N t3_block mean t3_block sd t3_block) format(%9.3f)
----------------------------------------------------------
      side |   N(t3_block)  mean(t3_block)   sd(t3_block)
----------+-----------------------------------------------
         1 |           10        36.556          0.442
         2 |           10        36.544          0.482
----------------------------------------------------------
-> ttest t3_block if group == 6, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10    36.55555    .1473008    .0919025    36.21588    36.89523
       2 |      10    36.54444    .1608233     .148247    36.17358     36.9153
---------+--------------------------------------------------------------------
combined |      20       36.55     .105796    .4488545    36.32679    36.77321
---------+--------------------------------------------------------------------
    diff |            .0111105    .2180864                -.451212    .4734331
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                     t =   0.0509
Ho: diff = 0                                  degrees of freedom =       18

    Ha: diff < 0                  Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 0.5200          Pr(|T| > |t|) = 0.9600          Pr(T > t) = 0.4800
-> table side if group == 6, c(N t4_media mean t4_media sd t4_media) format(%9.3f)
----------------------------------------------------------
      side |   N(t4_media)  mean(t4_media)   sd(t4_media)
----------+-----------------------------------------------
         1 |           10        36.851          0.452
         2 |           10        36.767          0.511
----------------------------------------------------------
-> ttest t4_media if group == 6, by( side )
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |      10    36.85111    .1508168    .0944505    36.79362    37.90586
       2 |      10    36.76667    .1703265    .0809796    36.71973    36.81361
---------+--------------------------------------------------------------------
combined |      20    36.80889    .1108285    .4702053    36.57506    37.04272
---------+--------------------------------------------------------------------
    diff |            .0844443    .2275013                -.397837    .5667255
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                     t =   0.3712
Ho: diff = 0                                  degrees of freedom =       18

    Ha: diff < 0                  Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 0.6423          Pr(|T| > |t|) = 0.7154          Pr(T > t) = 0.3577
```

Temperature correlations

```
. tab group
     group |      Freq.     Percent        Cum.
------------+-----------------------------------
         1 |         20       17.54       17.54
         2 |         20       17.54       35.09
         3 |         20       17.54       52.63
         4 |         20       15.79       68.42
         5 |         20       15.79       84.21
         6 |         20       15.79      100.00
------------+-----------------------------------
     Total |        114      100.00
```

```
. pwcorr t1_amb t2_device t3_block t4_media, sig obs (all)
             |   t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
             |      114
   t2_device |   0.1245   1.0000
             |   0.1868
             |      114       114
    t3_block |   0.2980   0.5469   1.0000
             |   0.0013   0.0000
             |      114       114       114
    t4_media |   0.1496   0.5392   0.7809   1.0000
             |   0.1122   0.0000   0.0000
             |      114       114       114       114
. pwcorr t1_amb t2_device t3_block t4_media if group == 1, sig obs
             |   t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
   t2_device |   0.1246   1.0000
             |   0.6006
             |       20        20
    t3_block |   0.4841  -0.0742   1.0000
             |   0.0305   0.7560
             |       20        20        20
    t4_media |   0.2530   0.0158   0.7400   1.0000
             |   0.2818   0.9474   0.0002
             |       20        20        20        20
. pwcorr t1_amb t2_device t3_block t4_media if group == 2, sig obs
             |   t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
             |       20
   t2_device |  -0.0710   1.0000
             |   0.7660
             |       20        20
    t3_block |   0.2570   0.4509   1.0000
             |   0.2740   0.0460
             |       20        20        20
    t4_media |   0.2817   0.0765   0.6832   1.0000
             |   0.2289   0.7485   0.0009
             |       20        20        20        20
. pwcorr t1_amb t2_device t3_block t4_media if group == 3, sig obs
             |   t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
   t2_device |  -0.1240   1.0000
             |   0.6024
             |       20        20
    t3_block |   0.2633   0.6835   1.0000
             |   0.2621   0.0009
             |       20        20        20
    t4_media |  -0.1209   0.5148   0.6987   1.0000
             |   0.6115   0.0202   0.0006
             |       20        20        20        20
. pwcorr t1_amb t2_device t3_block t4_media if group == 4, sig obs
             |   t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
             |       20
   t2_device |   0.0873   1.0000
             |   0.7304
             |       20        20
    t3_block |   0.0925  -0.6459   1.0000
             |   0.7150   0.0038
             |       20        20        20
    t4_media |   0.2541   0.5824  -0.5386   1.0000
             |   0.3089   0.0112   0.0211
             |       20        20        20        20
```

```
. pwcorr t1_amb t2_device t3_block t4_media if group == 5, sig obs
             |    t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
             |       20
   t2_device |  -0.2022   1.0000
             |   0.4209
             |       20       20
    t3_block |  -0.6724   0.4366   1.0000
             |   0.0022   0.0701
             |       20       20       20
    t4_media |  -0.4799  -0.0702   0.0749   1.0000
             |   0.0439   0.7820   0.7678
             |       20       20       20       20
. pwcorr t1_amb t2_device t3_block t4_media if group == 6, sig obs
             |    t1_amb t2_dev~e t3_block t4_media
-------------+------------------------------------
      t1_amb |   1.0000
             |       20
   t2_device |  -0.0898   1.0000
             |   0.7230
             |       20       20
    t3_block |  -0.1972   0.8511   1.0000
             |   0.4328   0.0000
             |       20       20       20
    t4_media |  -0.3289   0.7694   0.9504   1.0000
             |   0.1826   0.0002   0.0000
             |       20       20       20       20
```

. *pH Pretoria

```
. table ca, c(N ph mean ph sd ph ) format(%9.2f)
------------------------------------------------
       CA |      N(ph)     mean(ph)      sd(ph)
----------+-------------------------------------
      1.2 |          6         7.42        0.06
      1.4 |          7         7.38        0.04
      1.6 |         12         7.32        0.05
      1.8 |         12         7.31        0.03
      2.0 |         10         7.26        0.06
      2.2 |         10         7.24        0.04
      2.4 |         12         7.18        0.06
      2.6 |          8         7.16        0.04
      2.8 |          6         7.13        0.07
      3.0 |          9         7.11        0.07
------------------------------------------------
. oneway ph ca, bonferroni

                        Analysis of Variance
    Source               SS          df       MS            F     Prob > F
-----------------------------------------------------------------------
Between groups      .801664251        9   .089073806      33.66    0.0000
 Within groups       .21696971       82   .002645972
-----------------------------------------------------------------------
    Total           1.01863396       91    .01119378

Bartlett's test for equal variances:  chi2(9) =   9.8681  Prob>chi2 = 0.361
                        Comparison of pH by CA
                             (Bonferroni)
Row Mean-|
Col Mean |     1.2        1.4        1.6        1.8        2.0        2.2
---------+------------------------------------------------------------------
     1.4 |  -.038786
         |     1.000
     1.6 |    -.0955   -.056714
         |     0.017      1.000
     1.8 |  -.106167   -.067381   -.010667
         |     0.004      0.326      1.000
     2.0 |    -.1635   -.124714     -.068   -.057333
         |     0.000      0.000      0.124      0.493
```
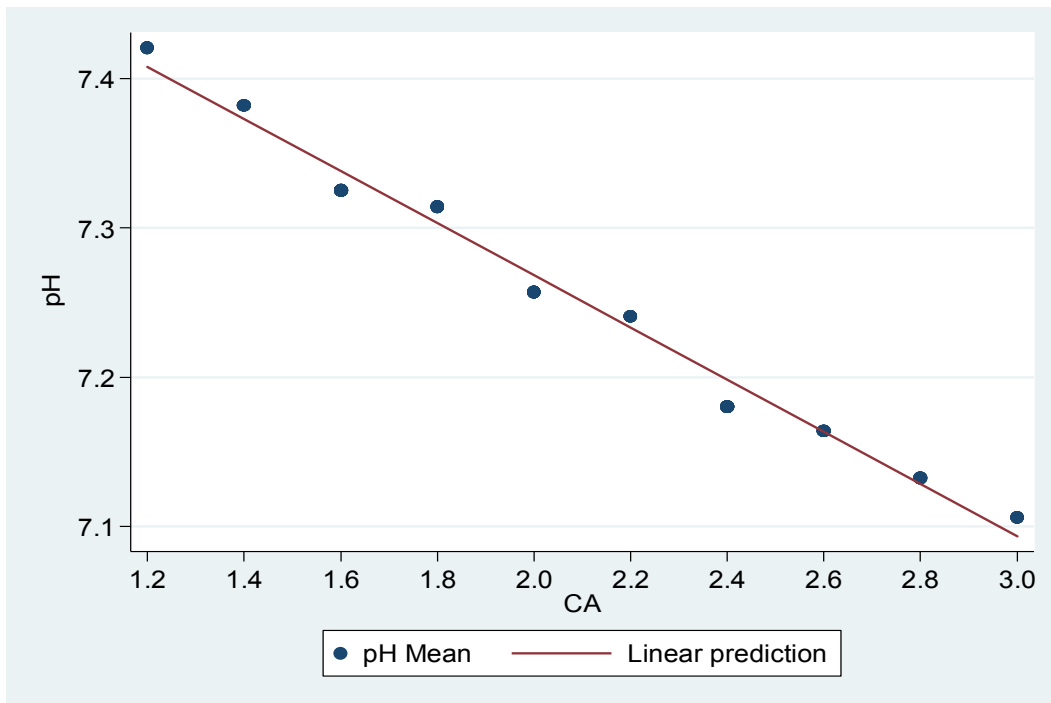
```
        2.2 |     -.1798   -.141014      -.0843   -.073633      -.0163
            |      0.000      0.000       0.011      0.056       1.000
        2.4 |   -.240167   -.201381    -.144667       -.134   -.076667   -.060367
            |      0.000      0.000       0.000       0.000      0.036      0.338
        2.6 |   -.256625   -.217839    -.161125   -.150458   -.093125   -.076825
            |      0.000      0.000       0.000       0.000      0.012      0.103
        2.8 |      -.288   -.249214      -.1925   -.181833     -.1245     -.1082
            |      0.000      0.000       0.000       0.000      0.000      0.005
        3.0 |   -.314611   -.275825    -.219111   -.208444   -.151111   -.134811
            |      0.000      0.000       0.000       0.000      0.000      0.000
Row Mean-|
Col Mean |        2.4        2.6         2.8
---------+------------------------------------
        2.6 |   -.016458
            |      1.000
        2.8 |   -.047833   -.031375
            |      1.000      1.000
        3.0 |   -.074444   -.057986    -.026611
            |      0.068      1.000       1.000
. scatter ph_mn ca || line ph_est ca
```



```
. *pH Water
. use "C:\Backup_2016\Boshoff_Gerhard\pH_Water_120516.dta", replace
. table ca water , c(N ph mean ph sd ph) format(%9.2f) row col
------------------------------
         |     Water
     CA  |    1     2   Total
---------+--------------------
     14  |    8     9     17
         | 7.43  7.28   7.35
         | 0.03  0.04   0.09
     17  |    8     9     17
         | 7.41  7.29   7.35
         | 0.06  0.06   0.08
     20  |    8     9     17
         | 7.34  7.34   7.34
         | 0.03  0.06   0.04
  Total  |   24    27     51
         | 7.40  7.30   7.35
         | 0.06  0.06   0.07
------------------------------
```
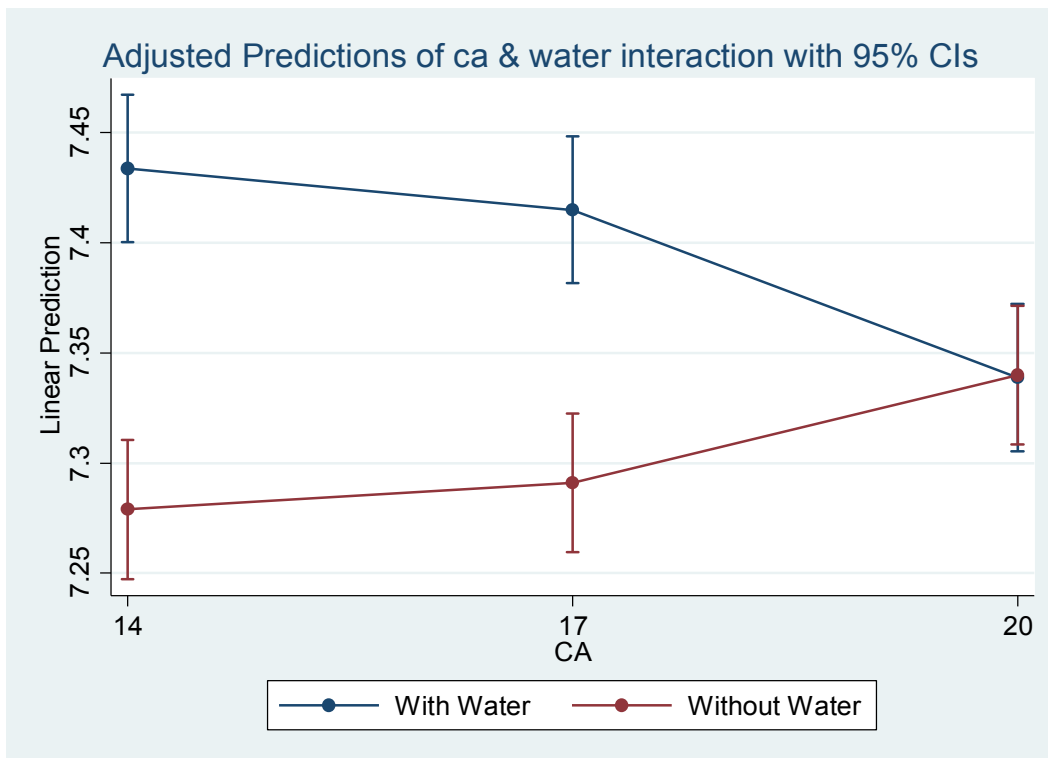
```
. anova ph ca##water
                          Number of obs =          51   R-squared     =  0.6292
                          Root MSE      =   .046917   Adj R-squared =  0.5879
                 Source | Partial SS        df         MS          F     Prob>F
            -----------+----------------------------------------------------------
                  Model |   .16804517         5   .03360903      15.27   0.0000
                        |
                     ca |   .00273839         2    .0013692       0.62   0.5414
                  water |   .10871471         1   .10871471      49.39   0.0000
               ca#water |   .05786772         2   .02893386      13.14   0.0000
                        |
               Residual |   .09905274        45   .00220117
            -----------+----------------------------------------------------------
                  Total |   .26709791        50   .00534196
. margins ca#water
Adjusted predictions                              Number of obs     =          51
Expression   : Linear prediction, predict()
--------------------------------------------------------------------------------
                |              Delta-method
                |    Margin   Std. Err.        t    P>|t|     [95% Conf. Interval]
   -------------+----------------------------------------------------------------
       ca#water |
          14 1  |   7.43375    .0165875   448.15   0.000     7.400341    7.467159
          14 2  |  7.278889    .0156389   465.44   0.000     7.247391    7.310387
          17 1  |     7.415    .0165875   447.02   0.000     7.381591    7.448409
          17 2  |  7.291111    .0156389   466.22   0.000     7.259613    7.322609
          20 1  |   7.33875    .0165875   442.43   0.000     7.305341    7.372159
          20 2  |      7.34    .0156389   469.34   0.000     7.308502    7.371498
--------------------------------------------------------------------------------
. marginsplot
  Variables that uniquely identify margins: ca water
```


Adjusted Predictions of ca & water interaction with 95% CIs

. *pH Temp(Time)

```
. table  time temp, c(N ph mean ph sd ph) format(%9.2f) row col
------------------------------------
          |           Temp
Time(hrs) |    15     25     37   Total
----------+-------------------------
      16  |            9     10     19
          |          7.50   7.46   7.48
          |          0.06   0.04   0.05
      18  |     9      8      9     26
          |   7.55   7.41   7.43   7.47
          |   0.10   0.03   0.06   0.10
      20  |    10      7            17
          |   7.51   7.43          7.48
          |   0.08   0.07          0.09
      22  |     9     10            19
          |   7.45   7.42          7.44
          |   0.07   0.04          0.06
      24  |    10      9      8     27
          |   7.45   7.43   7.44   7.44
          |   0.09   0.07   0.06   0.07
      30  |     7      5      6     18
          |   7.42   7.42   7.44   7.43
          |   0.11   0.07   0.07   0.08
   Total  |    45     48     33    126
          |   7.48   7.44   7.44   7.45
          |   0.10   0.06   0.05   0.08
------------------------------------
. anova ph time##temp
                        Number of obs =        126   R-squared     =  0.2612
                        Root MSE      =   .070622    Adj R-squared =  0.1680
              Source | Partial SS         df        MS         F     Prob>F
          -----------+----------------------------------------------------
               Model |  .1957439         14   .01398171      2.80   0.0013
                time |  .06368016         5   .01273603      2.55   0.0316
                temp |  .06237176         2   .03118588      6.25   0.0027
           time#temp |  .07779271         7   .01111324      2.23   0.0371
            Residual |  .55360311       111   .00498742
          -----------+----------------------------------------------------
               Total |  .74934701       125   .00599478
. margins time#temp
Adjusted predictions                          Number of obs     =        126
Expression  : Linear prediction, predict()
--------------------------------------------------------------------------------
            |            Delta-method
            |    Margin   Std. Err.      t    P>|t|     [95% Conf. Interval]
------------+-------------------------------------------------------------------
  time#temp |
      16 15 |         .   (not estimable)
      16 25 |  7.502222   .0235405   318.69   0.000    7.455575    7.548869
      16 37 |     7.464   .0223325   334.22   0.000    7.419747    7.508253
      18 15 |  7.554444   .0235405   320.91   0.000    7.507797    7.601092
      18 25 |   7.41125   .0249685   296.82   0.000    7.361773    7.460727
      18 37 |  7.425556   .0235405   315.44   0.000    7.378908    7.472203
      20 15 |     7.507   .0223325   336.15   0.000    7.462747    7.551253
      20 25 |      7.43   .0266925   278.36   0.000    7.377107    7.482893
      20 37 |         .   (not estimable)
      22 15 |  7.454444   .0235405   316.66   0.000    7.407797    7.501092
      22 25 |     7.423   .0223325   332.39   0.000    7.378747    7.467253
      22 37 |         .   (not estimable)
      24 15 |     7.454   .0223325   333.77   0.000    7.409747    7.498253
      24 25 |  7.433333   .0235405   315.77   0.000    7.386686     7.47998
      24 37 |   7.43625   .0249685   297.83   0.000    7.386773    7.485727
      30 15 |  7.421428   .0266925   278.03   0.000    7.368536    7.474321
      30 25 |     7.424    .031583   235.06   0.000    7.361416    7.486584
      30 37 |      7.44   .0288312   258.05   0.000    7.382869    7.497131
--------------------------------------------------------------------------------

. marginsplot
```

Adjusted Predictions of Time & Temp with 95% CIs
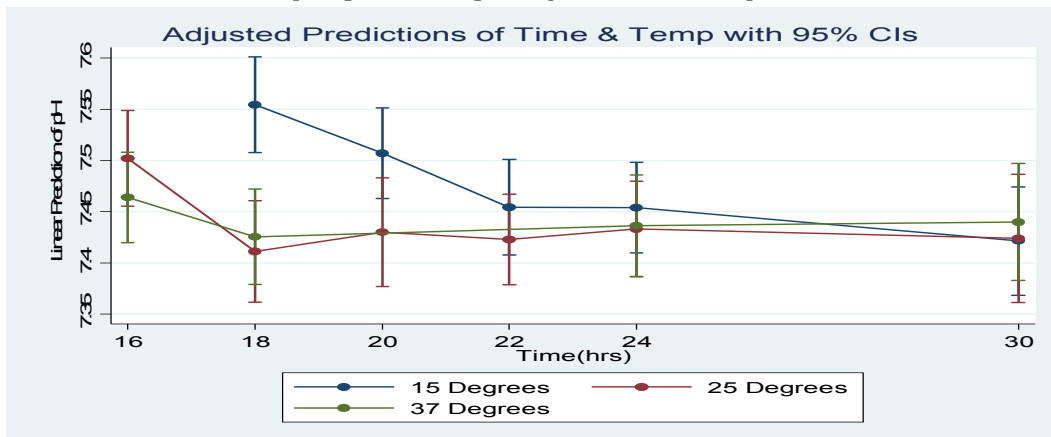
pH Altitude

```
. table vol altitude, c(N pH mean pH sd pH) format(%9.3f) col row
```

		altitude				
vol	30	350	925	1326	1627	Total
12	8	8	8	7	7	38
	7.445	7.482	7.455	7.449	7.431	7.453
	0.018	0.030	0.021	0.024	0.041	0.031
14	7	10	5	6	3	31
	7.390	7.391	7.394	7.397	7.350	7.388
	0.035	0.023	0.026	0.026	0.017	0.028
16	8	7	8	7	7	37
	7.339	7.349	7.340	7.330	7.323	7.336
	0.035	0.018	0.029	0.039	0.032	0.031
18	4	7	8	3	7	29
	7.232	7.279	7.310	7.227	7.327	7.287
	0.029	0.020	0.027	0.050	0.034	0.046
20	4	6	2	9	4	25
	7.157	7.270	7.185	7.242	7.220	7.227
	0.044	0.035	0.007	0.038	0.041	0.052
22	2	2	1	4	6	15
	7.160	7.260	7.220	7.160	7.168	7.181
	0.000	0.014		0.052	0.045	0.051
Total	33	40	32	36	34	175
	7.330	7.357	7.356	7.315	7.309	7.334
	0.108	0.083	0.080	0.103	0.096	0.096

```
. anova pH vol##altitude
```

| | | | Number of obs = | 175 | R-squared | = 0.9093 |
| | | | Root MSE | = .031526 | Adj R-squared | = 0.8911 |

Source	Partial SS	df	MS	F	Prob > F
Model	1.4440199	29	.04979379	50.10	0.0000
vol	1.1647966	5	.23295932	234.39	0.0000
altitude	.043032858	4	.010758214	10.82	0.0000
vol#altitude	.08571911	20	.004285955	4.31	0.0000
Residual	.14411496	145	.000993896		
Total	1.58813486	174	.009127212		

```
margins vol
```

Predictive margins   Number of obs   =   175
Expression   : Linear prediction, predict()

		Delta-method				
	Margin	Std. Err.	t	P>|t|	[95% Conf. Interval]	
vol						
12	7.453498	.0051411	1449.79	0.000	7.443337	7.463659

```
  14  |     7.38456    .0060335   1223.92    0.000       7.372635     7.396485
  16  |    7.336336    .0052305   1402.61    0.000       7.325998     7.346674
  18  |     7.27439    .0063077   1153.25    0.000       7.261923     7.286857
  20  |    7.217814    .0069396   1040.08    0.000       7.204098      7.23153
  22  |    7.195448    .0096765    743.60    0.000       7.176322     7.214573
-----------------------------------------------------------------------------
. marginsplot, ylabel(7(.1)7.5)
  Variables that uniquely identify margins: vol
```
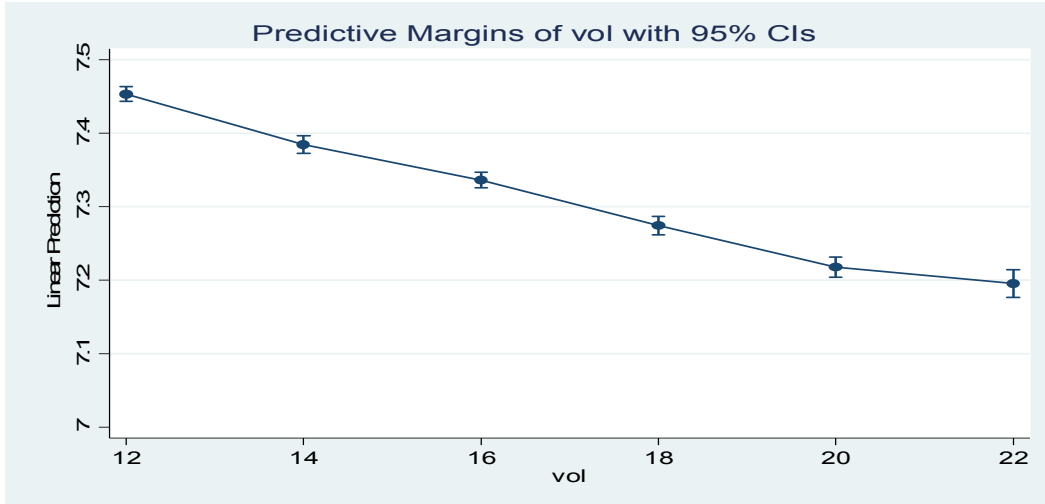

Predictive Margins of vol with 95% CIs

```
. margins  altitude

Predictive margins                                Number of obs    =        175
Expression   : Linear prediction, predict()
-----------------------------------------------------------------------------
              |            Delta-method
              |    Margin   Std. Err.        t    P>|t|    [95% Conf. Interval]
--------------+--------------------------------------------------------------
     altitude |
          30  |    7.312079    .0056047   1304.64    0.000       7.301001     7.323156
         350  |    7.354753    .0051239   1435.38    0.000       7.344625      7.36488
         925  |    7.337137    .0062052   1182.41    0.000       7.324873     7.349402
        1326  |    7.323322    .0056051   1306.55    0.000       7.312243       7.3344
        1627  |    7.324012    .0057987   1263.05    0.000       7.312551     7.335473
-----------------------------------------------------------------------------
. marginsplot, ylabel(7(.1)7.5)
  Variables that uniquely identify margins: altitude
```


Predictive Margins of altitude with 95% CIs

```
. margins vol##altitude
Predictive margins                              Number of obs   =        175
Expression   : Linear prediction, predict()
--------------------------------------------------------------------------------
              |            Delta-method
              |     Margin   Std. Err.      t    P>|t|    [95% Conf. Interval]
--------------+-----------------------------------------------------------------
          vol |
           12 |   7.453498   .0051411  1449.79   0.000    7.443337    7.463659
           14 |    7.38456   .0060335  1223.92   0.000    7.372635    7.396485
           16 |   7.336336   .0052305  1402.61   0.000    7.325998    7.346674
           18 |    7.27439   .0063077  1153.25   0.000    7.261923    7.286857
           20 |   7.217814   .0069396  1040.08   0.000    7.204098     7.23153
           22 |   7.195448   .0096765   743.60   0.000    7.176322    7.214573
     altitude |
           30 |   7.312079   .0056047  1304.64   0.000    7.301001    7.323156
          350 |   7.354753   .0051239  1435.38   0.000    7.344625     7.36488
          925 |   7.337137   .0062052  1182.41   0.000    7.324873    7.349402
         1326 |   7.323322   .0056051  1306.55   0.000    7.312243      7.3344
         1627 |   7.324012   .0057987  1263.05   0.000    7.312551    7.335473
 vol#altitude |
        12 30 |      7.445   .0111462   667.94   0.000     7.42297    7.46703
       12 350 |     7.4825   .0111462   671.31   0.000     7.46047    7.50453
       12 925 |      7.455   .0111462   668.84   0.000     7.43297    7.47703
      12 1326 |   7.448571   .0119158   625.10   0.000     7.42502    7.472122
      12 1627 |   7.431429   .0119158   623.66   0.000    7.407878     7.45498
        14 30 |       7.39   .0119158   620.19   0.000    7.366449    7.413551
       14 350 |      7.391   .0099694   741.37   0.000    7.371296    7.410704
       14 925 |      7.394   .0140989   524.44   0.000    7.366134    7.421866
      14 1326 |   7.396667   .0128705   574.70   0.000    7.371229    7.422105
      14 1627 |       7.35   .0182016   403.81   0.000    7.314025    7.385975
        16 30 |    7.33875   .0111462   658.41   0.000     7.31672    7.36078
       16 350 |   7.348571   .0119158   616.71   0.000     7.32502    7.372122
       16 925 |       7.34   .0111462   658.52   0.000     7.31797    7.36203
      16 1326 |       7.33   .0119158   615.15   0.000    7.306449    7.353551
      16 1627 |   7.322857   .0119158   614.55   0.000    7.299306    7.346408
        18 30 |     7.2325   .0157631   458.83   0.000    7.201345    7.263655
       18 350 |   7.278571   .0119158   610.84   0.000     7.25502    7.302122
       18 925 |       7.31   .0111462   655.83   0.000     7.28797    7.33203
      18 1326 |   7.226667   .0182016   397.03   0.000    7.190692    7.262641
      18 1627 |   7.327143   .0119158   614.91   0.000    7.303592    7.350694
        20 30 |     7.1575   .0157631   454.07   0.000    7.126345    7.188655
       20 350 |       7.27   .0128705   564.86   0.000    7.244562    7.295438
       20 925 |      7.185   .0222923   322.31   0.000     7.14094    7.22906
      20 1326 |   7.242222   .0105087   689.16   0.000    7.221452    7.262992
      20 1627 |       7.22   .0157631   458.03   0.000    7.188845    7.251155
        22 30 |       7.16   .0222923   321.19   0.000     7.11594    7.20406
       22 350 |       7.26   .0222923   325.67   0.000     7.21594    7.30406
       22 925 |       7.22   .0315261   229.02   0.000     7.15769    7.28231
      22 1326 |       7.16   .0157631   454.23   0.000    7.128845    7.191155
      22 1627 |   7.168333   .0128705   556.96   0.000    7.142895    7.193771
--------------------------------------------------------------------------------

. marginsplot, ylabel(7(.1)7.5)
  Variables that uniquely identify margins: vol altitude
```


Predictive Margins with 95% CIs

. *pH Water

```
. use "C:\Backup_2016\Boshoff_Gerhard\pH_Water_120516.dta", replace
. table ca water , c(N ph mean ph sd ph) format(%9.2f) row col
-------------------------------
          |        Water
     CA   |    1       2    Total
----------+--------------------
     14   |    8       9      17
          | 7.43    7.28    7.35
          | 0.03    0.04    0.09
     17   |    8       9      17
          | 7.41    7.29    7.35
          | 0.06    0.06    0.08
     20   |    8       9      17
          | 7.34    7.34    7.34
          | 0.03    0.06    0.04
  Total   |   24      27      51
          | 7.40    7.30    7.35
          | 0.06    0.06    0.07
-------------------------------
. anova ph ca##water
                    Number of obs =        51    R-squared     =  0.6292
                    Root MSE      =  .046917    Adj R-squared =  0.5879
              Source | Partial SS      df       MS          F     Prob>F
         -----------+----------------------------------------------------
               Model | .16804517        5   .03360903     15.27   0.0000
                  ca | .00273839        2    .0013692      0.62   0.5414
               water | .10871471        1   .10871471     49.39   0.0000
            ca#water | .05786772        2   .02893386     13.14   0.0000
            Residual | .09905274       45   .00220117
         -----------+----------------------------------------------------
               Total | .26709791       50   .00534196
. margins ca#water
Adjusted predictions                            Number of obs     =        51
Expression   : Linear prediction, predict()
------------------------------------------------------------------------------
             |            Delta-method
             |    Margin   Std. Err.      t    P>|t|    [95% Conf. Interval]
-------------+----------------------------------------------------------------
    ca#water |
        14 1 |   7.43375   .0165875   448.15   0.000    7.400341   7.467159
        14 2 |  7.278889   .0156389   465.44   0.000    7.247391   7.310387
        17 1 |     7.415   .0165875   447.02   0.000    7.381591   7.448409
        17 2 |  7.291111   .0156389   466.22   0.000    7.259613   7.322609
        20 1 |   7.33875   .0165875   442.43   0.000    7.305341   7.372159
        20 2 |      7.34   .0156389   469.34   0.000    7.308502   7.371498
------------------------------------------------------------------------------
. marginsplot
  Variables that uniquely identify margins: ca water
```


Adjusted Predictions of ca & water interaction with 95% CIs

HZA Counts

```
. table insem_cnt, c(N hzi mean hzi sd hzi) format(%9.3f)
----------------------------------------------
Insem_Cnt |    N(hzi)   mean(hzi)    sd(hzi)
----------+-----------------------------------
      500 |       18      13.948      10.163
     1000 |       18      20.533      10.530
     2000 |       18      35.314      17.491
     5000 |       18      39.638      18.475
    10000 |       15      62.824      26.716
    20000 |       17      73.256      26.763
----------------------------------------------
. oneway hzi insem_cnt, bonferroni
                      Analysis of Variance
     Source             SS         df      MS            F      Prob > F
-----------------------------------------------------------------------
Between groups     46052.1316       5   9210.42633     25.01    0.0000
 Within groups     36096.3954      98   368.330565
-----------------------------------------------------------------------
    Total          82148.527      103   797.558515
Bartlett's test for equal variances:  chi2(5) =  25.9523  Prob>chi2 = 0.000
                      Comparison of HZI by Insem_Cnt
                            (Bonferroni)
Row Mean-|
Col Mean |     500       1000       2000       5000      10000
---------+-------------------------------------------------------
    1000 |   6.5851
         |   1.000
    2000 |  21.3663    14.7812
         |   0.018      0.344
    5000 |  25.6907    19.1056     4.32438
         |   0.002      0.053      1.000
   10000 |  48.8765    42.2914    27.5102    23.1858
         |   0.000      0.000      0.001      0.012
   20000 |  59.3088    52.7237    37.9425    33.6181    10.4323
         |   0.000      0.000      0.000      0.000      1.000
. oneway hzi_r insem_cnt, bonferroni
                      Analysis of Variance
     Source             SS         df      MS            F      Prob > F
-----------------------------------------------------------------------
Between groups     56245.2712       5   11249.0542     29.41    0.0000
 Within groups     37484.7288      98   382.497232
-----------------------------------------------------------------------
    Total          93730          103   910
Bartlett's test for equal variances:  chi2(5) =   2.1491  Prob>chi2 = 0.828
                  Comparison of rank of (hzi)      by Insem_Cnt
                            (Bonferroni)
Row Mean-|
Col Mean |     500       1000       2000       5000      10000
---------+-------------------------------------------------------
    1000 |  11.0556
         |   1.000
    2000 |  32.2222    21.1667
         |   0.000      0.024
    5000 |  38.2222    27.1667        6
         |   0.000      0.001      1.000
   10000 |  58.7222    47.6667     26.5       20.5
         |   0.000      0.000      0.003      0.052
   20000 |  65.134     54.0784    32.9118    26.9118    6.41176
         |   0.000      0.000      0.000      0.001      1.000
```

Calculated:
99% CI 5000 insemination:

1. Degrees of freedom n-1: 18-1=17; (99%CI 1-0.099)/2: α=0.001 – t-distrubution table 17df, 0.001α: **2.898**
2. SD/square root n: 18.475/RT(18) = 18.475/4.242640687119285 = **4.354599260807155**
3. (1)x(2): 2.898x4.354599260807155=**12.619628565781914**
4. Mean±(3)=99%CI: 39.638-12.619628565781914=27.02; 39.638+12.619628565781914=52.26

99% CI: (27.02 – 52.26)

HZA 50ul vs 1ml (5000 insemination)
```
->  ttest test_15 = contr_15
```
Paired t test
```
-------------------------------------------------------------------------------
Variable |    Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+---------------------------------------------------------------------
 test_15 |     14    12.78571    2.070671     7.74774    8.312302    17.25913
contr_15 |     14    22.35714    1.353088    5.062793    19.43397    25.28031
---------+---------------------------------------------------------------------
    diff |     14   -9.571429    2.638919    9.873931   -15.27247   -3.870391
-------------------------------------------------------------------------------
     mean(diff) = mean(test_15 - contr_15)                    t =  -3.6270
 Ho: mean(diff) = 0                          degrees of freedom =       13

Ha: mean(diff) < 0          Ha: mean(diff) != 0          Ha: mean(diff) > 0
 Pr(T < t) = 0.0015       Pr(|T| > |t|) = 0.0031        Pr(T > t) = 0.9985
```

Calculated:
9.571429/22.35714=0.428115
42.81% less binding
ROS Generation
```
. xtreg ros i.method, i(sample) mle
Random-effects ML regression          Number of obs      =        12
Group variable: sample                Number of groups   =         6
Random effects u_i ~ Gaussian         Obs per group:
                                                    min =         2
                                                    avg =       2.0
                                                    max =         2
                                      LR chi2(1)        =       0.05
Log likelihood  = -90.725431          Prob > chi2       =    0.8184
-------------------------------------------------------------------------------
      ros |     Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
----------+--------------------------------------------------------------------
 2.method |   61.66667   268.3256    0.23   0.818   -464.2418    587.5751
    _cons |   923.1667   189.7348    4.87   0.000    551.2932     1295.04
----------+--------------------------------------------------------------------
  /sigma_u |         0   288.4625                          .           .
  /sigma_e |  464.7567   94.86643                    311.5142    693.3834
      rho |         0  (omitted)
-------------------------------------------------------------------------------
LR test of sigma_u=0: chibar2(01) = 0.00          Prob >= chibar2 = 1.000
. margins method
Adjusted predictions                  Number of obs      =        12
Model VCE    : OIM
Expression   : Linear prediction, predict()
-------------------------------------------------------------------------------
         |            Delta-method
         |    Margin   Std. Err.      z    P>|z|     [95% Conf. Interval]
---------+---------------------------------------------------------------------
  method |
       1 |  923.1667   189.7348    4.87   0.000    551.2932     1295.04
```

```
          2 |     984.8333    189.7348      5.19   0.000      612.9599    1356.707
-----------------------------------------------------------------------------------
. table method, c(N ros mean ros sd ros med ros)
-------------------------------------------------------------
    method |     N(ros)    mean(ros)     sd(ros)     med(ros)
----------+--------------------------------------------------
         1 |          6      923.167     194.8655          929
         2 |          6      984.833     693.1266          758
-------------------------------------------------------------
. *(1 variable, 6 observations pasted into data editor)
```

Paired t test

```
-----------------------------------------------------------------------------------
Variable |     Obs         Mean     Std. Err.    Std. Dev.    [95% Conf. Interval]
---------+-------------------------------------------------------------------------
   Ros_c |       6    923.1667     79.55351     194.8655     718.6679    1127.665
   Ros_t |       6    984.8333     282.9678     693.1267     257.4415    1712.225
---------+-------------------------------------------------------------------------
    diff |       6   -61.66667     324.1757     794.0652    -894.9869    771.6536
-----------------------------------------------------------------------------------
      mean(diff) = mean(ros - ros_01)                              t =  -0.1902
 Ho: mean(diff) = 0                              degrees of freedom =        5

Ha: mean(diff) < 0           Ha: mean(diff) != 0              Ha: mean(diff) > 0
 Pr(T < t) = 0.4283        Pr(|T| > |t|) = 0.8566           Pr(T > t) = 0.5717
. signrank ros = ros_01
Wilcoxon's matched pairs signed-rank test
         sign |     obs   sum ranks    expected
--------------+--------------------------------
     positive |       3          12        10.5
     negative |       3           9        10.5
         zero |       0           0           0
--------------+--------------------------------
          all |       6          21          21
unadjusted variance        22.75
adjustment for ties         0.00
adjustment for zeros        0.00
                       ----------
adjusted variance          22.75
Ho: ros = ros_01
            z =    0.314
   Prob > |z| =    0.7532
. means ros1500 if time == 0 & method == 1
   Variable |     Type          Obs         Mean       [95% Conf. Interval]
-----------+------------------------------------------------------------------
    ros1500 | Arithmetic          6     1626.167      1075.497     2176.837
            | Geometric           6      1563.63       1142.11      2140.72
            | Harmonic            6     1509.841      1174.276     2113.922
-----------+------------------------------------------------------------------
. means ros1500 if time == 18 & method == 1
   Variable |     Type          Obs         Mean       [95% Conf. Interval]
-----------+------------------------------------------------------------------
    ros1500 | Arithmetic          6     2512.833      2310.945     2714.722
            | Geometric           6     2506.897      2317.746     2711.484
            | Harmonic            6     2501.162      2323.309      2708.5
-----------+------------------------------------------------------------------
. means ros1500 if time == 0 & method == 2
   Variable |     Type          Obs         Mean       [95% Conf. Interval]
-----------+------------------------------------------------------------------
    ros1500 | Arithmetic          6     1648.167      792.8857     2503.448
            | Geometric           6     1444.459      757.7482     2753.501
            | Harmonic            6     1204.256      665.8726     6289.792
-----------+------------------------------------------------------------------
. means ros1500 if time == 18 & method == 2
   Variable |     Type          Obs         Mean       [95% Conf. Interval]
-----------+------------------------------------------------------------------
    ros1500 | Arithmetic          5       2085.8       275.176     3896.424
            | Geometric           5     1521.577      420.5186     5505.572
            | Harmonic            5     919.6569            .            .
Missing values in confidence intervals for harmonic mean indicate
```

```
that confidence interval is undefined for corresponding variables.
Consult Reference Manual for details.
. means ros1500 if time == 66 & method == 2
    Variable |    Type          Obs      Mean      [95% Conf. Interval]
-------------+--------------------------------------------------------------
     ros1500 | Arithmetic        6    2347.167      1619.729    3074.604
             |  Geometric         6    2271.261      1703.091    3028.979
             |   Harmonic         6    2205.04       1743.226    2999.725
-------------------------------------------------------------------------
. means ros1500 if time == 114 & method == 2
    Variable |    Type          Obs      Mean      [95% Conf. Interval]
-------------+--------------------------------------------------------------
     ros1500 | Arithmetic        6    2869.833      1970.672    3768.995
             |  Geometric         6    2746.815      1923.529    3922.474
             |   Harmonic         6    2607.676      1858.168    4370.587
-------------------------------------------------------------------------
. table time method, c(N ros1500 mean ros1500 sd ros1500 med ros1500) format(%9.3f)
      time |      1         2      Total
-----------+----------------------------
         0 |      6         6         12
           | 1626.167  1648.167  1637.167
           |  524.730   814.991   653.606
           | 1468.500  1688.500  1619.000
        18 |      6         5         11
           | 2512.833  2085.800  2318.727
           |  192.378  1458.224   958.543
           | 2469.500  2049.000  2427.000
        66 |                6          6
           |           2347.167  2347.167
           |            693.170   693.170
           |           2180.500  2180.500
       114 |                6          6
           |           2869.833  2869.833
           |            856.805   856.805
           |           2800.000  2800.000
     Total |     12        23         35
           | 2069.500  2244.348  2184.400
           |  596.984  1012.845   886.669
           | 2342.500  2049.000  2318.000
```

DNA Fragmentation (200ul vs 1ml)

DNA Fragmentation Paired t test results

P value and statistical significance:

  The two-tailed P value equals 0.9007

  By conventional criteria, this difference is considered to be not statistically significant.

Confidence interval:

  The mean of Control minus Test equals -0.33

  95% confidence interval of this difference: From -6.86 to 6.19

Intermediate values used in calculations:

  t = 0.1313 ; df = 5

  standard error of difference = 2.539

  Group   Control   Test

Mean   78.83   79.17

SD   2.71   4.02

SEM   1.11   1.64

N   6   6

7.2. Information leaflet and informed consent for non-clinical research

INFORMATION LEAFLET AND INFORMED CONSENT FOR NON-CLINICAL RESEARCH

TITLE OF STUDY

Investigating a novel *in vitro* embryo culture system - The simplified Walking Egg *in vitro* fertilization

1) INTRODUCTION

You are invited to participate in a research study. This information will help you to decide if you would like to participate. Before you agree to take part in the study you should fully understand what is involved. If you have questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator Mr G. Boshoff. You should not agree to take part unless you are completely satisfied about all the procedures involved.

2) THE NATURE AND PURPOSE OF THE STUDY

The objective of this study is to look at an easier and cheaper system of growing human embryos and to test specific parts of the system such as temperature and pH control. A second part of the study is to determine the lowest number of sperm that would still show fertilization of eggs.

You as a participant would provide the cells needed to perform the second part of the study. These cells will be sperm cells if you are a man or eggs that cannot be used for assisted reproduction, if you are a woman.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves the testing of a cheaper way of growing embryos. The tests that will be performed will not include actual growing of embryos, but will use scientific methods to test specific parts of the growing system. First the temperature of the system will be tested by using different heating methods. The next step would be to test the pH (acidity) of culture media when using the system. Lastly the binding of sperm to eggs will be tested while using the mentioned system. Eggs that did not fertilize and will not grow into an embryo will be cut into two halves and only the outer edge of the eggs, known as the zona, will be used. The zona halves will be mixed with sperm and the amount of sperm sticking to the zona's will be counted.

4) RISK INVOLVED

There is no risk in participating in the study. If you are a man, you will be asked to provide us with a semen sample to be used with this study. If you are a woman you will be asked to give permission that i) your eggs in the laboratory that did not form embryos and ii) the cumulus cells surrounding the eggs that is

Page 1 of 3

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not used during the procedure be used for the study. No embryos will be made using the sperm or eggs and all the used sperm, eggs and cumulus cells will be thrown away after the study is completed.

5) POSSIBLE BENEFITS OF THE STUDY

The testing of this new embryo growing system will give information to show if it can be used to grow embryos. This system can be used to provide cheaper IVF treatment to patients. This will help patients that cannot afford standard IVF to also attempt to fall pregnant with the help from a laboratory using this system.

6) WHAT ARE YOUR RIGHTS AS A PARTICIPANT?

Your participation in this study is entirely voluntary and **you can refuse to participate or stop at any time without stating any reason.** Your withdrawal will not affect you in anyway.

7) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This study has received written approval from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria. A copy may be obtained from the investigator should you wish to view it.

8) INFORMATION AND CONTACT PERSON

The contact person for the study is Mr G. Boshoff. If you have any questions about the study please contact him at Tel: 012 354 2061.
Alternatively you may contact his supervisor Prof. C. Huyser at Tel: 012 354 2208.

9) COMPENSATION

Your participation is voluntary. No compensation will be given for your participation.

10) CONFIDENTIALITY

All information that you give will be kept strictly confidential. Once we have analysed the information no one will be able to identify you. Research reports and articles in scientific journals will not include information that may identify you.

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CONSENT TO PARTICIPATE IN STUDY

Investigating a novel *in vitro* embryo culture system - The Walking Egg Affordable Assisted Reproductive Technology

I confirm that the person asking my consent to take part in this study has told me about the nature, procedure, risk, discomfort and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participating in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect me in anyway.

Volunteer's name: _____ (Please print)

Volunteer's Signature: _____ Date: _____

Investigator's name: _____ (Please print)
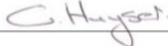
Investigator's Signature:_____ Date: _____

Witness's name: _____(Please print)

Witness's signature: _____ Date: _____

7.3. Standard operative procedures

7.3.1. SOP F1.16.2 Sperm processing for therapeutic procedures

Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Sperm processing for therapeutic procedures - Puresperm	SOP nr	F1.16.2
Category	Procedures	Author	GM Boshoff
Approved by HOD	*C. Huyser.*	Approved date	04/12/2014

Standard Operating Procedure:

Sperm processing with Puresperm - Guidelines for therapeutic procedures

Contents

Aim

Materials

Equipment

Method

    Preparation

    Sperm processing for therapeutic procedures

    For IUI

    For IVF

    For ICSI

Aim

The correct processing of a semen sample for a therapeutic procedure is a crucial step in the fertilization process. It will be helpful to read through SOP F1.2.1 Basic semen analysis, SOP F1.11.1 Morphology staining and evaluation and SOP F1.19.1 Media preparation for sperm processing before continuing. The following SOP refers to processing with PureSperm density gradients, however other media may be substituted.

Materials

- Semen sample
- White laboratory coat
- Latex free disposable gloves i.e. Nitrile gloves
- Disposable overshoes
- PureSperm40 (100PS40PE28) (See SOP F1.19.1 Media prep for sperm processing)
- PureSperm80 (100PS80PE28) (See SOP F1.19.1 Media prep for sperm processing)
- PureSperm Wash (100PSWPL03) (See SOP F1.19.1 Media prep for sperm processing)
- Gassed Fertilization media (K-SIFM-50) (See SOP F1.19.1 Media prep for sperm processing)

Equipment

- Makler counting chamber
- Neubauer counting chamber

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- Light microscope e.g. Axio*star* plus or Axioskop 40
- Bioflow stocked for processing semen (See SOP F1.2.1 Basic semen analysis)

Please note that for therapeutic procedures NO alcohol should be used in the bioflow or near the semen sample. Sterile water may be used to wipe down the bioflow before use.

Method

Preparation

- Always wipe the bioflow with water and a paper towel before processing and change the test tube racks
- Wipe the pipettes in the bioflow with sterile water and a paper towel before processing
- Gradients may be prepared the day before, however if this is not done, gradients can be taken out while waiting for the patient to produce the sample
- Check the previous spermiogram to ensure the correct volume of media is taken out i.e. in case of parallel processing
- Remember to check at what temperature specific gradients should be used e.g. PureSperm requires room temperature
- Switch on all heated equipment prior to use
- Run the centrifuge so that it can warm to 25°C before spinning the sample

Sperm processing for therapeutic procedures

- The entire semen volume must be used for any therapeutic processing
- A maximum of 1.5ml of semen may be used when processing with PureSperm gradients
- If the total semen volume exceeds 1.5ml, then parallel processing must be done
- Layer the gradients and semen immediately after liquefaction
  - Layer 2ml of 80% density gradient first
  - Layer 2ml of 40% density gradient on top of 80% layer
  - Layer semen (max 1.5ml) on top of 40% layer
  - Centrifuge for 20min at 300xg (using swing-out buckets and no break when stopping)
- Check pre-counts while the sample is spinning
- Prepare a disposable pipette before the end of the first spin to remove the top layers of media
- When the centrifuge stop, carefully remove the test tube without mixing the layers or pellet
- Remove top layers with the disposable pipette until only a bit of the last gradient and the pellet remains
- When removing the supernatant, be careful not to disturb the sperm pellet
- Using a 100 – 1000µl pipette, remove a 400µl pellet carefully without mixing the contents of the test tube to prevent excess debris from being transferred to the wash media
- Place only the tip of the pipette into the wash media
- Add the pellet slowly to the wash media, allowing the sample to mix with the media

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- Do not tip the test tube to mix the sample, rather run the test tube over your fingers a few times
- Spin the sample for 10min at 500xg (using swing-out buckets and no break when stopping)
- Label a pH tube with the patients name, the date and the procedure
- Prepare a disposable pipette
- When the second spin is complete, remove the supernatant in the same fashion as the first time
- Assess and determine the volume of the pellet
- Set a 100 – 1000µl pipette to a volume that will remove the whole pellet (400µl if performing an IUI)
- Carefully aspirate the pellet with the 100 – 1000µl pipette while not mixing the washing media
- Place the pellet into the labelled pH tube using the 100 – 1000µl pipette

For IUI
- The sperm pellet must always be 400µl in volume for an IUI
- Perform post counts on the sperm in the pH tube
- Ask the head of the laboratory or section to co-sign the IUI form
- Prepare a polystyrene cup with cotton wool inside
- Label the cup with the patients name and IUI
- Place sample in pH tube inside polystyrene cup with cotton wool surrounding the pH tube for insulation
- Hand the sample to the clinic in the polystyrene cup and check that it is placed in their warming oven if the IUI is not performed immediately
- Ask the person receiving the sample to please sign the spermiogram form
- If anything unusual was noted during processing e.g. a large amount of epithelial cells or high round cell count, inform the clinic of this
- Make a copy of the form for the clinic at this time
- Return to the lab to clean up only once the sample has been handed over

For IVF
- Determine sperm concentration by means of a Neubauer haemocytometer
  - The concentration should be close to 10×10^6 sperm/ml ($8 – 12 \times 10^6$ sperm/ml)
  - If the concentration is higher, add small amounts (e.g. 50µl) of PureSperm Wash media to dilute the sample
  - If the concentration is very high, remove a small volume of the sperm (e.g. 50µl-100µl) into a labelled pH tube and dilute by adding PureSperm Wash media to the sample
  - Check the concentration by means of a Makler counting chamber after each dilution until the correct concentration is obtained

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- Determine the percentage of progressively motile spermatozoa by performing a differential count
  - Grade the sperm motility as progressively motile (PR), non-progressively motile (NP) or immotile (IM) according to the WHO 2010 manual
  - The percentage of progressively motile sperm is used in further calculations
- Determine the number of progressively motile spermatozoa/ml
  - **Percentage progressively motile sperm x sperm concentration**
  - Example:

Progressive motility	= 80%
Sperm concentration	= 12×10^6 sperm/ml
No. of progressively motile	= $80\% \times 12 \times 10^6$
	= 9.6×10^6 sperm/ml

- Determine the volume to inseminate 50 000 spermatozoa by cross multiplying

Concentration of progressively motile spermatozoa (e.g. 9.6×10^6/ml)　　　　　　1000µL

Required sperm number to inseminate (e.g. 50 000)　　　　　　X µl
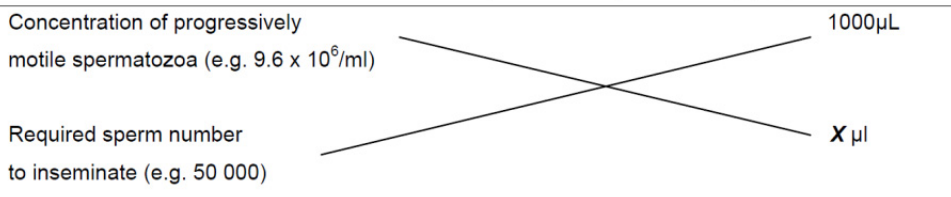
Therefore volume (X) to inseminate is:

$$\frac{9.6 \times 10^6}{50\ 000} = \frac{1000}{X}$$

$X \times 9\ 600\ 000 = 1000 \times 50\ 000$

$$X = \frac{50\ 000\ 000}{9\ 600\ 000}$$

$$X = \frac{50}{9.6}$$

$X = 5.2083$

Inseminate 5.2µl

- The test tube with spermatozoa should be stored in the workstation with the tube capped (PureSperm Wash does not need to be gassed)
- If only a small volume of sperm pellet was obtained, place the pellet in a mouse dish and cover with oil
  - Leave the dish on the workstation out of harms way

Note: The volume inseminated must be ≥ 5µl to avoid the insemination of incorrect numbers of sperm which can occur more easily when lower volumes are inseminated. When the volume to be

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inseminated is < 5µl, the sperm suspension should be diluted accordingly and a new insemination volume must be determined.

For ICSI

- Determine sperm concentration by means of a Neubauer haemocytometer
  - The concentration should be $20 - 30 \times 10^6$ sp/ml
  - If the concentration is higher, add small amounts e.g. 50µl of PureSperm Wash media to dilute the sample
  - Check the concentration by means of a Makler counting chamber after each dilution until the correct concentration is obtained.
- The test tube with spermatozoa should be stored in the workstation with the tube capped (PureSperm Wash does not need to be gassed)
- If only a small volume of sperm pellet was obtained, place the pellet in a mouse dish and cover with oil
  - Leave the dish on the workstation out of harms way

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7.3.2. SOP F2.7.1 Hemi-zona Assay

Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Hemizona Assay	SOP nr	F2.7.1
Category	Procedures	Author	JM Fourie
Approved by HOD	C. Huysel.	Approved date	26/06/2013

Standard Operating Procedure:

Hemizona Assay

Contents

Aim

Method

    Collection and cryopreservation of nonviable oocytes

    Setup of micro-blades

    Bisection of oocytes

    Sperm-zona incubation

    Removal of loosely bound spermatozoa

    Determination of the hemizona index (HZI)

Notes

    Reference

Aim

The hemizona assay is used to compare test sperm with control donor sperm (with known good sperm zona binding).

Materials

- Non-fertilized oocytes
- Sperm
- Micro blade (BD Micro-Sharp™, 377530)
- HEPES buffered media such as PureSperm®Wash (Nidacon International)
- Oocyte cryopreservation media:
    - 2.49ml DMSO (Sigma-Aldrich, D5879)
    - 7.51ml Culture media (Hams F10, Sigma-Aldrich, N6635))
    - 0.8g/10ml Sucrose (Sigma-Aldrich, S1888)
    - 300mg/10ml BSA (Sigma-Aldrich, 9048-46-8)
- Cryopreservation straw (Mini Tub distributed by IEPSA)
- Medical Freezer (-80°C, Sanyo Ultra low)
- Petridish for ICSI (BD-Falcon 353001)
- Petridish for insemination (BD-Falcon 353004)
- Inverted microscope (Axiovert, Zeiss)
- Micromanipulators (Transferman, Eppendorf)

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- Flexipette tips (Cook, K-FPIP-1080 &1130)
- Culture oil (FertiCult™, Fertipro)

Method

Collection and cryopreservation of nonviable oocytes

- Non fertilized **IVF (not ICSI)** oocytes are collected into HEPES buffered media and stored in fridge at 4°C until cryopreservation
- Cryopreservation must occur **within one week** after failed fertilization
- Add oocytes to cryopreservation media for 3 minutes
- Aspirate oocytes into cryopreservation straw and mark straw accordingly
    - See SOP G1.3.1. Blastocyst slow freeze for loading of straws
- Put straw in freezer at -80°C

Setup of micro-blades

- Set the angle of the micromanipulator's motor module on the right hand side at a 35 degree angle and swivel the motor module out of the way (to the right)
- Screw a micro blade into the micro blade holder
- Insert the micro blade holder into the motor module of the micro manipulator
- Put a petri dish on the microscope stage
- Move the motor module now containing the blade to it's top position by turning the respective controller clockwise
- Swivel the motor unit back into position
- Use the 5x objective to focus on the micro blade
- Turn the micro blade holder until the blade is totally straight and orientated horizontally
- Lower the blade unto the surface of the petri dish and make a cut from left to right by using the controller, if the cut line is not straight adjust the blade further until a straight horizontal cut can be made

Bisection of oocytes

- Thaw the straws containing the oocytes at room temperature (2 minutes) and transfer oocytes to HEPES buffered media using a 130µl flexipet
- Use an petri dish (BD-Falcon 353001) and add a 100µl droplet of HEPES buffered media to the centre of the dish
- Pipette an oocyte in the centre of the droplet and place the dish on the stage of the inverted microscope
- Swing the right-hand motor holding the blade into position
- Lower the blade onto the bottom surface of the petri dish and cut a horizontal groove in the bottom surface of the petri dish by moving the manipulator control from left to right
- By using the blade gently role the oocyte the on top of the groove, the groove will hold the oocyte in position during the cutting process

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- Lower the blade on top of the oocyte ensuring that the blade is exactly in the middle of the oocyte (as pressure is applied, the oocyte will start to bulge out from under the blade)
- Continue to adjust the position of the blade to ensure that equal halves of the oocyte bulge out from under the blade on both sides (see Figure 1: Diagrammatic illustration of blade orientation during bisecting of oocytes)
- When the blade touches the bottom of the petri dish the blade will start to move forward
- Bisect the oocyte by cutting from left to right
- Bisect the required number of oocytes and keep halves from the same oocyte together
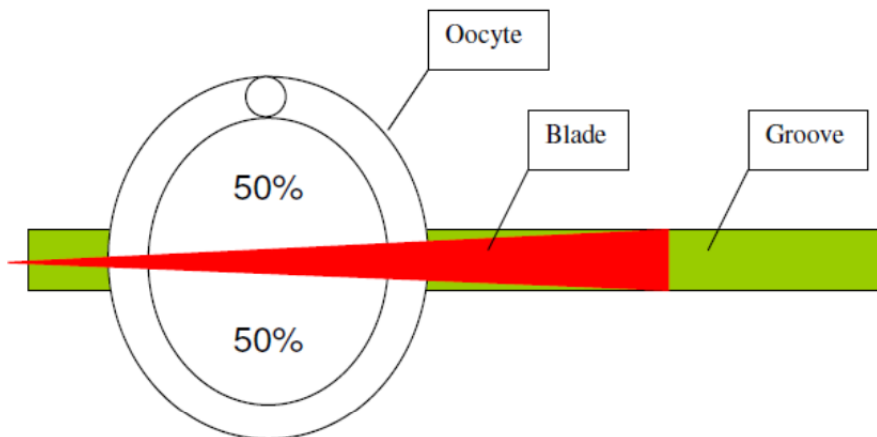
Figure 1: Diagrammatic illustration of blade orientation during bisecting of oocytes

Sperm-zona incubation

- Split the sperm sample in the respective test and control samples
    - Subject the test spermatozoa to the specific treatment to be tested
    - No treatment must be given to control sperm
- Prepare the petri dish by making two rows of 50µl droplets with HEPES buffered media
- Cover the drops with culture oil
- Inseminate containing 100 000 test spermatozoa (TS) or 100 000 control spermatozoa (CS) (see Figure 2: Diagrammatic illustration of droplet layout during the hemi zona assay)
- By keeping the hemi-zonae in pairs, add one hemi zona to each droplet containing sperm (see Figure 2: Diagrammatic illustration of droplet layout during the hemi zona assay)
- Ensure two halves of one oocyte are placed in a TS and CS drop next to each other
- Incubate the sperm with hemi zonas for 2 hours at 37°C in ambient air

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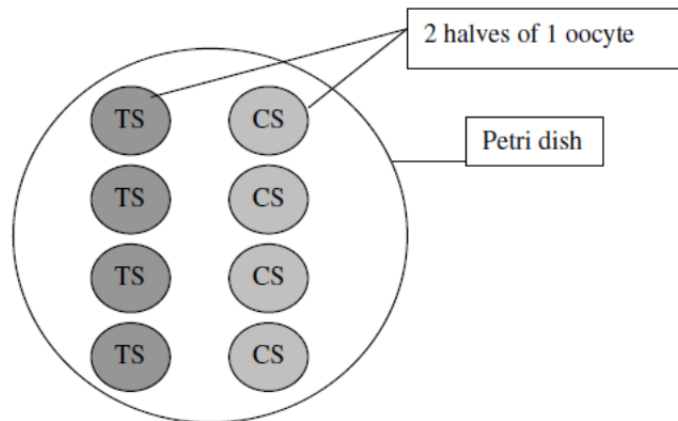
Figure 2: Diagrammatic illustration of droplet layout during the hemi zona assay

Removal of loosely bound spermatozoa

- Insert an 80µm flexi tip (Cook, K-FPIP-1080) in a flexitip handle
- Prepare a petri dish containing the same number and orientation of droplets as the dish that was used for zona-sperm incubation
- View the hemi-zonae under a stereo microscope
- Remove all loosely bound spermatozoa from the zona surfaces by gently pipetting the hemi-zonas in and out of the 80µm pipette tip twice
- Transfer the hemi-zonas with the 80µm pipette tip to the newly prepared dish, still keeping the hemi zonas in pairs

Determination of the hemizona index (HZI)

- View the hemi zonae under an inverted microscope at 400x magnification
- Focus on top of the hemi zona
- Count all spermatozoa in every focus plane while focusing through the hemi zona towards the bottom (repeat this step x3)
- The 3 counts per each hemi zona must be similar (5% difference), if not repeat the counting step
- More than 20 control spermatozoa must be bound to a hemi zona in order to use the data from a specific oocyte
- The following formula is used to determine the HZI:

  HZI = {Number of test sperm bound / Number of control sperm bound} x 100

- **The HZI must be >30% for good fertilization potential** (Franken *et al.*, 1993)

4

Notes:

Reference

Franken, D.R., Acosta, A.A., Kruger, T.F., Lombard, C.J., Oehninger, S. & Hodgen, G.D. 1993,
   "The hemizona assay: its role in identifying male factor infertility in assisted reproduction.",
   Fertility & Sterility, vol. 59, no. 5, pp. 1075-1080.

7.3.3. SOP F1.6.1 Toluidine Blue staining technique

Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Toluidine Blue	SOP nr	F1.6.1
Category	Procedures	Author	M Marais
Approved by HOD	C. Huysd.	Approved date	29/01/2015

Standard Operating Procedure:

Toluidine Blue staining technique

Contents

Aim

Bio-hazardous warning

Materials

Equipment

Method

    Preparing stains

    Staining

    Permanent preparation

    Evaluation

Notes

References

Aim

Toluidine Blue (TB) is a planar nuclear dye used to stain the metachromatic and orthochromatic of chromatin. The Toluidine Blue assay measures DNA integrity as free phosphate residues in the DNA backbone that will bind to the TB dye. Darkly stained cells have many binding sites, which indicate that there are structural errors in the DNA that allow the TB dye to bind. Lightly stained cells reveal structurally intact DNA.

Bio-hazardous warning

Tertiary butanol and **Xylene** (vapour and liquid) is highly flammable. Exercise caution in use, handling and disposal of the material and equipment exposed to the product.

Materials

Citric Acid (Sigma C-0759)

Na_2HPO_4 (Sigma S-5136)

Sterile water

96% Ethanol

Acetone (48358 *SUPELCO*)

Nigrosin powder (Sigma N-4763)

Hydrochloric acid (Sigma H-1758)

Toluidine Blue (Sigma T-3260)

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Tertiary butanol (Sigma 360538)

Xylene (Sigma 95670)

DPX (Sigma 06522)

Equipment

Light Microscope

Fume hood

Fridge (fridge next to fume hood)

Stage warmer

Method

Preparing stains

McIlvaines Buffer

- 0,1 M Citric Acid: 2,101 g in 100 ml sterile water

- 0,2 M Na_2HPO: 7,21 g in 200 ml sterile water

- **McIlvaines Buffer pH 3.5**: mix 33.9 ml Citric Acid + 16.1 ml Na_2HPO

- Store in sealed bottle in a fridge

0.1% Nigrosin stain

- Add 0.1 g of Nigrosin powder to 100ml sterile water

Store in a sealed bottle in a fridge (next to fume hood)

0.1N Hydrochloric acid (HCL)

- Add 1ml of HCl to 99 ml sterile water

- Store in a sealed bottle in a fridge (next to fume hood)

Fixative

- Add 96% ethanol to acetone in a ratio 1:1

- Store in sealed bottle in a fridge (next to fume hood)

Toluidine Blue staining solution

- Add **0.05g** of TB powder to **100ml** McIlvaines Buffer pH 3.5

- Stain can be stored for 1 year at 4˚C

Staining

- Make a semen smear (refer to Smear techniques SOP F1.12.1) and allow to air dry

- Place in fixative for 30 minutes at 4˚C, and allow to air dry

- Apply a weak nigrosin (0.1%) background stain and allow to air dry

    - Take air dried smear slide after fixations

    - Pipette 10µl of the 0.1% Nigrosin

    - Smear Nigrosin with cover slip over slide

- To hydrolyze, place the slides in a container with 0.1 N HCl for 15 minutes, in the fume hood at room temperature

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- Rinse slides in distilled water for 2 minutes
- To stain, place the slides in a container with the 0.05% TB solution for 15 minutes, in the fume hood at room temperature

Permanent preparation

- To dehydrate:
    - Place slides in tertiary butanol for 2 x 3 min at 37°C
    - Place 2 x 3 min in Xylene at room temperature
- Leave slides to air dry
- Mount slide using DPX:
    - Place drop of DPX on slide
    - Place cover slip and press on tissue to expel the excess DPX
- Slides can be stored indefinitely

Evaluation

- Slides are evaluated using a light microscope
- Place the slide onto the stage of the microscope
- Find a field in the stained area that has an evenly dispersed, surplus amount of spermatozoa under 20X magnification (Phase Contrast)
- Place a drop of immersion oil on the specific field and change to the 100 X phase contrast objective
- Focus and count 100-200 sperm, distinguishing between light blue and purple stained cells
    - Sperm with good chromatin integrity: light blue
    - Sperm with diminished integrity: deep violet (purple)
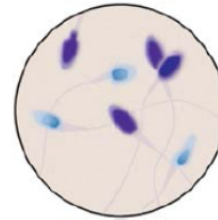
Notes

- All powdered reagents are stored alphabetically in the Chemical cabinet Q cart in the Biochemistry laboratory (room 72344)
- The other reagents are stored in the Chemical cabinet in the Biochemistry laboratory
- All stains and solutions are stored in the fridge in the Biochemistry laboratory
- When preparing stains and staining, work in the fume hood and at all times work aseptically with sterile equipment
- Warm tertiary butanol on a slide warmer (37°C), inside the fume hood

References

- In: Gardner DK, Weissman A, Howles CM, Shoman Z, eds. 2012 Textbook of Assisted Reproductive Technlues. 4th edition. Boca Raton. Taylor & Francis; Chapter 6; 80-1.

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7.4. Reporting of Results

Results obtained from the research were presented as posters and presentations at various conferences and meetings. Posters and presentations follow in this section in the order it was presented. At least two articles to be submitted to peer-reviewed journals are planned, with the first draft article presented as a last addendum. The list of addendums are as follows:

7.4.1. Presentation: tWE in Cape Town – Global access to infertility (2014)

From the 24th to 25th of November 2014, The Walking Egg foundation hosted a Conference on Reproductive Health: "*Global access to infertility care: The Walking Egg project*", which was held at Tygerberg Hospital, University of Stellenbosch, South Africa. At this conference, the current study was presented as a proposed research project, with the title: "*tWE Laboratory parameters evaluation: A proposal.*" Slides from this presentation can be seen in Addendum 7.4.1.

7.4.2. SASREG National Congress (2015)

On 30 October to 1 November 2015, at the national congress of the Southern African Society for Reproductive Medicine and Gynaecological Endoscopy, two sets of results from the research was presented. First, a poster titled: "*How many sperm to inseminate with? Results from a pilot study.*" was presented with an associated oral presentation on the last day of the conference. In Addendum 7.4.2.1., the poster as well as slides from this presentation can be found. This poster received an award (best abstract) in the junior student category. Second, a poster, titled: "*Set-up of a tWE basic ART laboratory*" was on exhibition, a copy of the poster is displayed in Addendum 7.4.2.2.

7.4.3. Oral presentation: Gauteng Special Interest Group Embryology (2016)

In June 2016, the Walking Egg foundation visited the Pentecost Fertility Centre in Accra, Ghana to assist in their procedures. Patients were seen and the simplified Walking Egg *in vitro* fertilization culture system used to culture embryos. The researcher accompanied the Walking Egg team and upon his return presented a talk on Ghana and the Walking Egg's involvement there at the bi-monthly Gauteng Special Interest Group: Embryology meeting. Slides from this presentation are in Addendum 7.4.3.

7.4.4. University of Pretoria, Faculty of Health Sciences faculty day (2016)

At the annual faculty day of the Faculty of Health Sciences, University of Pretoria, an oral titled "Sperm-Oocyte insemination during Assisted Reproduction – How low can you go?" was presented and slides of this presentation can be seen in Addendum 7.4.4. At the same time, a copy of the poster presented at the Southern African Society for Reproductive Medicine and Gynaecological Endoscopy National conference in 2015 (Addendum 7.4.2.2) was also on display.

7.4.5. Draft article – Facts, Views & Vision in ObGyn (2017)

The data gathered in section 1 of the current research (Quality control of the simplified tWE IVF culture system) is being compiled into an article to be submitted to Facts, Views & Vision in ObGyn by June 2017. A copy of the draft article as it is written so far is attached in Addendum 7.4.5.

7.4.1. Presentation: tWE in Cape Town – Global access to infertility (2014)

How many sperm to inseminate with?
Results from a pilot study

Gerhard Boshoff[1], Willem Ombelet[2] and Carin Huyser[1]

[1]Department of Obstetrics and Gynaecology, Reproductive Biology Laboratory, University of Pretoria, Steve Biko Academic Hospital, South Africa; [2]Department of Obstetrics and Gynaecology, Genk Institute for Fertility Technology, Ziekenhuis Oost Limburg, Belgium

Introduction

- Standard *in vitro* fertilization procedures employ the combined effect of multiple spermatozoa to dissociate cumulus cells surrounding the oocyte during insemination, attachment to the zona pellucida and ultimately fertilization of the oocyte.
- Supra or sub-minimum sperm concentrations per insemination can compromise fertilization outcome *in vitro*.
- The hemi-zona bioassay (HZA) was selected to portray the binding of spermatozoa to oocytes.
- Two identical zona halves are used with the HZA to compare test versus control sperm samples.

Aim

Determining the minimum number of spermatozoa that would still provide adequate sperm-zona binding as indicated by the hemi-zona bioassay.

Materials and Methods

- Semen samples from 5 donors was processed by double density gradient centrifugation and diluted to appropriate concentrations
- Unfertilized non-viable oocytes (n=107) were bisected using a micromanipulator where after the zona halves were isolated and randomly allocated to test and control fertilization media (50µl)
- Six different sperm concentrations (0.05, 0.1, 0.2, 0.5, 1.0 & 2.0 x 10^4) were tested against a control of 5.0 x 10^4 motile sperm (Figure 1)
- Incubating of zona and sperm was at 6% CO_2 for 18 hours
- The number of sperm bound to each zona half was counted independently by two qualified embryologists
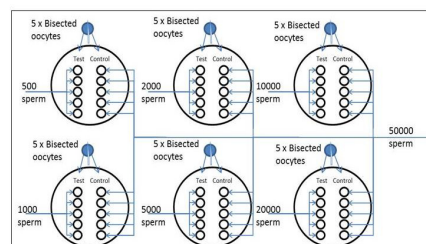
Figure 1: HZA with increasing sperm concentrations

Results

Control hemi-zonae with <50 bound spermatozoa were excluded, along with data from corresponding test hemi-zonae. The average number of sperm bound can be seen in Figure 2. Percentage of zonae with >20, >30 and >50 sperm bound is shown in Figure 3 and Figure 4 depicts the average number of sperm bound after 50 000 sperm insemination as correlated with sperm parameters for each donor.
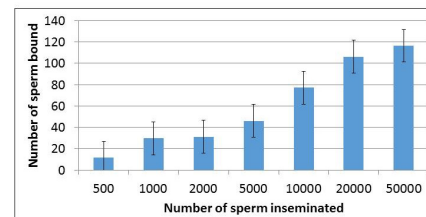
Figure 2: Average number of sperm bound to hemi-zonae vs. sperm inseminated during HZA
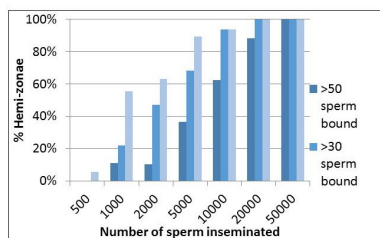
Figure 3: Percentage hemi-zonae with >20, >30 and >50 sperm bound for different insemination doses
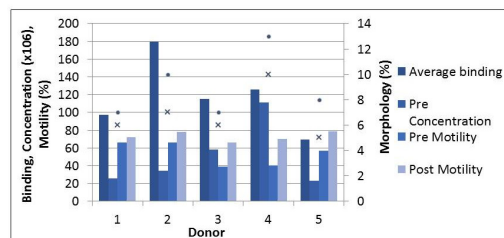
Figure 4: Sperm parameters vs. average sperm-zona binding (50 000 sperm)

Discussion

A direct association between insemination concentration and sperm bound to the zona pellucida was observed. Insemination with 0.5 x 10^4 spermatozoa has a 37% possibility to present with >50 spermatozoa bound to the hemi-zonae, and a 89% likelihood >20 bound spermatozoa when comparing to a standard insemination number of 5 x 10^4 spermatozoa. The average number of sperm bound to a hemi-zona after insemination with a dose of 0.5 x 10^4 spermatozoa was 46 ± 27.

It is possible that insemination with <0.05 x 10^4 spermatozoa may negatively influence fertilizing potential *in vitro*. Using a minimum of 20 sperm-zona binding as a cut-off indicator (Franken DR, Oehninger S *et al*. 1991), it is advisable that no less than 0.1 x 10^4 spermatozoa should be used for insemination.

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How many sperm to inseminate with?

Results from a pilot study

G Boshoff – University of Pretoria, South Africa
W Ombelet – The Walking Egg Foundation, Belgium
C Huyser – University of Pretoria, South Africa

01 November 2015 SASREG National Congress

Background

- Pilot study as part of MSc research (registered 2015)
- Determine minimum number of sperm for insemination
- Modified hemi-zona bio assay
- Semen donors (n=5)
  - G-Pattern morphology (Tygerberg Strict criteria[1])
  - Concentration >15x10^{6} (WHO 2010 lower reference limit[1])
  - Progressive motility >32% (WHO 2010 lower reference limit[1])
- Oocytes (n=107)
  - Non-fertilized IVF oocytes
- Cut-off control <50 sperm bound

Figure 1: Hemi-zona assay design[2]

1. World Health Organization (2010) Laboratory manual for the examination and processing of human semen 5th Ed.
2. Henkel R, Muller C et al. (1999) Use of failed-fertilized oocytes for diagnostic zona binding purposes after sperm binding improvement with a modified medium. Journal of assisted Reproduction and Genetics 16 (No. 1), 24 – 29.

Hemi-zona assay experimental design

Figure 2: Hemi-zona assay with increasing sperm concentrations

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Hemi-zona assay pilot study preliminary results

Table 1: Results from Hemi-zona assay pilot study

Number of sperm inseminated	500	1000	2000	5000	10000	20000	50000
>50 sperm bound	0%	11%	11%	37%	63%	88%	100%
>30 sperm bound	0%	22%	47%	68%	94%	100%	100%
>20 sperm bound	6%	56%	63%	89%	94%	100%	100%
Average bound	12	30	31	46	77	106	116

Figure 3: Average number of sperm bound to hemizonae

Figure 4: Percentage hemizonae with sperm bound for different insemination doses

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Conclusion

- 5000 sperm advised cut-off
- 1000 sperm absolute cut-off
- Lower ROS generation and less metabolites
- Semen with low concentration
- Continue study with semen with P-pattern morphology[1]

1. World Health Organization (2010) Laboratory manual for the examination and processing of human semen 5th Ed.

Thank you

Set-up of a ᵗWE basic ART laboratory

Gerhard Boshoff[1], Willem Ombelet[2] and Carin Huyser[1]

[1]Department of Obstetrics and Gynaecology, Reproductive Biology Laboratory, University of Pretoria, Steve Biko Academic Hospital, South Africa; [2]Department of Obstetrics and Gynaecology, Genk Institute for Fertility Technology, Ziekenhuis Oost Limburg, Belgium

Introduction

Parallel increases in Assisted Reproduction Technology (ART) regulations and specifications as well as growth in the commercial ART industry has occurred in the last decade, with a concurrent escalation in cost. The question arises what is the suitability and requirements of an ART procedure for its intended purpose or outcome? Deviations outside of the physico-chemical boundaries of culture media/conditions, i.e. osmolality, pH, temperature will impact negatively on clinical outcome. The Walking Egg (a non-profit organization), designed a ᵗWE lab ART method specific for low resource settings, whereby medical gases, complex incubation equipment and infrastructure are not required.

Aim

Investigating some of the physico-chemical parameters of the ᵗWE lab ART method, i.e. temperature and pH stability during culture.

Materials and Methods

Experiment 1:
- Stoppered glass tubes (n=150) with 1ml culture medium in aluminium blocks (Five tubes per block, 10 repeats) (Figure 1)
- Three heating devices generally used in an ART Laboratory set at set-point 37°C
  - Single surface heater – a slide warmer (Adamas SW85)
  - Closed - a warming oven (Quincy Lab Model 10-140E)
  - Partially closed - a dry bath (K-Systems DB-006)
- The temperatures of the culture media monitored in situ using a calibrated electronic thermometer (Greisinger Digital Thermometer GMH G230) equipped with a wire probe

Experiment 2:

Incubation of culture media with different CO_2 gas volumes
- Ten volumes of Citric Acid (CA) (1.2ml – 3.0ml at 0.2ml increments) added to excess amount bicarbonate of soda (BoS) ($C_6H_8O_7 + 3NaHCO_3 \rightarrow 3H_2O + 3CO_2 + Na_3C_6H_5O_7$)
- Sibling glass tubes with CO_2 generated connected via tubing to culture media containing tubes (Figure 2)
- Incubation of tubes at 37°C for 18H, pH of the culture media measured with blood gas analyser (Radiometer ABL 800 Flex)
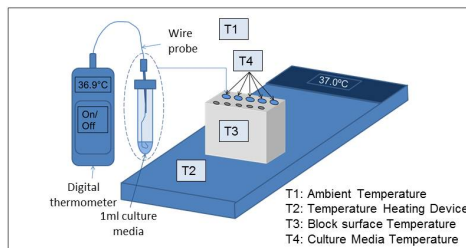
Figure 1: Experiment 1 setup

T1: Ambient Temperature
T2: Temperature Heating Device
T3: Block surface Temperature
T4: Culture Media Temperature
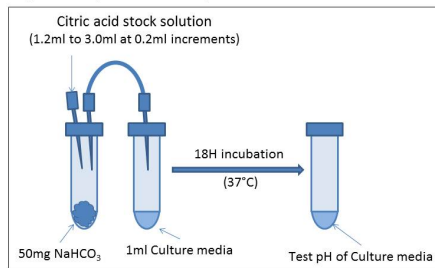
Figure 2: Experiment 2 setup
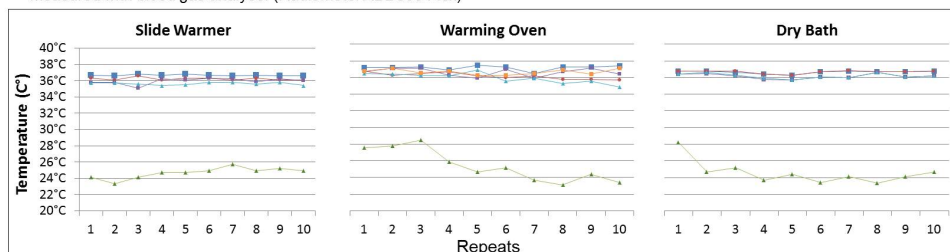
Figure 3: Temperature readings from different heating devices : ■ Media positions 1 and 2; ■ Block surface positions 1 and 2; ■ Incubator air; ■ Ambient air
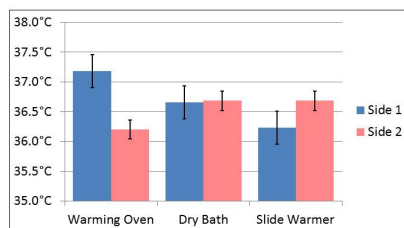
Figure 4: Average media temperature at two each of the three heating devices
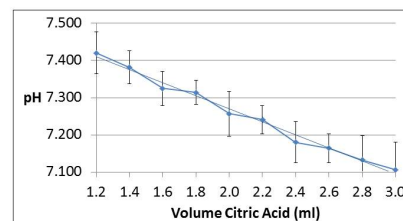
Figure 5: pH of culture media after gassing with CO_2 produced by increasing amount of CA added to BoS

Discussion

Optimal temperature regulation was achieved using the dry bath with blocks (Figures 3 & 4), which is a cost-efficient device. The fluctuation in temperature of the warming oven may be due to indirect heating through air, while direct heat transfer can be controlled more accurately using a slide warmer or dry bath. Temperature variances per temperature regulating device occurs more frequently than anticipated in the ART Laboratory.

Gassing of culture media with increasing volumes of CO_2 provides a linear decline in pH (Figure 5). The choice of media and location of the laboratory will impact on the setup of the culture system, with practical aspects influencing the pH values of culture media. The ᵗWE system design was addressed to prevent fluctuations, especially preventing leaking of CO_2 gas during embryo culture.

7.4.3. Oral presentation: Gauteng Special Interest Group Embryology (2016)

Training Genk, Belgium 2015

June 2016 Pentecost Hospital Fertility Centre, Ghana
Patient stimulation summary

Acknowledgements

Prof Willem Ombelet, ZOL, Belgium

Prof Carin Huyser, University of Pretoria, SA

Dr Nathalie Dhont, ZOL, Belgium

Jan Goossens, ZOL, Belgium

Ingrid Van der Auwera, Belgium

The Walking Egg Organization

IVF team, Accra, Ghana

Thanks !!

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7.4.4. Oral presentation: UP Faculty of Health Sciences faculty day (2016)

Zona pellucida sperm binding

Figure 7: Glycoprotein zona pellucida with bound spermatozoa

Background

- Pilot study as part of MSc research (registered 2015)
- Determine minimum number of sperm for insemination
- Modified hemi-zona bio assay
- Semen donors (n=5)
  - G-Pattern morphology (Tygerberg Strict criteria[1])
  - Concentration >15x10⁶ (WHO 2010 lower reference limit[1])
  - Progressive motility >32% (WHO 2010 lower reference limit[1])
- Oocytes (n=107)
  - Non-fertilized IVF oocytes
- Cut-off control <50 sperm bound
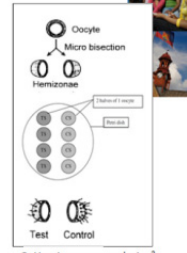
Figure 8: Hemi-zona assay design[2]

1. World Health Organization (2010) Laboratory manual for the examination and processing of human semen 5th Ed.
2. Henkel R, Muller C et al. (1999) Use of failed-fertilized oocytes for diagnostic zona binding purposes after sperm binding improvement with a modified medium. Journal of assisted Reproduction and Genetics 16 (No. 1), 24 – 29.

Experimental design
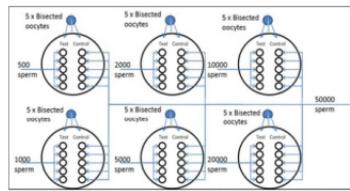
Figure 9: Hemi-zona assay with increasing sperm concentrations

Hemi-zona assay pilot study preliminary results

Table 1: Results from Hemi-zona assay pilot study

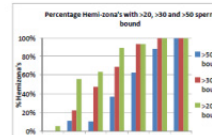| Number of sperm inseminated	500	1000	2000	5000	10000	20000	50000
>50 sperm bound	0%	11%	11%	37%	63%	88%	100%
>30 sperm bound	0%	22%	47%	68%	94%	100%	100%
>20 sperm bound	6%	56%	63%	89%	94%	100%	100%
Average bound	12	30	31	46	77	106	116

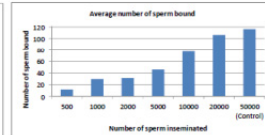Figure 10: Percentage hemi-zona's with sperm bound for different insemination doses

Figure 11: Average number of sperm bound to hemi-zona's

Conclusion

- 5000 sperm advised cut-off
- 1000 sperm absolute cut-off
- Lower ROS generation and less metabolites
- Semen with low concentration
- Continue study with semen with P-pattern morphology[1]

1. World Health Organization (2010) Laboratory manual for the examination and processing of human semen 5th Ed.

Thank you

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7.4.5. Draft article – Facts, Views & Vision in ObGyn (2017)

Title

Draft article – Setting up a tWE laboratory

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Abstract

Abstracts should not exceed 250 words clearly summarizing the findings of the manuscript. Note that online abstracts are published for viewing in isolation to the main body of the manuscript and should be self-explanatory.

Key words

Affordable IVF; Laboratory setup; Assisted Reproduction; Simplified Infertility treatment; Accessible IVF; Low-cost IVF; The Walking Egg Project

Introduction

New ideas are always looked upon with suspicion. Designing a new, innovative method of culturing human embryos without the use of an incubator, regulated gas or filtered air laboratory (Van Blerkom *et al.* 2013) definitely falls into this category. An IVF laboratory should be geared towards minimalizing environmentally induced stress and maximizing assisted reproductive outcome (Swain, 2014). Does the continuously developing and improving assisted reproductive technology (ART) laboratory provide for these demands? Embryologists would like to believe so. At least as close as possible with current technology, with a constant strive to always improve (Boone, *et al.*, 2010; Swain, 2013; Swain, 2014). This perpetual search for better reproductive outcome comes at a cost, literally. Laboratory costs can attribute up to 35-48% of ART fees payable by patients (Huyser & Boyd, 2013).

Less than 20% of the global and approximately 1% of the projected infertility treatment need in developing countries is being met according to Vayena *et al.* (2009). Childlessness is especially burdensome for lower income populations from developing countries, where assisted reproductive services are limited and expensive (Agarwal *et al.* 2015, Gerrits 2012, Inhorn and Patrizio 2015). A need exists for the cost of ART to be reduced in order to increase accessibility to the services rendered (Hammarberg and Kirkman, 2013; Vayena, *et al.*, 2009; Teoh and Maheshwari, 2014).

The Walking Egg ([t]WE) Foundation focuses on advancing affordable and accessible reproductive health treatment worldwide (Dhont, 2011, Ombelet, 2013, www.thewalkingegg.com). The development of the [t]WE lab system forms part of their affordable treatment plan (Van Blerkom *et al.* 2013). The lab system was designed to provide a basic, but optimal environment for human embryo culture without the use of complex high-tech incubation equipment, medical gasses and infrastructure typical of IVF laboratories in high resource settings (Van Blerkom *et al.* 2013). Key parameters considered were the provision of a culture environment with culture media at the desired temperature and pH, without risk of external influences on air quality and media osmolality. This culture environment must be created at a reduced cost, but without compromising on any of the parameters mentioned (Van Blerkom *et al.* 2013).

The setup of a ^{t}WE lab system in a developing country was investigated at a tertiary academic institution in South Africa to evaluate environmental variables when utilizing the culture system. Performing the investigation at this institution provided a platform that is situated in a developing country, therefore has experience of the challenges of providing ART services in such a setting. The laboratory at the institution is fitted with similar equipment as found in laboratories from developed countries and made it possible to perform a direct comparison between an established ART laboratory and the ^{t}WE lab system. Parameters that were evaluated included temperature control by various methods as well as the effect on culture media pH by location, addition of water to citric acid and gassing temperature. Ethical approval for the study was provided by the University of Pretoria Research Ethic Committee on 26/11/2015 with reference number 460/2015.

Materials and Methods

1. Temperature measurements

All experiments were performed at the Steve Biko Academic Hospital in Pretoria, South Africa unless indicated otherwise. Altitude at the laboratory is 1326 meters above sea level and ambient temperature was regulated between 22 and 26°C. The culture media tubes with stoppers supplied for the ^{t}WE lab system (referred to as "^{t}WE tubes") were kept at 37°C using aluminium blocks. These blocks were specifically designed and manufactured for the ^{t}WE lab system to maintain optimal culture temperature when heated to 37°C. The blocks fully encompass the ^{t}WE tubes to avoid condensation.

Six heating devices (Quincy Lab Model 10-140E Incubator; K-Systems Dry bath DB-006; Adamas Slide Warmer SW85; Labcon CPM 200 Waterbath; Thermo Electron Corporation Forma Series II Water Jacketed CO_2 incubator (no CO_2 gas connected) and K-Systems Mobile IVF workstation L13) were used to heat the blocks to 37°C. Global® Total® for Fertilization (LifeGlobal® Group, Guilford, CT, USA; www.LifeGlobal.com) culture media (1 ml) was injected into the ^{t}WE tubes (n=60, provided by The Walking Egg CVBA) using a calibrated 1000 µl

pipette (Thermo Scientific Finnpipette F2 100-1000 µl) and sterile pipette tips (Thermo Scientific Finntip 1000) and the tubes were closed off.

The temperature of the media inside the tubes was measured by passing a wire probe through a 16 gauge needle inserted through the stopper (Figure 1). The wire probe was connected to a calibrated electronic thermometer accurate to one decimal (Greisinger Digital Thermometer GMH G3230). The tubes were kept *in situ* in the warming block when temperatures were measured, ensuring accurate measurements while simulating actual culture conditions. Each warming device contained two blocks with five ^tWE tubes each (n=10) to determine variance between warming blocks heated by the same device as well as differences between devices. The following temperatures were measured (Figure 1): T1 - ambient temperature, T2 - actual temperature of the heating device, T3 - warming block's surface temperature and T4 - temperature of media in each of the five tubes in the block. All measurements were repeated three times a day for three days to determine the stability of temperature for each device over the experimentation period.
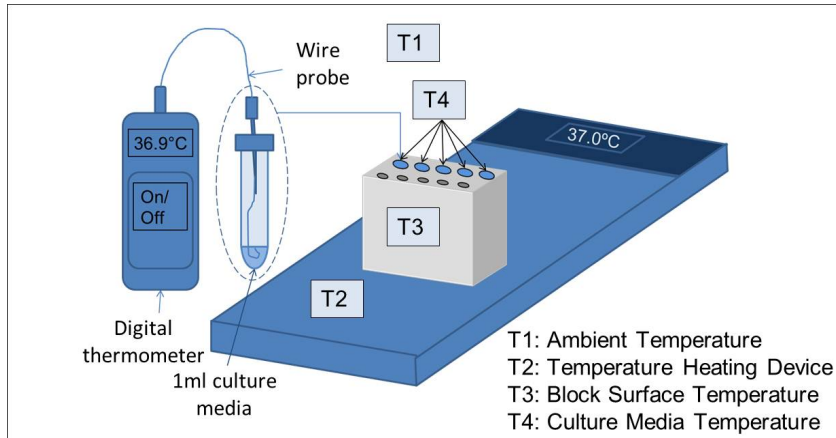

Figure 1: Diagram of different temperature measurements taken for each heating device.

2. pH measurements

The pH of culture media in the ^tWE tubes is influenced by injection of CO_2 gas. The CO_2 gas is aseptically produced by the reaction of citric acid (CA) and bicarbonate of soda (BoS) ($C_6H_8O_7 + 3NaHCO_3 \rightarrow 3H_2O + 3CO_2 + Na_3C_6H_5O_7$) (Van Blerkom et al. 2014). Different sub-studies were performed to fully investigate pH values under different conditions. Firstly a basic setup at a tertiary academic institution in South Africa was performed to compare the amounts of CA

needed to produce the pH of the culture media similar to the [t]WE lab system standard. The information gathered was used to better understand the interactions of CA and BoS to affect culture media pH and therefore indirectly the results from the sub-studies. The first sub-study was an investigation into the necessity of diluting CA with water in order to have an exact 3 ml volume in the gassing tube. The second and last sub-studies evaluated the pH of culture media when gassed at 37°C vs. room temperature and compared pH values obtained at different altitudes, respectively.
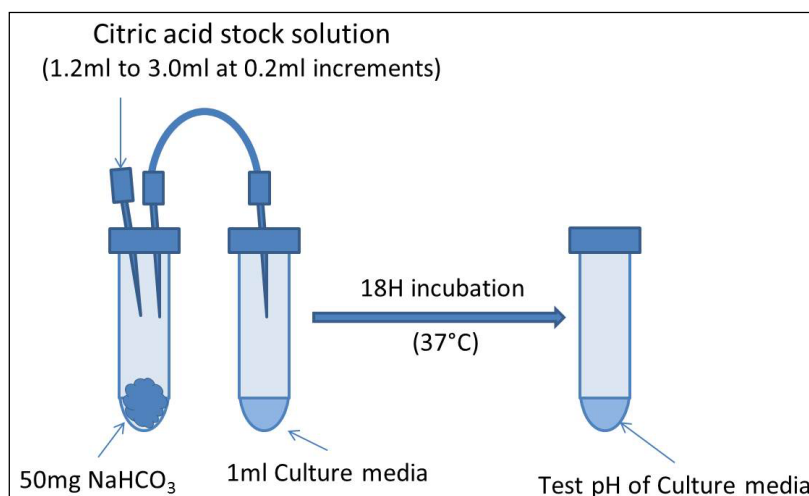

Figure 2: Image indicating the experimental design to test culture media.

2.1. Determining citric acid volume

According to the design of the [t]WE lab system, an excess amount of BoS mixed with 3 ml CA (chemicals and formula for CO_2 generation supplied by The Walking Egg CVBA, Genk, Belgium) produced the necessary amount of CO_2 gas to reach an optimal pH of 7.30 ±0.05 at The Ziekenhuis Oost-Limburg Campus Sint-Jan (site of the prototype [t]WE lab system testing).

Sets of [t]WE tubes (n=100: 10 sets of 10 tubes each) with 1 ml Global® Total® for Fertilization culture media were connected via connection tubes and needles to adjacent [t]WE tubes (Figure 2) containing a BoS pellet as described by Van Blerkom *et al.* (2014). Each set of tubes containing BoS were injected with increasing volumes of CA (concentration as supplied by The Walking Egg CVBA, 1.2 – 3 ml in 0.2 ml increments) and CA/BoS volumes were toped up to a total of 3

ml using sterile water. The ^{t}WE tubes were then placed in a warming block at 37°C for an 18 hour incubation period to allow for the formation and movement of CO_2 gas. After incubation, the needles were removed from the ^{t}WE tubes, and kept at 37°C. A 300 µl sample of the media was withdrawn from the ^{t}WE tube, using a syringe with a needle, and the pH was measured directly on a calibrated blood gas machine (Radiometer ABL 800 Flex; accurate to three decimals with the ability to compensate for temperature difference if the sample is not at 37°C). The data gathered was used to determine the volume of citric acid needed to obtain a pH of 7.30 ±0.05 in the culture media.

2.2. Sub-study 1: Comparing citric acid diluted with and without water

The same experimental setup as described in 2.1. was repeated to investigate the use of water to dilute CA. Six sets of ten ^{t}WE tubes (n=60) with BoS and three volumes of CA (1.4, 1.7 and 2.0 ml – one set of tubes test and control each for different volumes) were used to produce CO_2 gas. The CA was diluted with sterile water to a final volume of 3 ml for the control samples or used without dilution for test samples. The pH of the culture media after 18 hours of incubation was measured with a portable pH meter (ICRI5209, Crison, Barcelona, Spain) connected to a glass, double junction, KCl filled pH probe (5029, Crison, Barcelona, Spain, accurate to two decimals).

2.3. Sub-study 2: Media gassing at different temperatures

The same experimental setup as described in 2.1. was again utilised to determine if media gassing would be possible at ambient temperature instead of 37°C. Thirteen sets of ten ^{t}WE tubes (n=130) with a CA volume of 1.7 ml was used to produce CO_2 gas. Four sets of tubes were kept at 37°C for 16, 18, 24 and 30 hours respectively (control). Six sets of tubes were incubated at 25°C for 16, 18, 20, 22, 24 and 30 hours respectively (test 1) and three sets of tubes were incubated at 15°C for 18, 25 and 30 hours respectively (test 2). After gassing for the indicated time periods, tubes with CA/BoS mixture and culture media were disconnected from each other. The tubes with culture media were heated to 37°C, by placing in heated aluminium blocks for 30 minutes, before pH was measured with the portable pH meter described previously. The pH results were compared to

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explore the necessity of maintaining a heated environment during gassing of culture media.

2.4. Sub-study 3: Altitude change and pH

Five national locations across South Africa at different altitudes (30, 350, 925, 1326 and 1627 meters above sea level) were visited, and a comparison of media pH after gassing with increasing volumes of CA was performed. Thirty sets of ten [t]WE tubes, with each set gassed with CO_2 produced by increasing CA volumes (1.2 to 2.2 ml at 0.2 ml increments) at each of the five locations (5 locations x 6 sets per location: n=300) were tested. Incubation was at 37°C for 18 hours for all sets and pH was measured with the portable pH meter described. Results were compared to determine whether CA volumes should be adjusted according to a change in altitude.

Results

1. Temperature measurements

Temperature values for each warming device were compared using a two-sample t-test with equal variances and a 95% confidence interval was determined. Mean temperature and 95% confidence interval values for each warming device (two placement positions per device) are displayed in Table 1 with a summarised display of device, block and media temperature in Figure 3.
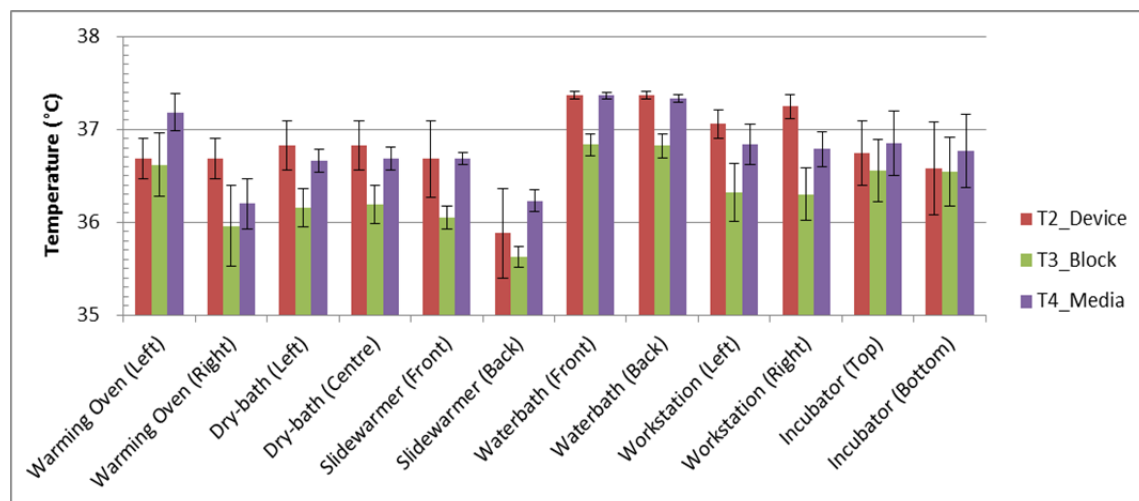

Figure 3: Mean temperatures of culture media, block surface temperature and device temperature as measured on two placement positions per device.

Table 1: Mean temperatures with 95% confidence interval (T1: Ambient temperature, T2: Measured device temperature, T3: Surface temperature of aluminium block and T4: Temperature of culture media in tWE tube) of six devices (two positions per device)

Equipment	Position	T1: Ambient		T2: Device		T3: Block		T4: Media	
		Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Warming Oven	Left	25.43	24.04-26.82	36.68	36.46-36.90	36.62	36.28-36.96	37.18	36.98-37.39
	Right	25.43	24.04-26.82	36.68	36.46-36.90	35.96	35.52-36.4	36.20	35.93-36.47
Dry-bath	Left	24.58	23.57-25.59	36.83	36.56-37.10	36.16	35.95-36.37	36.66	36.53-36.79
	Centre	24.58	23.57-25.59	36.83	36.56-37.10	36.19	35.98-36.4	36.68	36.56-36.81
Slide warmer	Front	24.65	24.17-25.13	36.68	36.26-37.10	36.05	35.92-36.18	36.68	36.62-36.75
	Back	24.65	24.17-25.13	35.88	35.40-36.36	35.63	35.52-35.74	36.23	36.11-36.35
Waterbath	Front	25.26	24.67-25.84	37.37	37.33-37.41	36.83	36.71-36.95	37.36	37.33-37.39
	Back	25.26	24.67-25.84	37.37	37.33-37.41	36.82	36.70-36.95	37.33	37.29-37.37
Workstation	Left	26.81	26.29-27.33	37.06	36.90-37.21	36.32	36.01-36.64	36.84	36.63-37.05
	Right	26.81	26.29-27.33	37.24	37.12-37.37	36.30	36.02-36.58	36.79	36.60-36.98
Incubator	Top	26.43	26.05-26.82	36.74	36.40-37.09	36.56	36.22-36.90	36.85	36.50-37.20
	Bottom	26.43	26.05-26.82	36.58	36.08-37.08	36.54	36.17-36.92	36.77	36.37-37.16

A Pearson's correlation test was performed to determine if there is a correlation between media temperature and the other parameters (block, device and ambient temperature). The results of this test are shown in Table 2.

Table 2: Correlation of culture media vs. block, device and ambient temperatures.

	T1: Ambient		T2: Device		T3: Block	
T4: Media	R	p	R	p	R	p
Warming Oven	0.25	0.282	0.02	0.947	0.74	0.000
Dry-bath	0.28	0.229	0.08	0.749	0.68	0.001
Slidewarmer	-0.12	0.612	0.51	0.020	0.70	0.001
Waterbath	0.25	0.309	0.58	0.011	-0.54	0.021
Workstation	-0.48	0.044	-0.07	0.782	0.07	0.768
Incubator	-0.33	0.183	0.77	0.000	0.95	0.000

Pearson's correlation coefficient(R) (0.1-0.3: small, 0.3-0.5: moderate, >0.5: strong correlation)
Statistical significance (p) (<0.05 significant)

2. pH measurements

2.1. Determining citric acid volume

Production of CO_2 by increasing volumes of CA (1.2 to 3.0 ml increasing with 0.2 ml increments) resulted in decreasing pH values of culture media. The average pH values were 7.42, 7.38, 7.32, 7.31, 7.26, 7.24, 7.18, 7.16, 7.13 and 7.11 respectively for different CA volume groups (Figure 4). A Bartlett's test for equal variances confirmed that the variances between groups were equal.
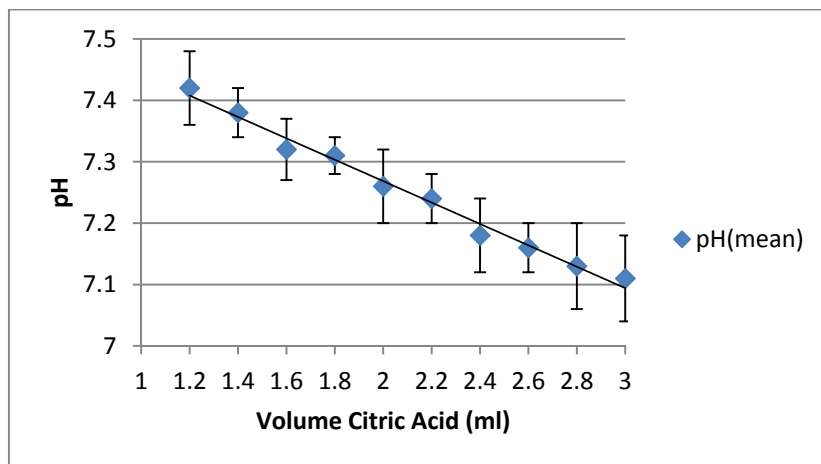

Figure 4: Mean pH values resulting from increasing CA volumes (1.2-3.0 ml at 0.2 ml increments)

2.2. Sub-study 1: Comparing citric acid diluted with and without water

Production of CO_2 by the reaction of increasing volumes of CA (1.4, 1.7 and 2.0 ml) diluted with water (control) or without dilution (test) presented mean pH values of 7.296, 7.287 and 7.24 (control), as well as 7.141, 7.163 and 7.202 (test), respectively. A linear prediction of pH values with 95% confidence intervals is presented in Figure 5.
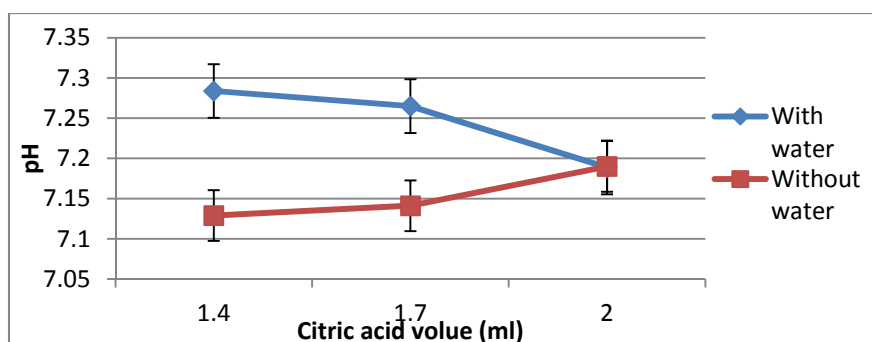

Figure 5: Linear prediction of pH values when gassing media by CO_2 produced with and without the addition of water.

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2.3. Sub-study 2: Media gassing at different temperatures

Gassing of culture media for increasing times (16, 18, 20, 22, 24 and 30 hours) by CO_2 produced with 1.7 ml CA, at different temperatures (15°C, 25°C and 37°C) produced mean pH values of: 7.40, 7.36, 7.30, 7.30, 7.27 (15°C at 18, 20, 22, 24 and 30 hours); 7.35, 7.26, 7.28, 7.27, 7.28, 7.27 (25°C at 16, 18, 20, 22, 24, 30 hours) and 7.31, 7.28, 7.29, 7.29 (37°C at 16, 18, 24 and 30 hours). A linear prediction of pH values with 95% confidence intervals is depicted in Figure 6.
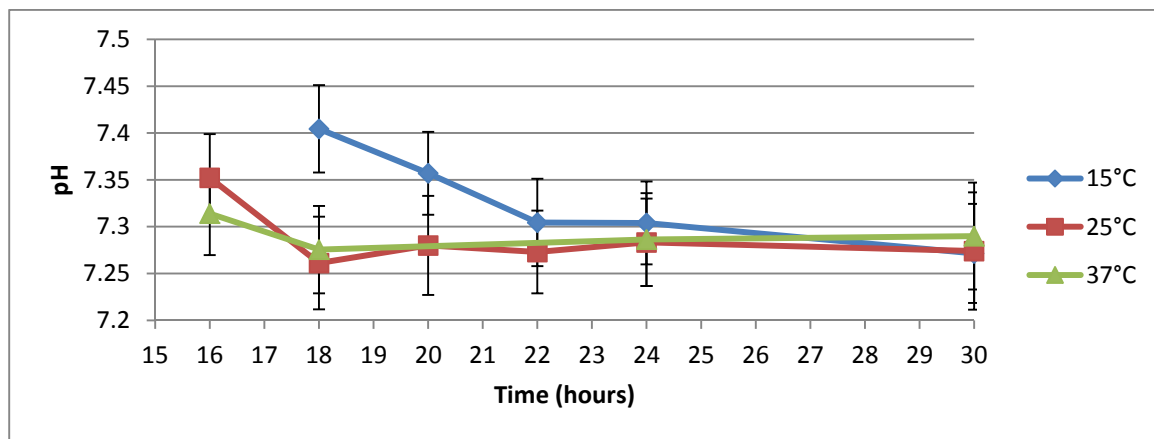

Figure 6: Linear prediction of pH values over time, media gassed at different temperatures.

2.4. Sub-study 3: Altitude change and pH

Mean pH values of culture media after gassing with CO_2 produced by increasing CA volumes (1.2-2.2 ml with 0.2 ml increments) at different altitudes (30, 350, 925, 1326 and 1627 meters above sea level) is displayed in Table 3. A linear prediction of mean pH values at each altitude can be seen in Figure 7.
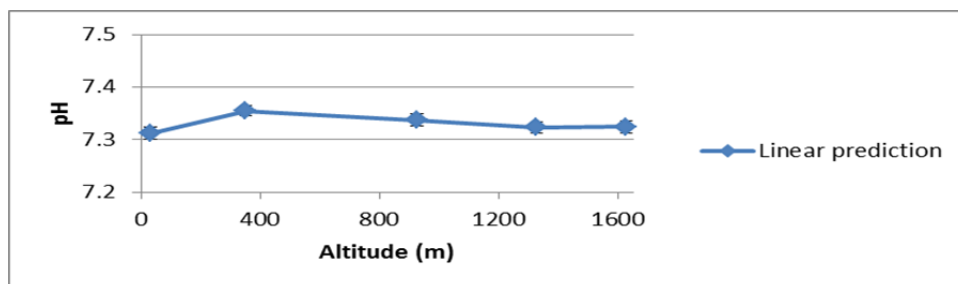

Figure 7: Linear prediction of mean pH values with 95% confidence intervals when gassing culture media at different altitudes.

Table 3: Mean pH values of culture media after gassing with CO_2 produced by increasing CA volumes at increasing altitudes.

Volume CA (ml)	Altitude (m)									
	30		350		925		1326		1627	
	pH (mean)	SD	pH (mean)	SD	pH (mean)	SD	pH (mean)	SD	pH (mean)	SD
1.2	7.445	0.018	7.482	0.030	7.455	0.021	7.449	0.024	7.431	0.041
1.4	7.390	0.035	7.391	0.023	7.394	0.026	7.397	0.026	7.350	0.017
1.6	7.339	0.035	7.349	0.018	7.340	0.029	7.330	0.039	7.323	0.032
1.8	7.232	0.029	7.279	0.020	7.310	0.027	7.227	0.050	7.327	0.034
2	7.157	0.044	7.270	0.035	7.185	0.007	7.242	0.038	7.220	0.041
2.2	7.160	0.000	7.260	0.014	7.220	0.000	7.160	0.052	7.168	0.045

Discussion

1. Temperature measurements

Constant media temperature is imperative during culturing of human embryos. Although the heating devices were set at 37°C, a drift from 37°C was seen from all of the devices and a difference in temperature at different places in/on the heating devices was also observed. A difference in temperature between areas in the same device was particularly noticed in the warming oven, slide warmer and workstation. When focussing on culture media temperature, all devices had a 95% confidence interval with a less than 0.8°C range and the slidewarmer, waterbath and dry bath had a 95% confidence interval with a less than 0.5°C range. Media temperature showed a strong correlation that was statistically significant with block temperature on all devices except the IVF workstation, although the block temperature was seen to be lower than both media temperature (average 0.47°C) and device temperature (average 0.5°C) for all devices. Device temperature also showed strong, significant correlations with media temperature for the slide warmer, waterbath and incubator. Ambient temperature did not have a significant correlation with media temperature for any of the heating devices except the IVF workstation. The large surface area of the IVF workstation is likely to be the reason for the correlation.

It is very important to understand equipment in one's laboratory and ensure calibration according to manufacturer's guidelines. Warm and cold areas in/on the heating device should be considered when placing blocks of [t]WE tubes for either embryo culture or gassing of tubes. Block surface temperature should not be used as a temperature indicator of culture media. A measured temperature of the heating device or if possible the actual measured temperature of culture media in a [t]WE tube in an aluminium block for QC purposes should be considered.

2. pH measurements

2.1. Sub-study 1: Comparing citric acid diluted with and without water

The standard practice of diluting CA with water to a set volume provides a specific volume of air in the [t]WE tubes that has to be filled with CO_2 gas. Measured pH values of diluted CA tubes were much higher than corresponding tubes that were not diluted. This was particularly observed in the tubes with lower amounts of CA, even though there is more air to be replaced with CO_2. Without water dilution, it appears as if the CA/BoS mixture has a more effective CO_2 production. The increased CO_2 production shows decreased culture media pH. Standardising the protocol to a set volume is essential and should not be varied upon, however it may be possible to reduce the standardised amount of CA to be used and use smaller volumes of CA without dilution with water.

2.2. Sub-study 2: Media gassing at different temperatures

Varying temperatures when gassing culture media showed a difference in culture media pH and a trend can be seen when this change in pH is also compared over time. Similar pH values are observed when values from approximately 4-6 hours longer gassing at 15°C than at 37°C is considered. This provides an avenue to consider for future research. Gassing the [t]WE tubes with culture media at room temperature will reduce the space needed in/on heating devices, which can then be dedicated to embryo culture.

2.3. Sub-study 3: Altitude change and pH

Due to the known effect of air pressure on the dissolving of CO_2 gas into culture media, it is necessary to adjust CO_2 levels according to altitude to obtain the same pH in culture media (Quinn and Cooke 2004). The pH values measured did not

appear to be influenced by a change in altitude, which was an unexpected outcome. It is theorised that a high pressure environment is created by the CO_2 production in the closed system of the two connected ^{t}WE tubes and the change in ambient pressure due to change in altitude is too small to have an effect on the final pressure and partial pressure of CO_2 in the tubes. A change in altitude therefore does not have to be considered when setting up a ^{t}WE culture system.

Conclusion

From the results obtained it can be concluded that the ^{t}WE lab system can affect culture media temperature and pH values conducive to human embryo culture. The device used to warm culture media must be carefully selected to be stable and accurate, but can be a flat surface heating device or a device that partially or fully contain the aluminium blocks. The culture media pH is not affected by a change in altitude and it should be possible to gas media at room temperature, but only when gassed for an increased time period. The volume of CA to be used should not be changed and if the protocol states that CA should be diluted with water, this should be adhered to.

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