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+ Review of ESHRE 2019

Vienna, Austria

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"As always, this congress was packed full of ground-breaking research and facilitated the sharing of knowledge across the field."

Spencer Gore, CEO

Editorial Board

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Aims and Scope

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Front cover and contents photograph: Vienna, Austria, home of the ESHRE 2019. © Brian Kinney / 123rf.com

EMJ Repro Health.

European Medical Journal 4.2 2019

By including articles from across the therapeutic spectrum, we hope to create fertile ground for the flowering of fresh ideas that will advance scientific knowledge and patient care.

VIEW ALL JOURNALS ←

Welcome

This year, the EMJ team travelled to Austria's historic capital city of Vienna for the fantastic European Society of Human Reproduction and Embryology (ESHRE) Congress. As always, this congress was packed full of ground-breaking research and facilitated the sharing of knowledge across the field. Our newest edition of *EMJ Reproductive Health* contains our review of this incomparable congress along with our peer-reviewed articles, history of contraception infographic, and interviews with industry experts.

Those who attended ESHRE this year know how difficult it is to see all of the fascinating sessions and thought-provoking abstract and poster presentations. Our review includes a hand-picked selection of abstracts from the congress, providing summaries penned by the authors themselves to give you a first-hand account. These cover a range of topics, including intracytoplasmic sperm injections in egg-sharing donation programmes, and anti-Müllerian hormone as a quantitative and qualitative marker of euploid blastocysts.

Along with our abstract summary section, we have our congress stories which delve into many topics, such as the effect of paternal smoking on offspring semen quality and the existence of a male biological clock. One story of particular interest looks at the quality and condition of sperm that is stored in outer space conditions. Additionally, this issue includes a range of congress features, including an interview with Dr Sarah Jarvis MBE on the panel she facilitated at ESHRE. Dr Jarvis explores social egg freezing, looking at the pressures on women to have children at a certain age, and the importance of fully educating women on their individual fertility windows.

EMJ Reproductive Health also showcases our usual array of peer-reviewed articles, including Tandulwadkar et al.'s paper 'Optimising the Outcome of Embryo Transfer', El Mahdi et al.'s paper on 'Fibroids and Infertility', and our Editor's Pick: 'Fertility Preservation in Women with Endometriosis: It is About Time We Talk About it!' by Carneiro et al.

Thank you to all our contributors for the fantastic range of papers, abstracts, and interviews, as well as to our peer reviewers and editorial board members who make this journal possible.

We hope to see you at next year's ESHRE congress in Copenhagen, Denmark, and please do get in touch with any thoughts on the journal.

Spencer Gore

Chief Executive Officer, European Medical Group

Foreword

Dear colleagues,

It is my pleasure to welcome you to *EMJ Reproductive Health 5.1*.

The ESHRE 2019 meeting was held in Vienna, Austria, from the 23rd–26th of June. The meeting was incredibly successful, with >12,000 participants coming from all over the globe, confirming the primary role of ESHRE meetings as a unique arena for scientists and operators of reproductive health to meet and exchange opinions and experiences. The programme this year, as usual, considered all the main themes regarding natural and assisted reproduction, with a particular focus on fertility preservation in women and men undergoing gonadotoxic treatments. The presentation from Dr Huang, which was very interesting and, I would add, reassuring, demonstrated that live birth can be obtained with spermatozoa preserved in the bank for a long time, reporting no differences in live birth rates using semen samples cryopreserved from 6 months or 5, 6, 10, and even 15 years in a large cohort of 119,558 donors. Dr de Geyter reported the latest annual (2016) data collected by ESHRE from European national registries. The data demonstrates a further rise in the cumulative use of *in vitro* fertilisation (IVF) in the treatment of infertility. However, it seems that the success rates after IVF or intracytoplasmic sperm injection have reached a peak, with pregnancy rates per started treatment calculated at 27.1% after IVF and 24.3% after intracytoplasmic sperm injection. According to the report, the use of frozen embryo cycles appears also to increase in Europe. Another important topic addressed during the meeting concerned the effect of air pollution on fertility. Prof La Marca presented results of the study "Ovarian Reserve and Exposure to Environmental Pollutants (OREXPo study)". He showed that many environmental chemicals, as well as natural and artificial components of the diet, have the potential to disturb the physiological role of hormones, interfering with their biosynthesis, signalling, or metabolism. An interesting result concerned levels of anti-Müllerian hormone found inversely related to environmental pollutants in women participating in the study, independent of age.

This edition of *EMJ Reproductive Health* contains a compendium of interesting peer-reviewed articles encompassing several important topics related to reproductive health, which I am confident you will enjoy.

Kind regards,

Elisabetta Baldi
University of Florence, Italy


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Congress Review

Review of the 35th European Society of Human Reproduction and Embryology (ESHRE) Congress 2019

Location: Reed Messe Wien Congress and Exhibition Centre, Vienna, Austria

Date: 23rd – 26th June 2019

Citation: EMJ Repro Health. 2019;5[1]:10-19. Congress Review.

Innovation and forward-thinking are not new concepts for the Austrian capital of Vienna; the city has been continuously producing a steady stream of brilliant minds for centuries. When we consider esteemed names such as Schrödinger, Asperger, Freud, and Schubert, we must also draw on them for inspiration in the disciplines that we partake. Reproductive health is increasingly vital for physical, mental, and socio-economic reasons, meaning that inspiration is more important than ever towards developing sustainable solutions that can change people's lives for the better. As such, we are proud to present this review of the 35th ESHRE Congress held in the stunning city Vienna.

Prof R.G. Edwards and Dr J. Cohen, from the UK and France, respectively, were the first pioneers to conceive the idea for ESHRE at a meeting in Helsinki, Finland, with their colleagues from the international reproductive health community. They expressed the need, from both patients and researchers, for the formation of a society in which the values of collaborative study and sharing of ideas are instilled for the betterment of the field. In 1985, following the small beginnings of several informal meetings, it was decided that the official annual meeting was to be held in Bonn, Germany. Since this time, the society's congresses have grown to become some of the largest gatherings of reproductive health specialists in the world.

There was no shortage of engaging stories of advancement in the field presented at this year's event. Perhaps most ambitious was a study involving the analysis of sperm characteristics in microgravity conditions. By boldly claiming that the research community has an obligation to realistically consider the feasibility of reproduction in space, and perhaps even colonisation of other planets, a precedent was set at the congress to dream big and push the envelope. Results from the OREXPO study were shared for the first time showing the effects that environmental influences such as air pollution have on female fertility markers: an especially

important study considering the increased urbanisation of human populations. Additionally, the delineation of a male biological clock in another study truly represents a paradigm shift in how reproductive success can be measured, highlighting the importance of the information we share with men considering parenthood.

As always, we have included a hand-picked selection of abstracts that caught our attention, which are penned by the authors themselves to provide first-hand summaries for your reading pleasure. A wide variety of important topics are covered, including but not limited to the influence of paternal age and ejaculatory abstinence length on intracytoplasmic sperm infection success, the identification of low β human chorionic gonadotropin concentration as an indicator of poor perinatal outcome in women, and the predictive value of the Sperm Zona-Adhesion Score for implantation outcome. These, along with the other fantastic summaries, are testament to the high calibre of work being presented at ESHRE.

Our congress review also includes a look at some of the thought-provoking sessions from ESHRE, such as 'The pressure paradox: can egg freezing tune out the tick tock of the biological clock?' facilitated by Dr Sarah Jarvis MBE, which looked the impact social egg freezing is having on age and pregnancy. Dr Jarvis, as our featured congress interview, gives us insight into what led her to become a general practitioner, as well as exploring the ESHRE session, highlighting the importance of educating women earlier on their fertility options and their individual fertility windows.

ESHRE 2019 was, as always, an incomparable event for those with a vested interest in reproduction and embryology. The team here at the European Medical Journal look forward to the 36th annual ESHRE meeting 2020 amongst the hustle and bustle of Copenhagen, Denmark, which is sure to be another unmissable event for the sharing of knowledge and best practice. Without further ado, we hope you enjoy our review of ESHRE 2019's congress highlights.

"Perhaps most ambitious was a study involving the analysis of sperm characteristics in microgravity conditions."

ESHRE 2019 REVIEWED →

"In the context of this emerging evidence for the deleterious effect of increasing paternal age, our data certainly support the importance of educating men about their fertility and the risks of delaying fatherhood."

Study Supports the Existence of the Male Biological Clock

THE BIOLOGICAL clock of women stops their fertility in its tracks at an average age of 51. For many years, despite known declines in sperm counts and increased sperm DNA damage, it was thought that fertility in men did not have such time restrictions. However, Dr Guy Morris, Centre for Reproductive and Genetic Health, London, UK, presented results on 26th June at ESHRE 2019 that suggested that men do indeed possess a biological clock.

The analysis from 4,833 *in vitro* fertilisation and intracytoplasmic sperm injection cycles performed at a single centre in London, UK, investigated patterns in age of males and outcomes of procedures. The male partners were grouped into five age groups for analysis: ≤35, 36–40, 41–44, 45–50, and >51 years of age. A male and female each aged <35 years were used as reference control groups for comparison.

Significant declines in success rates of procedures were observed as the age of the males in the groups increased: <35 = 49.9%, 36–40 = 42.5%, 41–45 = 35.2%, 46–50 = 32.8%, and >51 = 30.5%. Even when adjusted to take into account the maternal age, the probability of pregnancy still decreased significantly with paternal age >51 years. Despite this drop in success, miscarriage rate was not affected by male age in this study. Interestingly, it was noted that 80% of the cohort's couples with male partners >51 years of age were treated with intracytoplasmic sperm injection, a treatment for male infertility.

Dr Morris concluded, "In the context of this emerging evidence for the deleterious effect of increasing paternal age, our data certainly support the importance of educating men about their fertility and the risks of delaying fatherhood."

Study Confirms Added Obstetric Risk with Blastocyst Transfer

BLASTOCYST transfer has become a routine procedure for *in vitro* fertilisation and intracytoplasmic sperm injection. This increase in embryo culture time of 5 or 6 days versus 2 or 3 days in traditional culturing before implantation is associated with an increase chance of pregnancy and live birth, but results from a long cohort study, presented on 24th June at ESHRE 2019, suggested that an increased obstetric risk may be associated with this procedure.

The study analysed almost 90,000 assisted reproduction babies born in the Nordic countries: Denmark, Norway, and Sweden. Of the total study population, 69,751 were singletons and 18,154 were twins. The singleton and twin cohorts comprised 8,368 and 1,167 babies born after blastocyst transfer and 61,383 and 16,987 babies born after using traditional 3-day embryo transfer, respectively.

The risk of the baby being large for gestational age was analysed, showing that there was a 23% higher risk of this occurring in singletons born after fresh blastocyst transfer compared to 3-day embryo transfer. In everyday terms, this would present as a minor increase in incidence from 3.7% to 4.3%. Although an increased risk in the chance of a baby being preterm was seen with the use of frozen blastocyst use, no changes was seen with the use of fresh blastocyst. Interestingly, the incidence of twins occurring increased from 2.3% following the fresh 3-day transfers to 4.0% following blastocyst transfer.

> *"Today, blastocyst culture plays a crucial role in ART treatment. It increases survival rates of frozen-thawed embryos and so supports the use of single embryo transfer to reduce twin births."*

Commenting of the findings, Dr Anne Laerke Spangmose, Copenhagen University Hospital, Copenhagen, Denmark, who presented the results, said "Today, blastocyst culture plays a crucial role in ART treatment. It increases survival rates of frozen-thawed embryos and so supports the use of single embryo transfer to reduce twin births. From our results, the greater use of frozen blastocyst transfer could offset the adverse effects of extended embryo culture."

Frozen Sperm Retain Characteristics in Outer Space Conditions

COLONISATION of other planets and reproduction outside Earth might sound like far-fetched or distant ideals, however research presented on the 24th June at this year's ESHRE meeting may have taken us a small step closer, attesting that frozen sperm samples display a distinct lack of difference in a variety of characteristics in microgravity conditions compared to those in ground conditions. Dr Montserrat Boada presented the findings from Dexeus Women's Health, Barcelona, Spain, in collaboration with microgravity engineers from the Polytechnic University of Barcelona.

Microgravitational effects have been previously studied in the context of cardiovascular, central nervous system, and musculo-skeletal function; however, little is known about how human sperm and eggs can be influenced. This is particularly true for frozen samples: the state in which they would eventually be transported into space.

During the study, a small aerobatic training aircraft (CAP10) capable of generating short-duration hypogravity conditions conducted 20 parabolic maneuvers, allowing 8 seconds of microgravity to which 10 on-board, frozen sperm samples taken from healthy donors could have functional measurements taken. These included motility, morphology, concentration, vitality, and DNA fragmentation. The results showed 100% concordance between microgravitational and ground samples for DNA fragmentation rate and vitality, and 90% for sperm concentration and motility. Minor discrepancies could feasibly be explained by heterogeneity of the sperm samples.

"If the number of space missions increases in the coming years, and are of longer duration, it is important to the study the effects of long-term exposure to space in order to face them. It's not unreasonable to start thinking about the possibility of reproduction beyond the Earth," explained Boada. She added that her group next plan to validate these preliminary results and move onto using larger sperm samples, both frozen and fresh, and to induce longer periods of microgravity.

"It's not unreasonable to start thinking about the possibility of reproduction beyond the Earth"

IVF and ICSI Pregnancy Rates have Peaked in Europe

PREGNANCY rates in Europe from *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) have peaked. The data, presented by at ESHRE on the 25th of June in Vienna, Austria, showed that success rates are at 27.1% pregnancy per IVF treatments and 24.3% following ICSI. These figures support the recent trend of a higher success rate in IVF over ICSI. Data were also presented on other trends in assisted reproductive fertility treatments.

The data were collected by ESHRE from the European national registries from 2016. IVF, ICSI, egg donations, and intrauterine insemination treatments were monitored, covering >800,000 cycles in 2016, resulting in 165,000 babies being born. This provides not just the largest, but the most accurate view of assisted reproduction treatments in Europe. Dr de Geyter, Chair of EHSRE's European IVF Monitoring Consortium, shared his estimate that the programme includes 84% of all assisted reproductive treatments in Europe. However, this was the first year to not include any UK data so far, which usually see around 60,000 treatments each year.

Results showed that the success rates are no longer rising as they seem to have reached a peak. Despite the success rate of IVF, European clinics favoured ICSI 2:1 over IVF, with 359,858 and 125,626 treatments, respectively. Another favoured strategy which has higher pregnancy rates is the use of blastocytes: 5-day-old rather than 3-day-old embryos.

"... success rates are at 27.1% pregnancy per IVF treatments and 24.3% following ICSI"

While this highlighted a peak in the rates of success from IVF and ICSI, one treatment that is showing rising success rates and cycle numbers is frozen embryo cycles: embryos are cryopreserved to be thawed and transferred in a later cycle. The treatment saw a rise in pregnancy rate per treatment of 1.3% from 2015 to 2016, where it reached a success rate 30.5%. The data analysis showed that around half were frozen embryo transfers, which is also a higher rate than in 2015.

Paternal Smoking can Impact Male Offspring Semen Quality

SPERM quality could be affected by paternal smoking, as found in the preliminary results from the Danish National Birth Cohort study. The results, presented on the 25th June 2019 at ESHRE, showed that low sperm counts and concentrations were associated with paternal smoking, irrespective of maternal smoking.

Dr Sandra Søgaard Tøttenborg, Bispebjerg Frederiksberg Hospital, Copenhagen, Denmark, said: "Our larger study does support these previous findings that paternal smoking is associated with sperm concentrations in male offspring independently of maternal smoking. We also found the association was independent of other preconceptional and prenatal risk factors for adult semen quality – including paternal age, alcohol, caffeine consumption, pre-pregnancy BMI, and household occupational status."

The researchers analysed 778 19-year-old men whose mothers were registered in the Danish National Birth Cohort between 1996–2002. The maternal report included paternal smoking information from gestational Week 16. The semen quality of these men was analysed using the World Health Organization (WHO) criteria. Sperm motility, concentration, morphology, and total sperm count were analysed.

The men who had fathers who smoked daily, and mothers who did not smoke, displayed a 9% lower sperm count and an 8% lower sperm concentration than the sons whose fathers were non-smokers. However, the impact was not as great as maternal smoking: "The effect of maternal smoking is much larger. If the mother but not the father smoked, the reduction was 26% for sperm concentration and 46% for sperm count. It's certainly worse for the boys if the mother smokes. Nevertheless, the circumstances in which the father smokes but the mother doesn't is much more prevalent, so this is still very relevant for public health," continued Dr Tøttenborg.

The researchers recognised that the observed impact of paternal smoking on sperm count and concentration was not dramatic compared with risk factors such as exposure to specific pesticides and urogenital diseases. However, it does sit in a similar range to that of smoking in the adult man.

New Understanding of Environmental Influences on Female Fertility Marker

RESULTS from the OORExPo study were reported in a ESHRE press release dated 25th June 2019 and could be the first steps towards understanding how environmental factors influence female fertility markers. The study examined levels of anti-Müllerian hormone (AMH) and its concentration level in women who were exposed to different levels of air pollution and to assess the hypothesis that environmental factors such as air pollution and environmental chemicals could disrupt the biosynthesis, signalling, or metabolism of hormones.

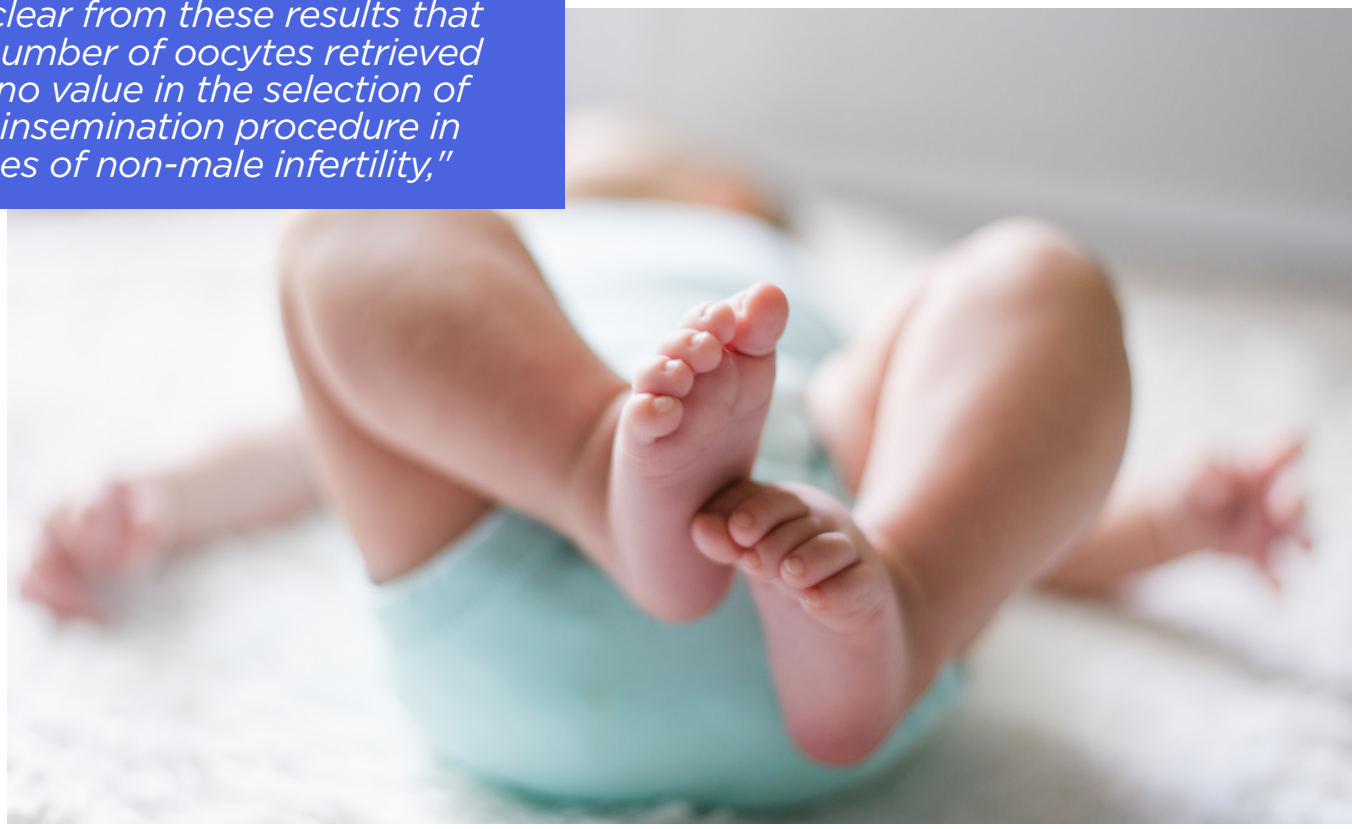
The study was led by Prof Antonio La Marca, University of Modena and Reggio Emilia, Emilia-Romagna, Italy, and looked at 1,463 AMH measurements that were taken from a total of 1,318 women who were living in Modena between 2007 and 2017. It considered daily particulate matter (PM), which was defined as PM2.5 or PM10, and nitrogen dioxide (NO_2). The levels of AMH were linked to the participant's age and residential address in the database, and the analysis used environmental data and a 'geo-localisation' estimate based on this data. The researchers grouped results into quartiles based on the concentrations of the PM and NO_2.

Results showed that even though age >25 years was inversely and significantly associated with lower serum AMH levels, the fourth quartile of results in terms of PM and NO_2 levels also demonstrated much lower concentrations of serum AMH than the lower quartiles. The participants who had the lowest serum level of AMH, i.e., 'severe ovarian reserve reduction', had been exposed to levels of PM10, PM2.5, and NO_2 of >29.5, 22, and 26 mcg/m^3, respectively. This was more frequent in the fourth quartile than the first three for PM10 (62% versus 38%), PM2.5, and NO_2.The European Union (EU)-recommended upper limits for these PM are 40, 25, and 40 mcg/m^3, respectively.

Prof La Marca explained: "This means by our calculations [...] that exposure to high levels of PM10, PM2.5, and NO_2 increases the risk of having a severely reduced ovarian reserve by a factor of between 2 and 3."

"...exposure to high levels of PM10, PM2.5, and NO_2 increases the risk of having a severely reduced ovarian reserve by a factor of between 2 and 3."

"It's clear from these results that the number of oocytes retrieved has no value in the selection of the insemination procedure in cases of non-male infertility,"



No Benefits Proven for ICSI over IVF in Non-Male Factor Cases

A LARGE, international study involving 5,000 patients from Belgium and Spain has shown that fresh and cumulative live birth rates in non-male factor cases are similar in those treated with intracytoplasmic sperm injection (ICSI) and *in vitro* fertilisation (IVF). The findings were presented in a press release on 26th June at the 2019 ESHRE Annual Meeting in Vienna, Austria.

Since its development in the 1990s, ICSI has quickly grown to become one of the predominate means by which infertility, particularly of male cause, has been tackled: before this time, it was near impossible for men to father children if their sperm were of poor quality or insufficient in number. Currently, the technique has expanded to include cases of female-cause infertility and as a practice outnumbers conventional IVF cycles by around two to one.

In explaining the increased trend of using ICSI for the treatment of fertility, Dr Panagiotis Drakapoulos, Centre for Reproductive Medicine (UZ Brussel), Belgium, commented that "the rationale for this seems to be that ICSI is associated with a higher likelihood of fertilisation and increased number of embryos: but this is controversial."

No overall differences in live birth rate, fertilisation rate, and cumulative live birth rate were observed between the two approaches, similarities that were evident across four different patient response categories characterised by poor (1–3) to high (>15) egg retrieval. "It's clear from these results that the number of oocytes retrieved has no value in the selection of the insemination procedure in cases of non-male infertility," stated Dr Drakapoulos. The study also considered patient response to ovarian stimulation.

In debunking misconceptions around the wide-spread preference of ICSI as opposed to IVF, the group hopes to inform clinicians to not blindly recommend one technique or the other to patients blanketed under the 'infertile' umbrella, and that the number of viable eggs is not as determining a factor as previously thought.

Sexual Dysfunction: The Psychological Burden

Kirstie Turner
Editorial Administrator

ESHRE's platform 'Focus for Reproduction' published an article before the congress, on 7th May 2019: 'Discussion, counselling and psychological support all necessary for treating sexual dysfunction in fertility care'. The key topics, which outline the links between sexual dysfunction and psychological health, are summarised in this congress feature.

INTRODUCTION

In an age when we are working towards having an open conversation around mental health, patients undergoing treatment for infertility caused by sexual dysfunction should not be exempt. ESHRE's platform 'Focus for Reproduction' delves into the issue in their precongress article calling for better psychological support for patients undergoing treatment in a fertility care setting.[1] We are only seeing the start of a much-needed focus on mental health, but this needs to go much further; psychological interventions could even be pivotal in treating sexual dysfunction that is hindering fertility. While research into this topic remains inconclusive, there is a suggestion that sexual dysfunction can be treated with discussion therapy or cognitive behavioural therapy as opposed to the use of medication.[1] It is time to address the burden of psychological issues and the impact these can have on couples trying to conceive. Infertility causes and intervention were, unsurprisingly, hot topics at EHSRE 2019, and along with their platform covering the issue, there was a session dedicated to 'Fertility Outcomes and the Male'.[2]

PSYCHOLOGICAL ROLE

As discussed by ESHRE's 'Focus for Reproduction' platform, Nobre's research[3] outlined the importance of cognitive factors, which can be significant predictors of sexual desire, and lack thereof resulting in sexual dysfunction in some cases. Negative connotations surrounding sexual activity were found to be influential on sexual desire; for males, the pressure of sexual performance was a psychological burden, and females displayed reservations surrounding sexual intercourse due to the perceived need to be conservative.

Nobre found discrepancies in sexual arousal indicators for females; subjective indicators, such as physical arousal, were not always concurrent with psychological feelings. This suggests that physical and mental aspects of sexual desire are different, and it is important to address both. If sexual dysfunction is being caused by a cognitive factor, such as the aforementioned concerns regarding sexual performance or conservativity, the treatment choice should reflect this. A lack of interest or desire for sexual activity could be a barrier to conceiving and confronting these issues could lead to a healthier mindset surrounding sex, resulting in an increase in sexual intercourse, raising the chances of pregnancy.

If the case of sexual dysfunction is caused by psychological issues, it makes sense for the first treatment port of call to be psychological therapy. Each individual's psychological wellbeing differs, and therefore any interventions should be handled on a case-by-case basis, taking these cognitive sex differences into consideration. Addressing concerns regarding sexual performance may be highly beneficial to a male patient but may not be to a female patient.

TREATMENT OPTIONS FOR SEXUAL DYSFUNCTION

Psychological Interventions

Premature ejaculation is considered to be the most common cause of sexual dysfunction, with a prevalence of >30% worldwide.[4] Psychosexual counselling can be an effective treatment option as many cases are psychogenic, caused by performance anxiety, low self-esteem, avoidance of sexual intercourse, partner hostility, and decreased quality of relationship.[5] Psychosexual counselling can have a range of means to diminish the problem, resulting in improve ability to reproduce: techniques to control ejaculation, increase sexual confidence, decrease performance anxiety, adapt to sexual repertories, overcome intimacy barriers, address interpersonal issues, face their feelings surrounding sexual dysfunction, and improve communication within the relationship.[5]

While psychological interventions within sexual dysfunctions have been studied, it is in limiting numbers. Brigitte Leeners, University of Zurich, Zurich, Switzerland, outlined the limitations of using small samples of patients. This renders conclusions insignificant. Cultural differences are also a challenge for comparing data from different countries, hindering the opportunity to compare and contrast the available data. Leeners outlines the therapy options surrounding low sexual desire, vaginismus, and fear of sexual intercourse.[6]

Pharmaceutical and Medical Interventions

Medical interventions for male patients experiencing sexual dysfunction include shockwave treatment, penile implant surgery, and oral inhibitors to treat erectile dysfunction: a

condition that affects 11–69% of men experiencing infertility.[1] Phosphodiesterase type 5 inhibitors can be used to alter semen parameters: motility and morphology see small increases. However, the heightened nitric oxide levels released during sexual stimulation can impact negatively upon this. Paolo Capogrosso, San Raffaele Scientific Institute, Milan, Italy, said these inhibitors are safe for use but cannot be recommended.

Clomiphene and anastrozole can be successful treatment options for normalising testosterone levels: an important factor considering there is a 45–69% prevalence of hypoactive sexual desire in infertile men. Flibanserin is U.S. Food and Drug Administration (FDA)-approved for treatment of hypoactive sexual disorder, but this pharmaceutical intervention has a limited effect.

EDUCATION SURROUNDING SEXUAL DYSFUNCTION

There is a call for better education of sexuality to help reduce infertility problems. Paul Ezlin, Leuven University Hospital, Leuven, Belgium, has recommended that clinicians work towards an open discussion on sexuality with couples. Barriers to opening this discussion include time constraints and a lack of sexuality education for doctors. Ezlin suggests using the permission, limited information, specific suggestions, and intensive therapy (PLISSIT) model for identification of issues, rather than waiting for problems to develop.

A significant step in recognising the psychological role in sexual dysfunction is improving communication with patients. Eline Dancet created 'Pleasure and Pregnancy' with colleagues, a 6-month programme designed to educate patients on a range of topics including psychosexual education, communication exercises, and mindfulness. The creation of this online tool came after research came to light that suggested that couples had an unmet desire for advice.

CONCLUSION

While opening a discussion surrounding sexual dysfunction is of key importance, it is vital to consider patient preferences. Ana Gomes, University of Porto, Porto, Portugal, expressed caution regarding over-diagnosis of sexual dysfunction. Some couples find therapy beneficial when they are experiencing sexual dysfunction implemented by pressure for 'sex on the clock'. On the other hand, some couples find a softer approach more appropriate, with a 'door open approach' to discussing dysfunction, and it is important to identify the correct approach on a personalised basis.

While pharmaceutical interventions offer some viable possibilities, there is a real chance of solving infertility caused by dysfunction through psychological therapies. Problems can be prevented from the very beginning if the right questions are asked during medical assessments, outlining the importance of the role of the physician. Mental health is not something we can ignore in infertility and it is time for cognitive treatments to be more widely considered, along with offering better psychological support for couples undergoing fertility treatment.

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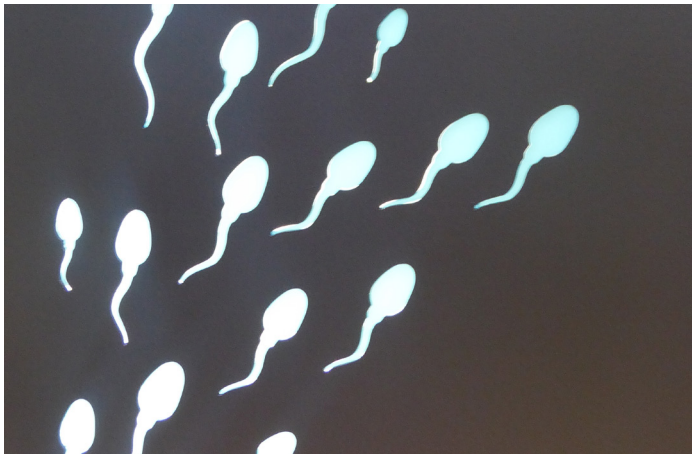

Congress Interview

Dr Sarah Jarvis MBE

General practitioner, Trainer, and Fellow of The Royal College of General Practitioners, and Medical Writer and Broadcaster, UK

ESHRE hosted a fascinating panel entitled 'The pressure paradox: can egg freezing tune out the tick tock of the biological clock?' facilitated by Dr Sarah Jarvis. The session looked at the relationship between age and pregnancy and how social egg freezing impacts this. We spoke to Dr Jarvis about what led her to become a GP and heard her insight into the panel discussion, delving into the topic of age and pregnancy and the importance of educating women on their individual fertility windows.

Q1 What first attracted you to a career in healthcare?

I'm quite unusual; I haven't just wanted to be a doctor since I was a child, I have wanted to be a general practitioner (GP) since I was 8 when the GP came to my house and gave my father an injection. He was wonderfully kind to me and let me delve around in his bag: he even gave me a needle and syringe and I practiced injecting my teddy bear. Ever since then, I knew that I wanted to be a GP. It never crossed my mind that there was any other kind of doctor because I come from a family where my parents were incredibly healthy and didn't believe in doctors. The only doctor I met was a GP and I wanted to be like him. When I decided I wanted to become a GP, it was all about making people better. I think the big difference is that in the intervening 30 years there has been much more focus on not just making people better, but keeping people well and on prevention. We have also started talking about the psychological distress associated with fertility problems.

Q2 Are we seeing a change in the relationship between age and pregnancy?

There is absolutely no question that we are having children later. Woman are doing it; the question is whether they understand the implications of that. If we look at Spain, as an extreme example, in the last 40 years they've gone from a mean age for having their first child of 25 to a mean age of 31. The average age in many European countries, such as Spain and Italy, is now over 30; as that is an average it means there is a significant number of people at the top end who are wanting to have their first baby over 35, over 38. I think there is a huge issue.

We've got women who are economically independent; they don't feel pressured to get married young, to become dependent on a

husband and have a baby. At the same time, it's getting increasingly difficult for them to afford their own homes due to the cost of housing. Long gone are the days where a couple could get married, the husband would work, the wife would stay at home, and they could still afford a very nice life. These days women have the want, and absolutely have the right, to work alongside the man. It is often not financially viable for just one half of the couple to be working. Women are putting off having babies when they are not financially stable or feel they're not; they could also perhaps be waiting longer to find the ideal partner.

Q3 Does social egg freezing increase or alleviate the pressure on women to have children at a young age, rather than when they are ready?

There is huge pressure within the media and from society to say, 'age is not a barrier anymore, 40 is the new 30'. In many respects, it is, but it does women no favours not to be more honest about the fact that that does not apply to reproduction. The problem is that women are very much unaware of the facts. The facts may not be palatable, but they are the facts, nonetheless. So, the likelihood of conceiving drops sharply from your early to mid-30s. At the same time, your likelihood of miscarriage increases dramatically and that is largely because the quality of the egg (and with it the likelihood of a successful pregnancy and taking home a healthy live baby) decreases significantly.

"There is absolutely no question that we are having children later. Woman are doing it; the question is whether they understand the implications of that."

We are looking at 20% of eggs being aneuploid (completely unviable) at the age of 20, compared to 60% by the time you reach the age of 40. The likelihood of a successful, natural conception and a healthy live birth reduces dramatically in your 30s. At the same time, the success rate of IVF is absolutely not what my patients expect. They really do believe that if they go through IVF, it will all be fine. Not only is it not guaranteed at any age, it gets less and less successful as time goes on. The Human Fertilisation and Embryology Authority (HFEA) data suggest it goes from about 43% success at age 18–34, by the time you get to 38 or 39 that figure is about 30%, and by the time you get to 43–44 the likelihood of successful IVF with a successful live birth has dropped to 10%.

Q4 How is the 'Be Ready, Whenever You're Ready' campaign helping to achieve this?

One of the really important points to make is that egg freezing is absolutely not for everyone and women have to be in a position where they can make an informed decision. They need all of the facts, and those facts have to be balanced and not skewed by the media. The 'Be Ready, Whenever You're Ready' campaign provides facts about why women need to start thinking about their fertility at a younger age, reducing the chances of them being disappointed. It also allows women to hear the experiences of other women who are in their position, who have gone through similar experiences. It is unbiased: I have gone through it with a fine-tooth comb to ensure it is absolutely unbiased information on the likelihood of success, for instance of natural conception, of IVF, of egg freezing. It empowers women to have a conversation with their healthcare provider. Women need to consider these facts before they reach the age where these factors kick in.

Q5 How important is it to fully educate women on their individual fertility window at an earlier age to ensure that they can make informed decisions and manage realistic expectations?

This is hugely important. Unfortunately, I still regularly see women coming in too late. In my experience, when I talk to women about their likelihood of having a successful pregnancy from IVF later in life, they can be hugely overly optimistic, and these are often highly educated women who don't recognise that their fertility starts to decline. They do not understand what egg freezing is. I think they need to understand that, for instance, if they are looking at egg freezing, what it is giving them is an opportunity

to freeze their eggs when they are of (and I hate the term higher quality), but unfortunately in terms of successful pregnancy, they are higher quality. They also need to be aware that even egg freezing needs to be thought of early; to put that into perspective, even if you do manage to get 10 oocytes, if you take them from a woman under the age of 35, the likelihood of having a successful pregnancy is 43%, if you take them from a woman who is over 35, that drops to 25%.

Q6 What is one of the biggest challenges to providing women with the correct information on fertility preservation options?

The short answer is the media. The mass media tells women that they do not need to think about it because we have got fertility options, we have got IVF, it has been 40 years since Louise Brown. Once women have got over that, we need to help women to understand that IVF is not the answer for all and that egg freezing is not suitable for everyone but that it is suitable for many women if they think about it at the right time.

Q7 Do you expect to see the average age of women having their first child to increase in the next few years?

I certainly do not think it is going to go down. I think it may increase further. Some of the increase, in some countries more than others, has been because of a drop in teenage pregnancy at the bottom end of the age spectrum. There is no question that there are fewer women having unwanted or unplanned pregnancies in teenage years. That is good news, but it is important to bear in mind that it will skew the figures to an extent. I think this continued increase is likely to slow down; however, there is a significant ongoing pressure on women to be financially independent to be in a position where you can have a baby as pregnancy and babies seem to be more expensive with every passing year. I do think women are going to continue evermore to delay the time to get to where they feel they are in a financially stable position.

Q8 Is there an area of fertility preservation that you hope to see gain more attention in the future?

There are two; the first is that we need to continue to try and educate women about the statistics on fertility decline. Secondly, I would very much like to see more awareness of the options including social egg freezing, and that is freezing for purposes other than medical reasons. Non-medical egg freezing does not mean there is no medical procedure involved; the term social egg freezing means freezing for non-medical reasons.

Q9 As someone with a wealth of experience in healthcare, what advice would you give to those starting out their career today?

Know the facts, whether they are palatable or not. Don't believe everything you read in the media about older mothers; it is definitely not as easy as it looks. Don't believe that we have fertility sussed and that IVF means that anybody can have a baby whenever they want. Do understand that options such as egg freezing aren't suitable for everyone, but it is essential to know all about it so you can make an informed decision. Do understand that even egg freezing will not work forever and that as with natural conception, the success of egg freezing reduces with age.

The Untapped Potential of Ultrasound in Reproductive Health: Interviews with Key Opinion Leaders

Interviewees: Nick Raine-Fenning,[1] Michaël Grynberg,[2] Stephen Hussey[3]

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2. University Hospital Antoine Béclère, Clamart, France
3. General Manager Women's Health Ultrasound Europe & RCIS, GE Healthcare, Amersham Place, UK

Disclosure: Dr Nick Raine-Fenning is a shareholder at Nurture Fertility and an advisor to GE Healthcare, Merck, Ferring Pharmaceuticals, and Pharmasure. Prof Michaël Grynberg has received fees from Merck Serono, Ferring, Gedeon Richter, GE Healthcare, Samsung, and Cook Medical.

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Ultrasound (US) has been a frequently-used tool in the field of reproductive health since the mid-1950s. It has been hugely successful, especially during pregnancy, in helping to detect issues such as fetal abnormalities. It is also a non-invasive procedure, working by bouncing ultrasonic soundwaves at body structures or tissues and detecting the echoes that bounce back. The quality of US scans has continually improved over the years, and with emergent technologies, such as Doppler and three dimensional (3D) imaging, the ability for physicians to use US to accurately visualise organs and structures within the female pelvis is extensive. However, across the broad spectrum of reproductive health physicians, there appears to be significantly more scope for US to be utilised in daily clinical practice, particularly in treating infertility and increasing the success rates of procedures such as *in vitro* fertilisation (IVF).

To better understand the extent to which US can be utilised, the European Medical Journal recently interviewed two leading figures in reproductive health who are particularly strong proponents of a broad use of US in clinical practice. These were Dr Nicholas Raine-Fenning, Clinical Associate Professor & Reader in Reproductive Medicine and Surgery, Faculty of Medicine & Health Sciences, University of Nottingham, Nottingham, UK, and Prof Michaël Grynberg, Head of the Department of Reproductive Medicine and Fertility Preservation at the University Hospital Antoine-Béclère, Clamart, France. As well as providing a general overview of US across reproductive health, each provided an analysis from the perspective of their main area of clinical expertise: Dr Raine-Fenning focussed primarily on the use of US in IVF, while Prof Grynberg gave insights on its role in diagnosing infertility. In addition, Mr Stephen Hussey, General Manager, Women's Health Ultrasound, Europe & RCIS, GE Healthcare, Amersham Place, UK, was invited to share a commercial perspective on the use of US technology in reproductive medicine.

INTRODUCTION

The importance of ultrasound (US) technology to the working practices of reproductive health physicians is difficult to overstate. "It is not so much a benefit, more an absolute necessity," explained Dr Nicholas Raine-Fenning. "You could not practice reproductive medicine without US, it is a central part of everything we do every day: all patients have a scan as part of their fertility investigation to see if there is an underlying issue, as looking for pelvic pathologies, such as cysts, fibroids, polyps, uterine abnormalities, swollen tubes, or polycystic ovaries is crucial."

"It is not so much a benefit, more an absolute necessity. You could not practice reproductive medicine without US."

Prof Michaël Grynberg strongly concurred with this assessment, appreciating the critical role that US has to play throughout the whole cycle of assessment and treatment that an infertile patient will typically undertake, stating: "It is probably one of the main ways a fertility expert can profile the assessment of female infertility. It is certainly important for us in helping diagnosis because it can directly locate the aetiology of the infertility." He added: "It is also important for planning the treatment strategy according to ovarian function because, from the results, we will be able to tell if a patient should be able to respond to the proposed fertility treatment which is primarily based around ovarian stimulation. It is important to measure and assess the ovarian response to our treatment."

Considering the technological advancements in US that have taken place in recent years, there is a great opportunity to enhance patient care in reproductive health, while at the same time improving efficiency and reducing costs if US is used more widely. Mr Stephen Hussey added: "There has been a great deal of investment in US technologies that, when combined with the correct training, can help improve diagnostic confidence and at the same time provide more patient comfort during examination." For instance, although there are no differences in patient preference for either procedure, hysterosalpingo contrast sonography (HyCoSy) is associated with less pain[1,2] and is better tolerated when compared with using hysterosalpingography (HSG) to assess tubal patency.[3]

Despite this, the use of US is not as widespread as it could, or arguably should, be across this area of medicine. "If someone is seen in a general gynaecology or fertility clinic, they do not all get a scan," explained Dr Raine-Fenning. "Unless they have got an indication for a scan, such as pain, heavy bleeding, etc., they tend not to be scanned. Rather, they tend to just get a vaginal examination, which has less sensitivity and specificity. I think all reproductive medicine professionals specialising in *in vitro* fertilisation (IVF) realise this and so tend to do scans at a lower threshold than in general gynaecology. In general gynaecology, US is often used as a baseline scan to exclude pathology in women with signs and symptoms; it is not used much after that. In IVF, it has a much more functional role."

ULTRASOUND IN CLINICAL PRACTICE

Dr Raine-Fenning's practice is primarily focussed on IVF, an area of reproductive health in which US underpins all aspects of treatment. Dr Raine-Fenning detailed how this plays a fundamental role in his day-to-day clinical work: "Before a patient even comes into the IVF unit, we scan them; that means when I first see them for their initial consultation, I can talk to them about any pathology that we have seen and whether they need surgery. Then when they start treatment, it is monitored by daily US scan, which tells us how they are responding and whether changes need to be made to maximise response and therefore outcome."

It is this ability to accurately measure responses to treatment that Dr Raine-Fenning believes has had the most profound impact on the chances of a successful IVF treatment. "It has completely changed our practice, as we are able to dose more accurately and confidently," he confirmed. "Ensuring they are on the right dosage of drugs from Day 1 reduces under and over-response. And that has revolutionised everything for us because, previously, we gave drugs to people based on their age and follicle-stimulating hormone level, which is a far inferior way to predict response."

At the Department of Reproductive Medicine, Hôpital Antoine Béclère, Prof Grynberg and his colleagues have likewise ensured that US is used to the fullest extent throughout the team, ensuring that everyone they work with, from gynaecologists to midwives, are trained to perform thorough US scans on patients which provide reliable results. He informed us that, as a result of this emphasis, there are between 80 and 100 US scans that take place in the hospital each day. The focus of US in this context is on helping the varying needs of patients with fertility problems. "We perform US to diagnose infertility and, in those fortunate to conceive, to check for pregnancy, but we also offer US to those patients thinking ahead who want to preserve their fertility," outlined Prof Grynberg.

"US is very helpful in enabling the physician to guide the patient to the best way of reaching the goal of fertility as quickly and as effectively as possible."

In a similar fashion to the influence US has in Dr Raine-Fenning's team, at Prof Grynberg's department the results of an US scan will ultimately be the biggest factor in the type of treatment that is decided upon in a given case. "The decision in the end will be taken according to the patient's age, sperm results, tubal patency, and ovarian function. Both tube patency and ovarian function will be assessed by US scan," he elucidated. "For example, if you have a patient with a large number of eggs who is quite young, intrauterine insemination will be discussed as an option. And, for another young patient who has a low number of eggs and a low follicle count, they may be a direct candidate for IVF. So, US is very helpful in enabling the physician to guide the patient to the best way of reaching the goal of fertility as quickly and as effectively as possible."

It is US's ability to assess tubal patency that provides a pertinent example of how it can improve upon the kind of procedures that would otherwise be used. "Through using US we are able to assess tubal patency because we can use the US to monitor the flow of contrast media injected through the cervix," said Prof Grynberg. The greater levels of safety and comfort patients have when US is used in this way compared with HSG examinations will be analysed later in this article.

Prof Grynberg then spoke about how US scans, which have improved in quality over the years, have been a major driver of improved outcomes at his clinical practice over the last 30 years. "We know we are more efficient in the diagnosis of infertility by better understanding the aetiology. And this leads to an overall improvement because it guides the physician in finding the best technique to offer to the patient, and probably provides the best method of measuring the efficiency of our treatments," he outlined. "This is the result of many changes in the laboratory, for sure, but it is also due to US enabling physicians to be more precise in deciding treatments for infertility and the monitoring of those treatments."

TECHNOLOGICAL ADVANCEMENTS

As previously alluded to, advancements in US technology are increasing the benefits these machines offer to reproductive health physicians. In Dr Raine-Fenning's view, the most important aspect, above all others, is ensuring image and resolution is clear and of the highest possible quality: "Some US machines are quite poor. When you scan on a machine that has got less power driving it, it is so much harder. So, it is not just the availability of US, it is also the quality: it makes a massive difference."

Likewise, Prof Grynberg sees machine quality as vital; making careful selection on the US scan being used should therefore be taken heavily into consideration by physicians. He stated: "I think the US machine is extremely important because, depending on the type of machine, you are able to see things that you would not see in others. So, with high quality machines, operator dependence will be much lower. The quality of the machine makes all the difference."

Automated Measurements

Dr Raine-Fenning and Prof Grynberg also see the potential benefits that new technological features provide to specific aspects of the role US can play. One of these, automated

measurements, has had a substantial impact for both of these experts, greatly enhancing their ability to count follicles. Dr Raine-Fenning said: "When it works, it is very good. Even though it is not exactly spot on in terms of accuracy, it is very good for reliability, because the beauty of automated US is that it does not count follicles twice; this really improves your ability to count. So, for an objective measure of the numbers, it is brilliant, but we still need to work on measurement of size and volume." Dr Raine-Fenning emphasised that good image quality is vital for this to occur.

Having an automatic measuring capability is also vital for the day-to-day work of Prof Grynberg. Again, machine quality is the defining factor in ensuring this capability works well. "We have a Voluson™ E8 [GE Healthcare, Illinois, USA] machine, which is one of the best for assessing infertility because we are able to get a very good picture of the ovaries, which allows us to have an automatic follicle count," he explained. "This follicle count is a key issue for us, both in planning for and follow-up of treatment."

Mr Hussey is similarly acutely aware of the impact this feature can have for physicians.[4] "Automated assessment tools, designed in collaboration with leading reproductive medicine specialists, can help enhance accuracy, improve independent user reproducibility, and shorten exam times. Such technologies are becoming increasingly important to help enhance the potential diagnosis, screening, and treatment success," he noted.

Three-Dimensional Technology

Another prominent feature that can be included with US machines is 3D technology, which provides more detail and images not possible with 2D. This has become a necessity for analysing the uterus, especially regarding the discovery and classification of uterine abnormalities. Prof Grynberg outlined how areas such as the uterine cavity cannot be viewed as extensively using a traditional 2D US. He went on to describe the ways in which 3D technology can be used to improve the reliability of automatic follicle counting, citing how the SonoAVC™ (GE Healthcare, Illinois, USA) follicle automatic counting model has substantially enhanced the measuring of ovarian follicles at his department: "When you do it with the 2D, we know we probably miss some of these follicles because it is not possible to make sure we are assessing the whole ovary. But with 3D we can observe the whole ovary even while moving the probe from one place to another. And by using automatic counting with the SonoAVC, we know that with a 3D view all the fluid structures can be counted with reliability."

Dr Raine-Fenning is also a proponent of 3D being used in this manner; however, he believes that there is limited recognition of this among reproductive health physicians. "It is the gold standard and the only way to diagnose uterine abnormalities with confidence," he commented. "It helps with everything; if you are doing a measurement, then the spatial orientation is much better with 3D compared with 2D. It is not used in that way by most people; most use it for uterine abnormalities alone, but it is also a great way of viewing data and measuring as you have three reference points."

Mr Hussey also shared his experience of the benefits that a 3D view can provide to physicians: "3D US of the pelvis can be used routinely to diagnose uterine abnormalities as it enables unprecedented views of the uterus and endometrium in a coronal plane."[5]

ENHANCING THE PATIENT EXPERIENCE

US scans in reproductive health are not just highly beneficial to clinicians; they can substantially improve the experience of patients in a clinic. There are several reasons for this. One is by reducing the need for invasive procedures such as manual vaginal examinations; another is that the more detailed information a physician can collect from US scans can allow them to better guide and explain to patients what their treatment options are, enhancing patient knowledge and thereby the role they can play in their own care.

To emphasise this point, and from his own personal experience, Dr Raine-Fenning has used US to correct misdiagnoses on a number of occasions: "We have lots of people who come from clinics and been told they might have a uterine abnormality but it is often unclear. We rarely have such disparity; we scan in 3D and can confidently tell a patient what their pathology is.

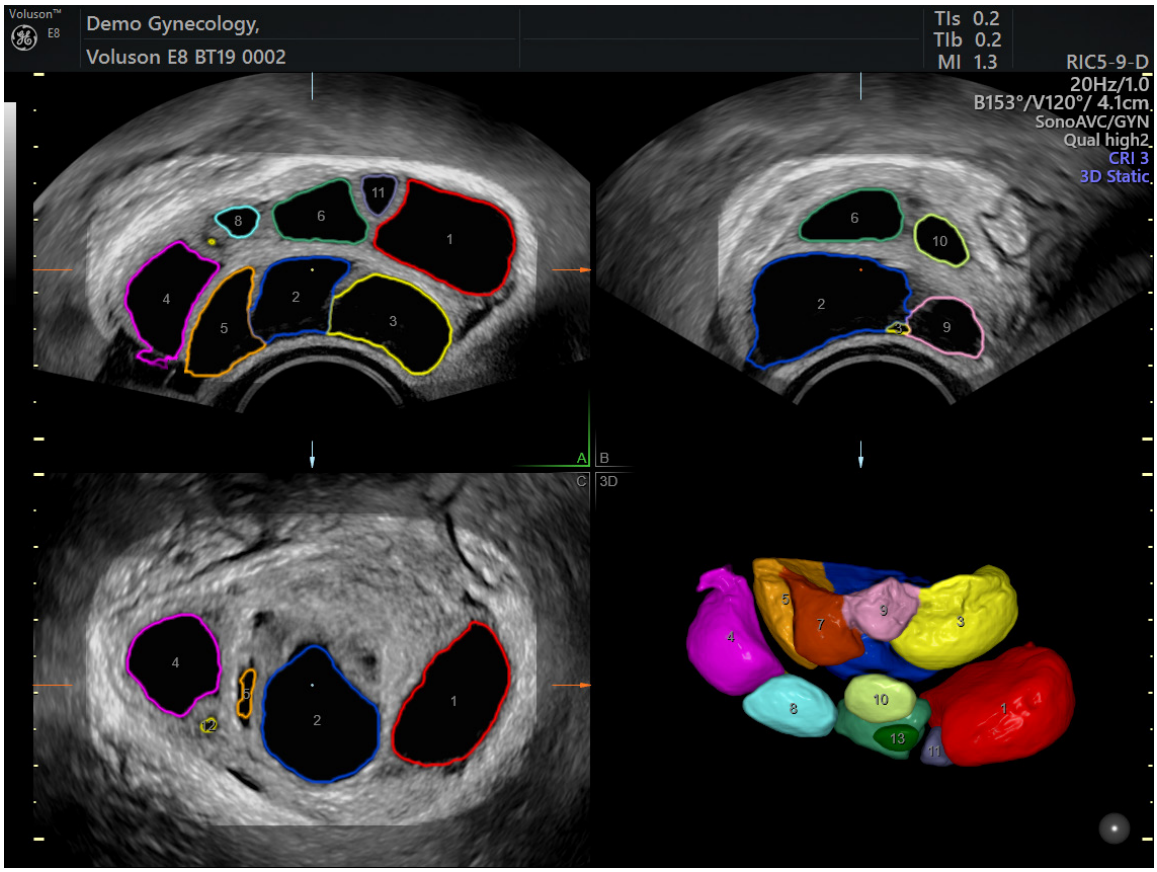

Figure 1: Example of a volume acquisition of the ovary using SonoAVC Follicle ™ - 3D volume technique that automatically counts and measures follicles.

Uterine anomalies can be diagnosed with 100% concordance and we can also subclassify the type removing any confusion. Patients appreciate this and leave with renewed confidence and complete understanding."

As alluded to earlier, analysing tubal patency is one area where using US can be far more beneficial to patients than a HSG X-ray examination. One reason for this is that it is much safer. "US techniques such as HyCoSy or hysterosalpingo-foam sonography, for example, can offer information on tubal patency without the harmful effects of radiation exposure necessary in HSG examinations," said Mr Hussey. Women undergoing HSG are exposed to pelvic radiation. The mean dose-area product (DAP) for a complete HSG examination is 2.05 Gy cm^2. In comparison, the mean DAP for a single posterior–anterior chest X-ray examination is 0.09 Gy cm^2.[6] In contrast, there is no exposure to radiation associated with HyCoSy and CAT.

As well as mitigating the need for radiation to be present, using US for the purpose of determining tubal patency can be a far less invasive, more comfortable, and less time-consuming process for the patient in comparison to HSG. "The HSG exam is difficult for the patient because it can be painful, so by using US and combining the administering of the foam gel, the procedure is definitely less invasive for the patient," commented Dr Grynberg. "In addition, it does not require any radiation exposure; these are all benefits for the patients and the physicians too because it allows us to have what we call a fertility 1-day check, where we assess the ovary, uterine cavity, and tube."

AWARENESS AND TRAINING

Despite the fact US can make such a profound impact on the work of clinicians, and indeed on the lives of patients, it is apparent that, particularly in certain areas of reproductive

medicine, it is not used to its full capacity, partly due to lack of awareness and partly due to lack of training. Dr Raine-Fenning outlined: "The training of obstetricians is engrained in US; every assessment they do is in US. But in gynaecology, for example, US does not seem to form a key part of training, so I think generally in gynaecology, the level of knowledge of US and its application is a lot poorer because there are so many people who are not brought up with scanning."

Drawing on his own experience of organising and teaching a 3D US scanning course, Dr Raine-Fenning has observed just how much room there is for the improvement of reproductive health specialists in terms of knowledge and skill in using US. "I have had doctors from all over the world come on my 3D course and they have all got potential to keep on learning," he commented. "Within a few hours I realise the standard level is quite low, and not just 3D US but basic 2D US as well. I think the machines are ahead of many of the users so training and standardisation in US is what is needed now. We simply must make the most of what we already have."

"We need to make sure that everybody is aware that US is probably the most important tool for the fertility worker."

Ensuring that the understanding of the full potential of US, as well as extensive training in its use, is part of the curriculum of medical students in the reproductive health field should therefore be a high priority. As a renowned university hospital, at the Hôpital Antoine-Béclère a major emphasis is placed by Prof Grynberg and his team on ensuring that all junior doctors learn the processes and skills they need to be proficient in using US. More generally, Prof Grynberg argues that a more thorough US training programme needs to take place at an early stage of a physician's development. "US is now a big aspect of the training of young medical students, particularly those who want to specialise in obstetrics. So, they know how to perform an overall US, but for those who want to go further in reproductive medicine, we need to train them more and this can be performed in universities.

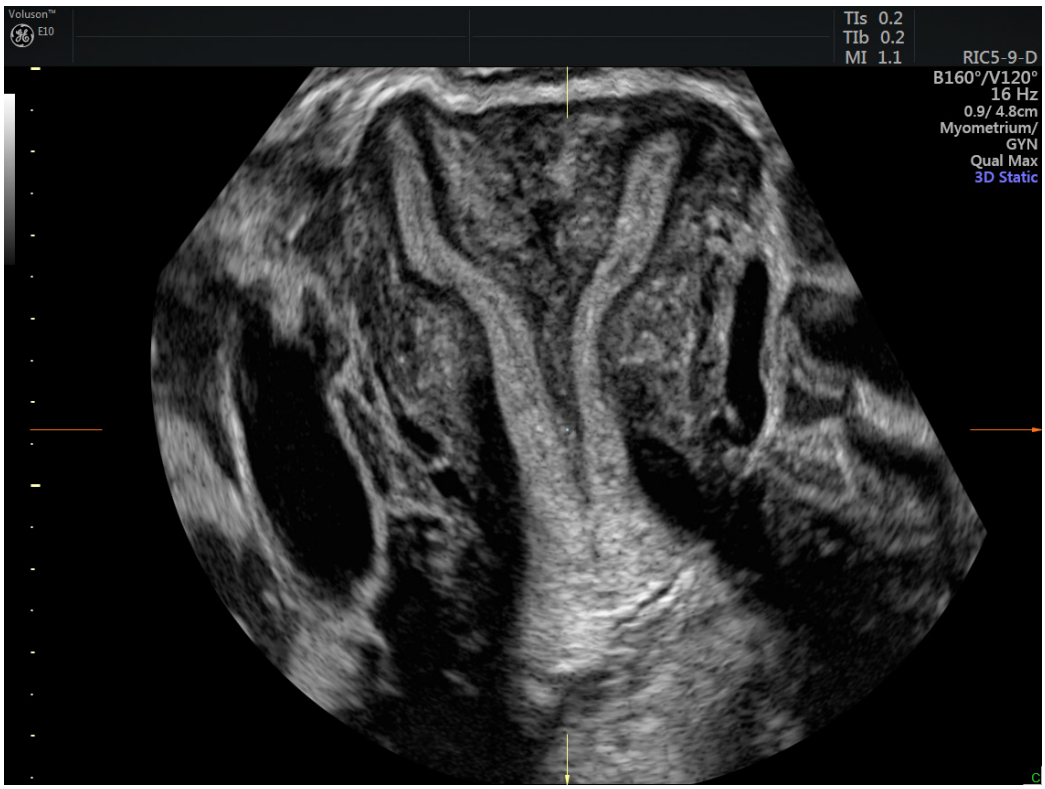

Figure 2: An image of the uterus demonstrating the endometrium in the coronal plane, acquired in 3D volume acquisition mode.

We need to make sure that everybody is aware that US is probably the most important tool for the fertility worker," he said.

Dr Raine-Fenning, with his vast experience in training others on US, noted that the key to becoming proficient in US scanning is constant practise. While online videos and simulation are helpful, nothing can replace hands-on experience in his view. That is his advice to physicians in this area: repetition. "I have always said the key to US is to be able to recognise normality, if you know it is not normal, then you can refer to the relevant person if necessary. The more you do it the quicker you will start recognising normality and therefore abnormalities: it is all about pattern recognition," he said.

CONCLUSION

While US has been a staple in reproductive health for several decades, advancements in machine quality and the advent of features such as 3D and automated measurements have enhanced the potential it has to be used across virtually all stages of a patient's cycle during pregnancy or when seeking fertility treatment. In the examples shared in this article, US has revolutionised the clinical work of Dr Raine-Fenning and Prof Grynberg in making diagnoses, discovering underlying pathologies, and predicting and assessing the impact of treatment. Increasing use of US in this area of medicine can not only improve patient outcomes but patient experiences too, with US being more accessible and far less invasive than alternative imaging methods. A greater emphasis on explaining its importance, as well as providing more opportunities for practical training of physicians in all areas of reproductive health, is clearly a major requirement moving forward.

Biographies

Dr Nicholas Raine-Fenning

Faculty of Medicine & Health Sciences, University of Nottingham; Clinical/Scientific Director, Nurture Fertility, The Fertility Partnership, Nottingham, UK

Dr Raine-Fenning is an internationally recognised expert in three-dimensional ultrasound and gynaecological imaging, regularly providing lectures and leading plenary sessions and workshops in this area. Among a host of prominent positions, he is a board member and Vice Chair of the Scientific Committee of the International Society for Ultrasound in Obstetrics & Gynaecology (ISUOG). In his clinical role, Dr Raine-Fenning manages women with benign gynaecological pathology and subfertility undergoing IVF treatment.

Prof Michaël Grynberg

Department of Reproductive Medicine and Fertility Preservation, University Hôpital Antoine-Béclère, Clamart, France

Prof Grynberg is considered a pioneer on the topic of female fertility preservation, having undertaken numerous research projects in this area. Other research interests include assessment of ovarian follicular status and the regulation of anti-Müllerian hormone (AMH). His clinical work is based around the diagnosis and treatment of subfertile or infertile couples.

Mr Stephen Hussey

General Manager Women's Health Ultrasound Europe & RCIS,
GE Healthcare, Amersham Place, UK

Mr Hussey has over 20 years' experience working in medical ultrasound sales, having started his career as a clinical applications specialist before progressing into sales and commercial product management. Previously, Mr Hussey trained as a radiographer and sonographer, gaining invaluable experience in obstetrics and gynaecology while working at both Hereford County Hospital and the Birmingham Women's Hospital.

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A Modern Approach to Progesterone Supplementation

Interviewee: Elena Labarta

Gynecologist and specialist in human reproduction, IVI RMA Valencia, Spain

Disclosure: Dr Labarta received a grant from Finox in 2016, has provided consultancy services for Ferring Pharmaceuticals and MSD, and is part of the Ferring Pharmaceuticals LIFE programme. During the past 12 months, she has received honoraria from Angelini/IBSA, MSD, and Ferring Pharmaceuticals for lecturing.

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Disclaimer: The opinions expressed in this article belong solely to the named interviewees.

Citation: EMJ Repro Health. 2019;5[1]:34-37.

At the end of December 2017, some colleagues and I reported results from a prospective cohort study (N=211) into the relationship between serum progesterone (P) and endometrial volume on the day of embryo transfer (ET) and ongoing pregnancy rates (OPR) in artificial endometrium preparation cycles.[1]

PROGESTERONE DEFICIENCY – A MINIMUM THRESHOLD LEVEL

Our study found that patients with serum P <9.2 ng/mL on the day of ET had a significantly lower OPR; but that endometrial volume was not related to OPR.[1] The implication is that, if outcomes are to be optimised, a minimal threshold of serum P values on the day of ET must be reached.

Until our study it was thought that the level of serum P would not reflect its effect in the uterus as there was no direct correlation between blood and uterine P levels.[2]

However, there were some limitations to this initial study. Only women with a healthy uterine cavity and appropriate endometrial thickness were included. A further study was needed. At ESHRE 2019, we were delighted to present our findings from a new investigation of 1,197 non-selected patients[3]. Our preliminary findings appear to be in line with the first study.[1]

There are some clear implications for current clinical practice; we found there was a minimum threshold of serum P below which the chance of having a live birth was decreased by around a fifth.[3]

MANAGEMENT OF LUTEAL PHASE SUPPORT – THE INDIVIDUALISED MODERN APPROACH

Although serum P is not the only predictor of implantation failure – there are other factors related to the success of the cycle[4] – the results demonstrate the importance of measuring serum P at ET. We now do this at our practice as a matter of routine. In this way, we can detect which patients have adequate levels of P, essential for embryo implantation and pregnancy maintenance. Until now, it was thought that a conventional dose would be sufficient for all patients during the luteal phase. However, it has become clear that many patients require

individualised luteal phase support (LPS); by routinely measuring serum P levels, we can detect which patients need raised P in order to optimise their pregnancy rates. In fact, we found that 30% of women showed inadequate levels of serum P after receiving the standard dose of vaginal P.[3]

The Addition of Subcutaneous Progesterone

Vaginal P via capsule, gel, or tablet is the most common route for LPS support in Europe.[5] In a retrospective analysis conducted at our centre (IVI RMA Valencia, Spain), which included almost 1,700 patients, we observed that by adding subcutaneous (sc) P to the vaginal administration for patients with low serum levels, we could overcome P deficiency and therefore deliver similar results to those patients with adequate levels.

We chose to administer sc P for a number of reasons: increasing the vaginal dose might increase the vaginal discharge (patients were already receiving four tablets per day); and the sc route to P supplementation could overcome the possibility of poor absorption by the vaginal route, where P is rapidly absorbed into the epithelium and endometrium and thus prevented from entering into circulation.[2] sc P, on the other hand, has been shown to very rapidly increase the levels of serum P[6] (see Figure 1). Herein lies the importance of pursuing alternative administration routes for P, especially considering the lack of positive correlation between vaginal administration and circulating blood levels.

Observed Benefits of Administering Subcutaneous Progesterone

We observed that almost all patients increased their levels of serum P when adding sc P; it is known that patients with pregnancy-related vaginal bleeding have significantly lower serum P levels. Furthermore, no additional procedural risks were detected using the sc method. It has yet to be demonstrated whether other ways of administering increased doses of P are as effective.

As well as the apparent difference this technique has made to pregnancy outcomes, we observed that patients appear to feel more confident in us as clinicians. We hypothesise that this is because by bringing more control to the procedure we are able to improve their results.

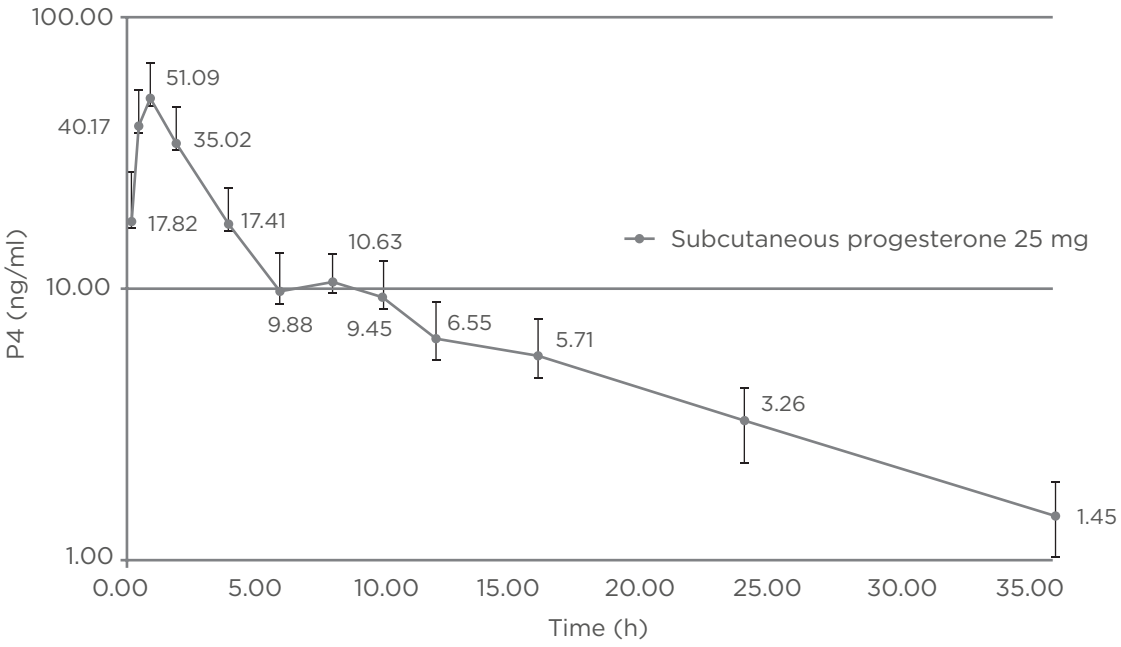

Figure 1: Mean (+SD) baseline corrected plasma progesterone concentrations (ng/mL) versus time profiles after single dose administration of subcutaneous progesterone 25 mg to 28 post-menopausal women. (Semi-log scale).

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GREATER PREDICTABILITY

We can predict quite accurately whether patients are going to have low serum P levels; in 80.5% of cases, patients show similar results in a second cycle when using the same doses of exogenous P.[7]

MAINTAINING THE SUCCESS RATES OF *IN VITRO* FERTILISATION CLINICS

We have seen that individualising the LPS in patients with inadequate exposure to exogenous P, by increasing the dosage using sc administration of water-soluble P, can overcome low serum levels and normalise pregnancy rates. Such improvements are essential if we are to maintain the success rates of *in vitro* fertilisation clinics. Using this strategy in our clinic, those patients who showed a smaller chance of having a baby due to low serum P levels have been able to improve their results significantly - this is an outstanding finding (Figure 2).

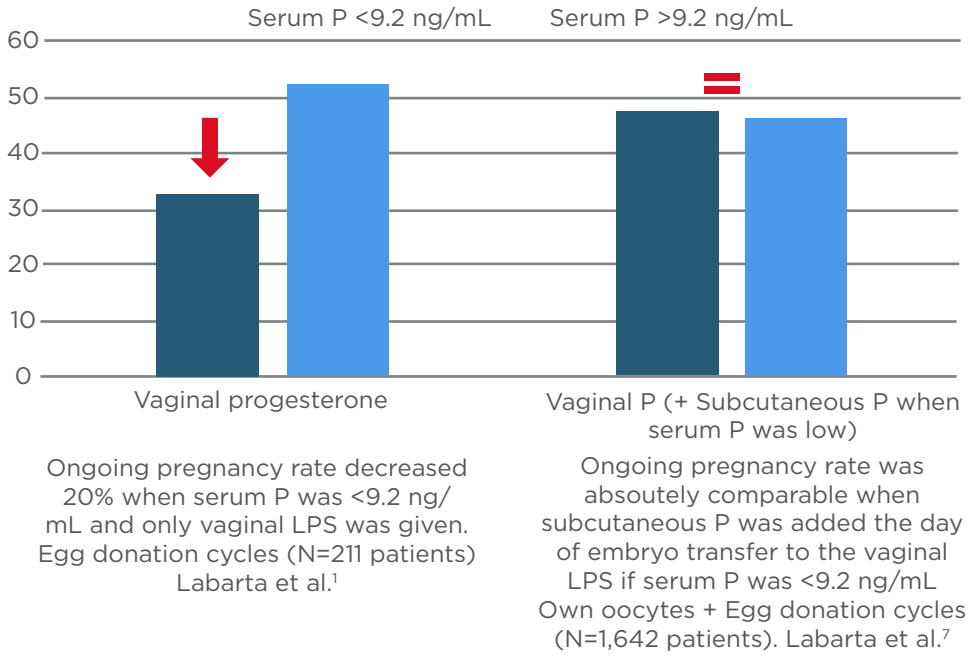

Figure 2: Ongoing pregnancy outcome according to serum progesterone levels on the day of embryo transfer.

LPS: luteal phase support; P: progesterone.

Biographies

Elena Labarta

Gynecologist and specialist in human reproduction, IVI RMA Valencia, Spain

Dr Labarta has worked at IVI Valencia since 2005. She has participated as a researcher in numerous clinical research projects, including Phase II and Phase III clinical trials, and has published many scientific articles and book chapters based on the field of *in vitro* fertilisation. She is a frequently invited lecturer in the most relevant meetings. Dr Labarta's research is now focussed on the predictive

value of mid-luteal serum progesterone levels in egg donation cycles; ovarian stimulation and embryo quality; use of 3D ultrasound for uterine evaluation in infertile patients; and the impact of autologous mitochondrial transfer on oocyte and embryo quality in *in vitro* fertilisation patients, among others.

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Interviews

Reproductive health is an ever-changing field that covers many facets. We interviewed Jacek Malejczyk from our editorial board, who works in histology and embryology, and Aonghus Nolan, who has retired from a career in assisted human reproduction, to gain insight into the field from two experts.

Featuring: Jacek Malejczyk and Aonghus Nolan

Dr Jacek Malejczyk

Department of Histology and Embryology, Medical University of Warsaw, Warsaw, Poland

Q1

What first inspired you to pursue a career in histology and embryology?

I was always interested in basic medical sciences: cell and tissue physiology and pathophysiology in particular. I started my scientific work as a student at the Department of Histology and Embryology at the Medical University of Warsaw and I found that this is the right place for me. This is both a teaching and research department, so the work here gave me an excellent opportunity for my personal development. I have several research interests including immunology and cancer cell biology; but what I have found the most exciting is biology and immunology of human reproduction and gynaecological disorders.

"With the exponentially developing numbers of different laboratory and clinical data there is an urgent need for people with well-developed bioinformatic skills."

Q2

ESHRE had >12,000 attendees this year. How does collaboration and sharing of knowledge at events such as this impact the progression of the reproductive health field?

This is indeed a huge conference giving an opportunity to learn about progress in different fields of reproductive biology and medicine.

This is also an excellent opportunity to meet colleagues and collaborators from other countries and to talk about recent progress in the common field of interest and new opportunities for further collaboration. Friendly meetings with a glass of wine are effective in particular. I think that meeting people is what makes things go forward.

Q3

There was a fantastic programme on offer at ESHRE this year. Was there a stand-out session that you were looking forward to?

The programme was indeed fantastic. Everyone from our huge ESHRE family will find something. I myself am the most interested in endometrium and endometriosis, so I attended all respective clinical and basic science sessions in the field.

Q1

Last year you co-authored the paper 'Recurrent miscarriage is associated with increased ghrelin mRNA expression in the endometrium- a case-control study.' What would you say is the take-home message from this study?

Ghrelin is an important peptide responsible for energy expenditure control and regulation of immune responses. We have previously reported that it is elevated and may play a role in the pathogenesis of endometriosis. In the above paper we found that mRNA for ghrelin and vascular endothelial growth factor A are elevated in endometrium from women with recurrent miscarriage, thus suggesting their role in the pathogenesis of this disorder. This was a preliminary report that still awaits confirmations in other studies.

Q5

How have you seen the field of embryology change over your career? Is there an area of the field you would like to see gain more attention?

The field has changed dramatically. Owing to the development of molecular biology methods, the greatest progress was observed in understanding the mechanisms of early human development. The most spectacular, however, was the introduction of *in vitro* fertilisation. Indeed, it was a true milestone in fighting infertility and, as such, helped millions of people to have a baby. There are still many areas that require more extensive investigations. For example, during the last decades we have learnt a lot about the pathogenesis and genetic background of endometriosis, but no significant strides in diagnosis and treatment of this horrible disease have happened, so far.

Q6

What advice would you give to someone starting out in the field today?

This is a difficult question. Everything depends on personal preferences. Knowledge of sophisticated molecular biology methods is today the must for basic research. With the exponentially developing numbers of different laboratory and clinical data there is an urgent need for people with well-developed bioinformatic skills. These are now the most wanted specialisations. However, irrespective of the field, anyone who wants to be successful must remember that science is a jealous lover and will not accept any opponents.

"...anyone who wants to be successful must remember that science is a jealous lover and will not accept any opponents."

Dr Aonghus Nolan

Dr Nolan began his career in assisted human reproduction in 1989. Before his retirement, he last worked at Galway Fertility Unit, Galway, Ireland.

Q1 What was it that first sparked your interest in IVF?

To be honest, I originally had no interest in the subject of assisted human fertility. I had just graduated with my PhD in Zoology and was applying for jobs. I mainly applied for academic posts. Unexpectedly I was called to interview for a post as an embryologist in the Fertility Unit of the Humana Hospital, St John's Wood, London, UK. I actually did not remember applying for the post in the first place, but I attended the interview, was offered the job. Thirty years later I am still an embryologist (albeit now retired).

Q2 Last year saw the 40th anniversary of the birth of Louise Brown. How has the field of IVF changed throughout your career?

In many ways, the field has not changed in that the goals remain the same, i.e., trying to achieve a successful pregnancy and live birth for people with fertility issues. When I began my career in assisted human reproduction (AHR) on 9th January 1989, the main treatments available to clients were *in vitro* fertilisation (IVF) and embryo transfer, gamete intrafallopian transfer (GIFT), and ovulation induction (OI), with or without intrauterine insemination (IUI). At the time, there were very few options for the treatment of male infertility other than the use of donor sperm. However, the first successful pregnancy from an intracytoplasmic sperm injection (ICSI) procedure in 1991, and birth in 1992, revolutionised the treatment of male infertility. It allowed sperm from men with very low, abnormal counts or even no ejaculate sperm to be used to inseminate their partner's eggs for IVF.

One of the earlier problems with IVF was the over transfer of embryos, in those days it was routine to transfer three to four embryos and sometimes more in the hope that one would implant. This could, and often did, lead to multiple pregnancies, which were unsafe both for the women and the implanted embryos and fetuses. Multiple embryo transfer is now considered unethical, with single transfer being the accepted norm. Additionally, there have been advances in optimising the length and incubation time for embryos. When I began, embryos were routinely transferred on Day 2 (four cell stage) or Day 3 (eight cell stage) post egg collection: the majority of embryos are now transferred at the blastocyst stage (Day 5). This extended culture has increased success rates, and the use of constant monitoring incubators allows more consistent tracking of embryo growth and development prior to transfer.

Q3 Throughout your scientific career, what was your biggest 'wow' moment?

If you are referring to my career in fertility, I cannot think of a particular moment, as there have been quite a few. I have often been surprised when, despite my doubts, people I thought would have had no chance of success actually achieved a baby. It is a lesson in humility if you think you know better than nature, which often has other plans. Rather than 'wow', I am going to use 'proud'. I was very proud to be the embryologist for the first testicular sperm biopsy carried out in this country, where the harvested sperm were used for ICSI, resulting in a pregnancy and baby. That couple had two subsequent children from the same biopsy. It was the first successful pregnancy from testicular biopsied sperm in this country.

My most memorable 'wow' moment was not from IVF but the first time I isolated human DNA from a blood sample. It was fascinating to see the little glob of DNA precipitate in the test tube and to think that I held the blueprint of a person in my hand. I suppose, having said that, every time I chose an embryo for transfer, I also held the blueprints for a person in my hand. Wow indeed.

"...every time I chose an embryo for transfer, I also held the blueprints for a person in my hand."

Q4

The Human Fertilisation and Embryology Authority recently signed a consensus statement that called for private IVF clinics to stop offering patients costly optional add-on treatments that have not been demonstrated to work. How would you like to see this issue tackled?

Legislation and patient education. Patients read about these overly priced, unproven add-ons and think they need them. They seem to think that paying more will give them a better chance. I have seen patients leave a unit to go to another more expensive one simply because of the add-ons. Paying more does not improve success rates. This area needs to be strictly legislated and the 'advertising' information that is passed to patients by units be controlled.

Q5

Following on from this, how can the medical community strike a balance between encouraging innovation and ensuring a solid evidence base?

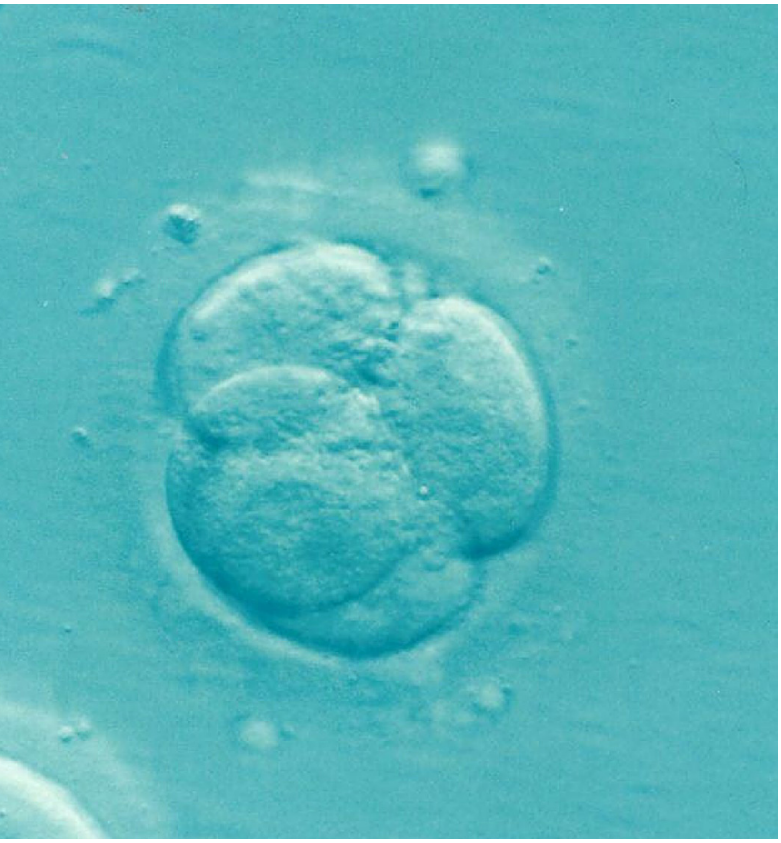
The medical community should not be asked to balance innovation with evidence based research and validation. That is akin to asking it to police itself. The balance should come from outside, from legislation.

According to the European Union (EU) Tissues and Cells Directive, all new procedures must be monitored for safety and efficacy. They must be safe and have sound scientific research backing before they can be offered to patients as a treatment option. The history of IVF shows new techniques being developed, picked up by the IVF community, and introduced into treatment before adequate data regarding efficiency and safety have been collected and presented. ICSI IVF is a case in point. Once the technique had been developed and published, it was rapidly incorporated into treatment regimes. It was offered to patients almost simultaneously with scientists trying to learn and adapt the procedure into the other work of the laboratory. There was very little time to assess the implications of using it and whether there were carry-on consequences for any of the children born as a result. Twenty-seven years on, it appears to be a very safe technology, but when first developed, that could not have been known or assumed.

Q6

Male infertility has recently been in the news regarding lack of adequate care. Do you agree with this perception and, if so, how do we improve this situation?

I completely agree with this perception. As I mentioned in answer to a previous question, when I began my career in AHR, there were very few options for treating male infertility. The use of donor sperm was the most common treatment offered. I believe that very little consideration was given to how the man might feel about not being the genetic father of his children. Most of my early research involved the study of the genetic causes of male infertility; latterly I became interested in the psychological effects of treatment on the male partner, both fertile and infertile. Many male patients reported that they felt their role in treatment was primarily supportive, which meant having to relinquish control of their emotions and 'put on a brave face'. Many felt left out when it came to the treatment regimen, with the primary consideration given to the female partner and her concerns. They felt excluded. Perhaps they should be offered separate counselling. During a treatment cycle, it is not unusual for the female partner to attend her scans alone, to avoid both having to take time off work. In these cases, the female partner may build up stronger relations with the unit staff, if the male partner does not attend, he loses out on these relationships and may feel excluded.



Q7 What are the pressing ethical issues facing the field of IVF today?

The speed with which new scientific procedures are introduced into treatments, many unproven from a safety perspective. Gene editing procedures need to be approached very cautiously, as there may be unseen consequences for the whole genome. There has been a lot of discussion about designer babies. In the not-too-distant future, couples may be able to choose specific phenotypic characteristics for their potential children, such as eye or hair colour, intelligence, and sex. A genetically deaf or blind couple may wish to have a genetically deaf or blind child. These choices may be the couple's but would they be the child's choice once they reach the age at which they can make autonomous decisions for themselves? Is it possible that the child might decide to take legal action against his/her parents or the treating clinic for allowing a disorder to be passed to him/her?

AHR has always been an ethical minefield, and there are always going to be ethical dilemmas and questions for those working in the area. When IVF began in 1978, it was denounced as unethical, evil, and against the laws of nature. The same goes for embryo cryopreservation, ICSI, pre-implantation genetic diagnosis, and pre-implantation genetic screening. It could be argued that all were introduced with undue speed, yet all these treatments are now routinely offered to couples. I do not know what new treatments or procedures will be developed or offered in the future, but I do know that there will always be ethical or moral objections to them.

Q8 In terms of your professional life, what have been some of your most satisfying moments?

Seeing the proud faces of people coming back to the unit to show off their babies and children, and perhaps remembering their initial trauma at the thoughts of having to have IVF. Also, learning new techniques and successfully applying them. I cannot think of specific satisfying moments. Satisfaction was in the job, the clinical work, and the patient interaction; it was in the ability to hold an egg cell (the size of a speck of dust) in one hand and a sperm cell (100 times smaller) in the other, to inject the sperm into an egg, see fertilisation, achieve an embryo, and watch it develop into a viable pregnancy and baby: to be there at the very beginning of a human life. That is a privilege not many people get to experience.

Q9 Finally, is there any advice you would like to share with those thinking of beginning a career in IVF?

IVF is a career, not a job. Although I am retired, I have had 30 wonderful years working in the area of AHR. There were times when I could not believe that I was being paid to do what I did. I looked forward to going into work in the morning and was often still in the laboratory long after the working day had ended. Thinking of a career in IVF? I started when it was a relatively new career path, and from the beginning I was lucky to be working with some wonderful people, friends I made at the very beginning of my career are still my friends today. Like many jobs and careers, it can be very stressful, the hours can be very long, and the pay can be relatively poor. As an embryologist, you will not live in a mansion, but the rewards are worth every last minute spent in the laboratory.

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History of Contraception

The History of Non-Barrier Contraception

Source: FPA, 2010; Planned Parenthood, 2015; Preceden LLC, 2019; International Agency for Research on Cancer, 1999

1909 — R Richter designs first IUD – a ring of silkworm gut inserted into the uterus

1929 — E Gräfenberg develops silver suture IUD

1950 — **WORK BY G PINCUS, J ROCK, AND C DJERASSI LEADS TO DEVELOPMENT OF FIRST ORAL CONTRACEPTIVES (OCP)**

1953 — K Junkman synthesises first injectable progestogen

1956 — Large scale clinical trials begin for OCP

1961 — Oestrogen/progestogen OCP become available in Great Britain on NHS for married women

1966 — The development of the implant begins in the USA as hormone-filled capsules

1967 — OCP becomes available on NHS for all British women. Worldwide, the number of OCP users nears 13 million

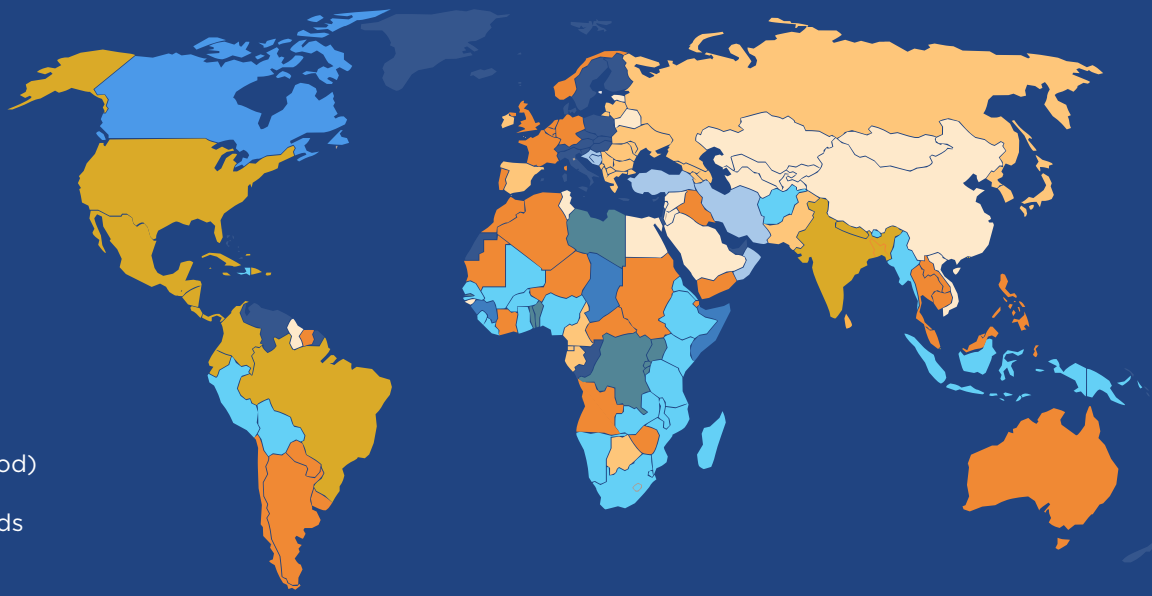
1968 — Introduction of copper IUD

1981 — Desogestrel OCP with new progestogens is developed, introducing the 'third generation' of OCP

Top Method of Contraception per Country

Source: United Nations, World Contraceptive U

- Oral Contraceptive pill
- Female sterilisation
- Male condom
- IUD
- Male sterilisation
- Injectable
- Withdrawal
- Rhythm (calendar method)
- Other traditional methods



Oral Contraceptives IUD Injectable Contraceptives Other

AN ESTIMATED 50–80 MILLION WOMEN ARE USING OCP WORLDWIDE

g PC4, the icensed rgency ception, is ed in Great ritain

1984

Injectable contraceptive Depo-Provera (the depo shot) obtains FDA approval

1992

THE ECONOMIST NAMES THE OCP ONE OF THE SEVEN WONDERS OF THE MODERN WORLD

1993

INTRODUCTION OF HORMONE RELEASING IUD

1996

YASMIN, THE COMBINED OCP, BECOMES FDA APPROVED

2001

The vaginal ring, NuvaRing, containing progestogen and oestrogen becomes available

The contraceptive patch Ortho Evra becomes available

2002

Emergency OCP, ellaOne, licensed in the EU for use <120 hours following unprotected sex

2009

Introduction of Skyla, a new low-hormone IUD; the first new IUD in 12 years

2013

Research/development of contraception continues + development of a male OCP

2019

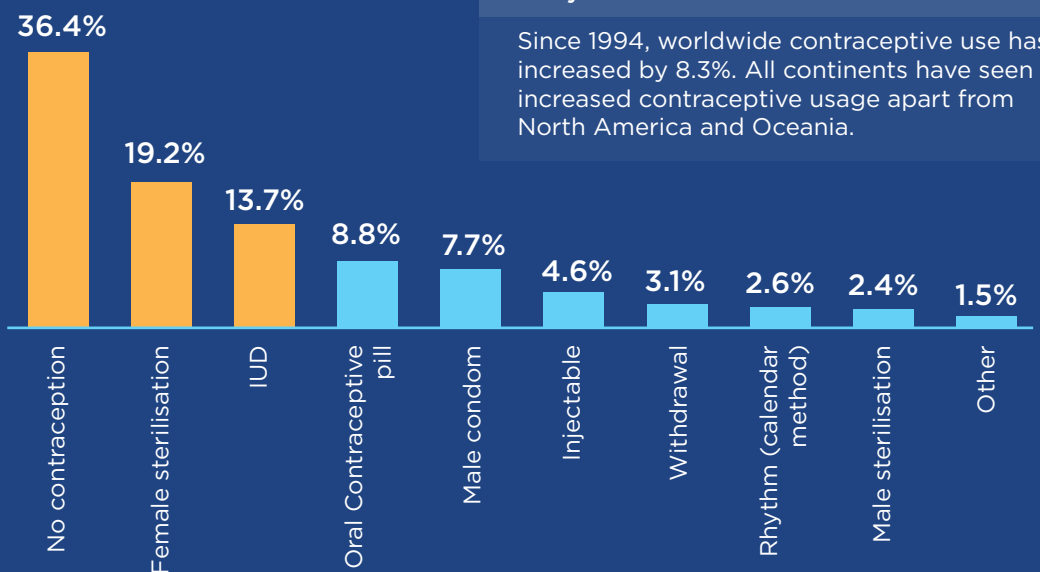
se, 2019

Contraceptive Use Around the World

Source: United Nations, World Contraceptive Use, 2019

Did you know:

Since 1994, worldwide contraceptive use has increased by 8.3%. All continents have seen increased contraceptive usage apart from North America and Oceania.



Abstract Reviews

ESHRE 2019 hosted an abundance of abstract presentations on a range of topics within reproductive health.

Predictive Value of the Sperm Zona-Adhesion Score for the Implantation Outcome

Authors: *Rumiana Ganeva, Dimitar Parvanov, Kristina Nikolova, Miriyana Gospodinova, Ivka Ivanova, Ivaylo Rangelov, Magdalena Vasileva, Georgi Stamenov

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Disclosure: The authors have declared no conflicts of interest.

Keywords: Implantation, male infertility, sperm test, sperm zona adhesion test, spermatozoa, zona pellucida.

Citation: EMJ Repro Health. 2019;5[1]:46-47. Abstract Review No. AR1.

ABSTRACT

One of the most frequent causes of male infertility is failure in sperm-zona recognition and adhesion, but that cannot be diagnosed by the routine semen analysis. Many tests have been developed to assess sperm functional abilities towards the zona.[1-3] It has been proven that these zona-binding tests are an effective method for predicting the fertilisation capacity of the spermatozoa and the outcomes of assisted reproductive technology.[4] However, the existing methods have some practical shortcomings, such as using different zona pellucida in each test, difficulties in sperm counting, and the necessity of a healthy donor.[4,5] The present study aimed to assess the predictive power of an optimised zona-adhesion assay and sperm zona-adhesion score (SZAS) on the implantation outcome.

Data on spermatozoa zona-adhesion ability and implantation success of 42 couples with male infertility factor and good quality oocytes were collected. Semen samples for standard intracytoplasmic sperm injection procedure were analysed for routine parameters according to the World Health Organization (WHO) 2010 guidelines and the SZAS was assessed. A zona-adhesion test was performed using donors' zonae pellucidae that were acid solubilised and coated on polystyrene plates. SZAS was evaluated in samples of 250,000 motile spermatozoa by calculating the number of adhered spermatozoa per mm^2 of zona-coated surface. Implantation was determined by peripheral blood human chorionic gonadotropin concentration on Day

14 after embryo transfer. Pearson correlation and t-test were used to analyse the results. Logistic regression with receiver operating characteristic (ROC) curve analysis were performed to evaluate SZAS as a predictor for the implantation outcome.

No statistical difference was observed in the common semen parameters (sperm count, concentration, motility, and morphology) between the successful (n=21) and unsuccessful (n=21) implantation groups. The evaluated SZAS did not correlate to the sperm concentration (p>0.05), sperm count (p>0.05), motility (p>0.05), or morphology (p>0.05) among the studied patients. However, as shown in Figure 1, the mean SZAS in the successful implantation group was found to be significantly higher (149±67) compared to the unsuccessful implantation group (84±37 [p=0.002]). By ROC curve analysis, SZAS predicted successful implantation with an area under the curve of 80.2% (95% confidence interval: 0.66–0.94). A cut off value for successful implantation was estimated at 65 with sensitivity 95.2% and specificity 52.4%. Moreover, logistic regression analysis identified SZAS as a significant parameter for the prediction of implantation outcome with 73.8% accuracy: 76.2% specificity and 71.7% sensitivity of the developed predictive model.

This study demonstrates the high predictive value of the sperm zona-adhesion ability for successful implantation after intracytoplasmic sperm injection. The evaluation of SZAS could be a useful tool in directing couples with male infertility to appropriate assisted reproduction therapy.

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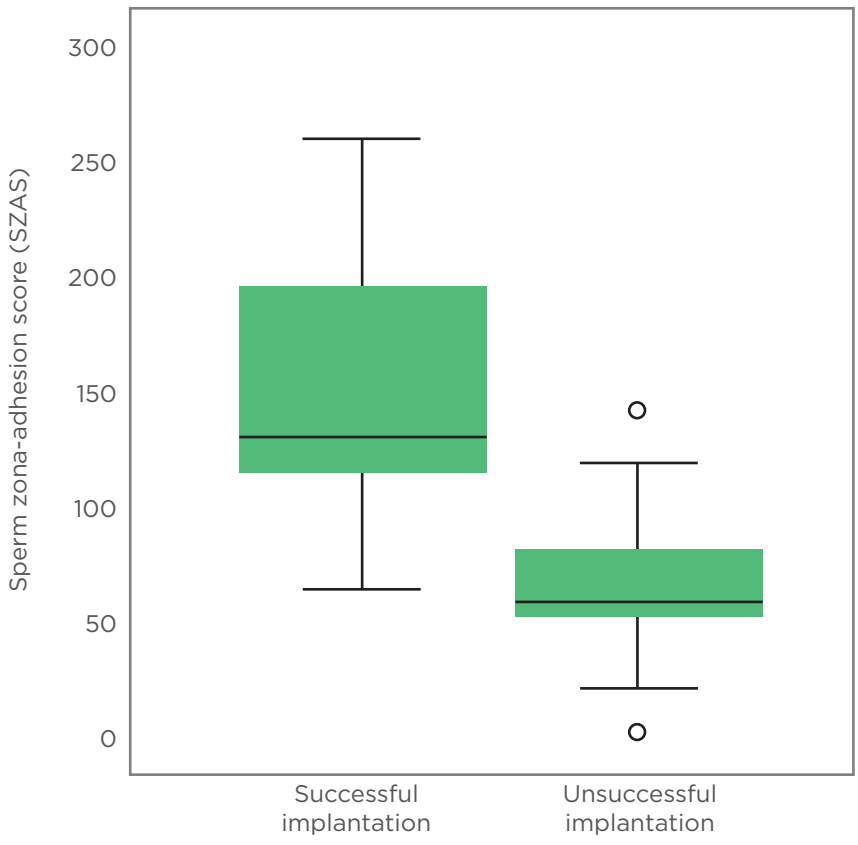

Figure 1: Sperm zona-adhesion score in the successful and the unsuccessful implantation groups.

Selection of Vacuole Free Spermatozoa with the Assistance of the Hyaluronic Acid-Binding Assay

Authors: *Nabil Saymé,[1] Kljajic Marija,[2] Thomas Krebs,[1] Dieter Maas[1]

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Disclosure: The authors have declared no conflicts of interest. This abstract has been approved by a local Institutional Review Board (IRB).

Keywords: Hyaluronic acid (HA)-binding, spermatozoa, sperm selecting, vacuoles.

Citation: EMJ Repro Health. 2019;5[1]:48. Abstract Review No. AR2.

BACKGROUND

The selection of normal spermatozoa during intracytoplasmic sperm injection does not enable the detection of nuclear defects. Sperm head nuclear abnormalities have previously been identified as vacuoles by motile-sperm organelle-morphology examination. In terms of the link between the presence of vacuoles and embryo development, it has been shown that the injection of morphometrically normal spermatozoa with no vacuoles is associated with significantly higher blastocyst rate, a smaller proportion of arrested embryos, and higher pregnancy rates.[1] According to previously obtained data in assisted reproduction, it is of importance to reliably select vacuole-free spermatozoa.[2]

METHODOLOGY

This was a prospective and blinded observational study. Hyaluronic acid (HA)-bound, standard morphologically (SM)-selected (200x), and unselected sperm (control) were collected from different persons. The evaluation of vacuoles was performed by Nomarski high-power differential interference contrast optics (600x–7,200x). The number of vacuoles in each sperm head was determined and the spermatozoa were placed into 4 groups: absence of vacuoles, the presence of one vacuole, the presence of two vacuoles, or the presence of >two vacuoles. Fifteen human semen samples were prepared by 80% density gradient. From each sample, a minimum of 20 spermatozoa per method (HA, SM selection) were collected in separated polyvinylpyrrolidone droplets. Additionally, 20 unselected sperm were collected from each sample and designated as controls. All samples were observed blind by the same person. Statistical significance was defined as $p<0.05$. One-way analysis of variance and post-hoc Tukey-Test were performed. All statistical evaluations were carried out using SigmaStat Version 3.5.

RESULTS

After completing statistical analysis, the number of spermatozoa without vacuoles found in HA-selected ($p<0.001$) and SM-selected ($p<0.01$) spermatozoa were significantly higher compared to the unselected. The number of sperms with one ($p<0.05$), two ($p<0.01$), and >two vacuoles ($p<0.001$) was significantly higher in the unselected spermatozoa. Furthermore, in HA-selected spermatozoa, the appearance of two and >two vacuoles was significantly lower compared to the SM-selected spermatozoa ($p<0.050$). Although the time necessary for the isolation of individual spermatozoa from each sample was one of the limiting factors for this study, both selection methods provided spermatozoa which contained a smaller number of vacuoles compared to the unselected samples, especially in the group with >two vacuoles. Therefore, HA selection may be an effective method to identify spermatozoa with a higher fertilisation potential to improve results in assisted reproductive technology procedures.

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The Effect of Advanced Paternal Age on Sperm Parameters and on the Outcome of *In Vitro* Fertilisation Treatments

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Keywords: Advanced paternal age, blastulation rate, fertilisation rate, intracytoplasmic sperm injection (ICSI), *in vitro* fertilisation (IVF), paternal ageing, pregnancy rate, sperm count, sperm motility, sperm parameters.

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BACKGROUND

Over the last decade, delayed childbearing has become a trend in developed countries. It is proven that advanced maternal age plays a dominant role in determining the success of *in vitro* fertilisation (IVF) treatments.[1] However, the impact of advanced paternal age on IVF outcomes is still poorly known. Recent studies found that a man's age can affect testicular functions and sperm parameters.[2,3] Paternal ageing was also found to be associated with increased incidence of DNA damage, chromosomal aberrations, and embryonic aneuploidy.[4] These can lead to decreased fertilisation and blastulation rate or increased miscarriage rate and thus a decreased live birth rate.[3,5,6] The following was investigated in this study: how does advanced paternal age affect sperm parameters and the outcome of intracytoplasmic sperm injection (ICSI) cycles.

METHOD

Data were collected retrospectively on all couples with a female age <35 years at the authors' private fertility clinic between January 2013 and December 2018. A total of 135 fresh embryo transfer cycles were analysed; two groups were formed based on the paternal age: <40 years group and ≥40 years group. Evaluation of all semen parameters was done according to World Health Organization (WHO) standard criteria (2010).[7] All embryos were fertilised by ICSI using fresh, frozen, or testicular sperm extraction sample. In the <40 years group (average age 34.00±3.79), 101 fresh blastocyst transfers were carried out, while in the ≥40 years group (average age 43.18±2.47), 34 blastocyst transfers were carried out. Cycles with pre-implantation genetic testing were excluded from the analysis. The measured IVF outcomes were fertilisation rate, blastulation rate, implantation rate, clinical pregnancy rate, and miscarriage rate.

RESULTS

The mean maternal age did not differ between the two examined groups (31.43±3.32 in the <40 years group and 31.76±4.29 in the ≥40 years group). The mean initial sperm count was significantly higher in the <40 years group than in the ≥40 years group (58.87 million/mL versus 44.38 million/mL, p=0.0464), and sperm motility was also significantly higher in the <40 years group (49.98% versus 37.18%, p=0.0054). The fertilisation rate was slightly higher in the <40 years group (70.11%) but not significantly different from the ≥40 years group (66.67%, p=0.4371). However, the authors found a significantly higher blastulation rate in the <40 years group compared to the ≥40 years group (60.87% versus 56.18%, respectively, p=0.02). The implantation rate (41.22% [54/131] versus 52.17% [24/46], p=0.1689) and clinical pregnancy rate (42.57% [43/101] versus 61.76% [21/34], p=0.0734) did not differ significantly in the examined groups after IVF-ICSI. Additionally, no significant difference in case of miscarriage rates (22.22% [12/54] in <40 years group versus 16.66% [4/24] in ≥40 years group, p=0.7634) was found.

CONCLUSION

Based on these results, the quality of sperm seems to decline with age, which demonstrates that male fertility also has a defined lifetime. However, IVF-ICSI can compensate for this. With the studies findings, the authors would like to draw men's attention to the increasing health risks associated with late childbearing.

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Effect of a High-Fat Diet on Male Fertility and Sperm Physiology in High Fertility Performance Mice

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Keywords: High-fat diet (HFD), fertilisation, male fertility, metabolic syndrome (MetS), sperm.

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The prevalence of metabolic syndrome (MetS) has increased in alarming proportions, affecting around 20–25% of the global population.[1] MetS has been defined as an increase in at least three of the following factors: abdominal obesity, blood pressure, triglycerides, cholesterol, and fasting glucose.[2] Although MetS had been originally associated with advanced age, changes in lifestyle have accelerated the appearance of the symptoms, coinciding with reproductive age and becoming a risk factor for fertility disorders. Therefore, the study of the effect of MetS on fertility emerges as a novel area of research. However, a direct correlation of MetS with male infertility still remains unclear. In the case of the mouse model, most of the studies published so far have been performed in the C57BL/6 strain.[3] Considering both that C57BL/6 mice are poor reproducers and that the multifactorial syndrome could be attributable to susceptibility genes modulated by the genetic background, the authors wondered whether the acquisition of a metabolic disorder could affect the fertility of males from a different mouse strain without pre-existing reproductive deficiencies. In view of this, the present study evaluates whether MetS

has a negative impact on fertility and sperm function of hybrid male mice with high reproductive performance.

To induce a MetS-like condition, C57BL/6xBALB/c F1 (B6CF1) male mice were fed a high-fat diet (HFD, 30% fat) for 19 weeks, while controls received a normal-fat diet (NFD, 6% fat). HFD-fed mice ingested a higher amount of fat ($p<0.01$) but less total food ($p<0.01$) and only 12% more calories than NFD-fed animals ($p<0.05$), indicating that HFD-fed animals received a poor-quality diet. Since Week 11 of treatment, HFD-fed mice gained more weight compared to NFD-fed mice ($p<0.001$). At the end of the treatment, serum triglyceride levels were similar in both groups, but there was a significant increase in cholesterol ($p<0.001$), fasting glucose levels ($p<0.05$), and glucose intolerance ($p<0.05$) in HFD-fed mice, compatible with MetS acquisition. When fertility was evaluated, there was no significant difference between groups in the *in vivo* fertilisation rate or in the percentage of embryos that developed *in vitro* to blastocysts. While testicular weight and morphology were similar in both groups, HFD-fed mice presented lighter epididymides and higher amounts of gonadal fat compared to controls ($p<0.01$). *In vitro* studies were performed as a more restricted condition to unveil sperm defects. Whereas there was no difference in sperm viability, motility, or acrosome reaction between groups, sperm count was lower in HFD-diet mice compared to control ($p<0.05$). Finally, *in vitro* fertilisation assays showed no differences in either fertilisation or embryo development rates between groups.

In summary, HFD-fed B6CF1 animals developed a physiological condition compatible with MetS. However, this metabolic condition did not impact on the reproductive performance of hybrid B6CF1 animals, albeit a significant decrease in sperm count. These findings support the possibility that fertility impairment in humans could be the result of a combination of different environmental and genetic factors that may act in a cumulative manner with other predisposing factors from any of the two members of the couple.

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Increasing Paternal Age and Ejaculatory Abstinence Length Negatively Influence the Outcomes of Intracytoplasmic Sperm Injection in an Egg-Sharing Donation Programme

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Keywords: Egg-sharing, infertility, intracytoplasmic sperm injection (ICSI), paternal age, pregnancy.

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INTRODUCTION

Infertility is experienced by an estimated 48.5 million couples worldwide.[1] Even though male factor infertility is known to play a role in 50% of the cases, the impact of male partner characteristics on *in vitro* fertilisation (IVF) is often ignored.[2] Indeed, male factor infertility is equally important for establishing the success of assisted reproduction cycles. However, few studies have focussed on the influence of male factors on IVF outcomes, with those that have producing conflicting results,[3-6] probably due to confound variables introduced in the analysis regarding autologous cycles. Considering this, the use of an egg-sharing donation programme may be extremely useful for studying the impact of partner characteristics and semen parameters on intracytoplasmic sperm injection (ICSI) outcomes. The objective of this study was to investigate if paternal age, ejaculatory abstinence length (EA), and semen quality influence ICSI outcomes in recipients' cycles in an egg-sharing donation programme.

MATERIAL AND METHODS

This study was performed in a private university-affiliated IVF centre. Data analysed in this historical cohort study were obtained via chart review of 268 vitrified oocyte donor ICSI cycles, as well as 321 oocyte recipients undergoing 427 oocyte recipient ICSI cycles, participating in an egg-sharing donation programme between January 2015 and May 2017. For this sample size, computed achieved post-hoc power was 95.7%. Oocyte donors were between the ages of 19 and 35 years old, and recipients were between the ages of 26 and 59 years old. General mixed models fit by restricted maximum likelihood, generated using covariates as fixed effects and egg-donors and egg-recipients as random effects, with unstructured covariance structure, were used to investigate the impact of paternal age, EA, and semen quality on recipients' ICSI outcomes. The results are expressed as regression coefficient (B), standard error (SE), exponentiation of B (ExpB), 95% confidence interval (CI), and p-value (P).

RESULTS

Fertilisation rate was negatively affected by paternal age and positively affected by sperm count. High-quality embryo rate on Day 3 was negatively correlated with paternal age and EA. Normal embryo development (cleavage speed) rate on Day 3 was negatively affected by paternal age and EA, and positively affected by the percentage of progressive sperm motility. Blastocyst development rate was negatively influenced by paternal age and EA, and positively influenced by sperm count and total motile sperm count. Paternal age was negatively correlated with high-quality blastocysts rate. Implantation rate was negatively affected by paternal age and EA, and positively affected by sperm count, progressive sperm motility, and total motile sperm count. Paternal age was associated with reduced odds of pregnancy. These results are expressed in Table 1.

CONCLUSION

Increasing paternal age and EA, as well as poor semen parameters, negatively impact ICSI outcomes, from fertilisation rate to pregnancy, in recipients' cycles. Further tracking of the impact of paternal characteristics on ICSI outcomes should be encouraged. Despite the fact paternal age is uncontrollable, and there are only so many things that can be done concerning semen quality, shortening of EA length could be used as a strategy to optimise ICSI outcomes.

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Table 1: General mixed model results for the associations between paternal factors and intracytoplasmic sperm injection outcomes.

Paternal variable		Fertilisation (%)	D3 high-quality embryos (%)	D3 normal embryo development (%)	Blastocyst development (%)	High-quality blastocysts (%)	Implantation (%)	Pregnancy chance	Miscarriage chance
Age	B	-0.276	-0.040	-2.750	-0.070	-44.058	-0.060	Exp(B) 0.664	Exp(B) 1.019
	SE	0.085	0.017	0.8625	0.035	20.248	0.007	0.187	0.052
	CI	-0.442 – -0.110	-0.072 – -0.006	-4.441 – -1.059	-0.139 – -0.002	-84.065 – -4.051	-0.075 – -0.045	0.457 – 0.967	0.918 – 1.131
	P	0.001	0.021	0.001	0.043	0.031	<0.001	0.033	0.718
EA	B	-0.083	-0.003	-0.300	-0.589	13.8125	-0.012	Exp(B) 0.051	Exp(B) 0.861
	SE	0.847	0.015	0.014	0.243	88.143	0.003	1.803	0.190
	CI	-0.442 – -0.110	-0.006 – -0.001	-0.058 – -0.020	-1.067 – -0.111	-160.341 – 187.966	-0.203 – -0.353	0.001 – 1.870	0.589 – 1.258
	P	0.765	0.028	0.036	0.016	0.876	<0.001	0.103	0.435
Sperm count	B	0.075	2.296	-0.884	2.155	-36.970	0.025	Exp(B) 0.920	Exp(B) 1.002
	SE	0.020	7.074	0.568	0.884	27.177	0.003	0.167	0.019
	CI	0.035 – 0.115	-11.587 – 16.179	-1.999 – 0.232	0.420 – 3.891	-90.666 – 16.727	0.020 – 0.031	0.658 – 1.284	0.966 – 1.040
	P	<0.001	0.746	0.120	0.015	0.176	<0.001	0.617	0.901
Sperm motility	B	-0.003	-1.573	0.017	0.412	-5.955	0.183	Exp(B) 1.037	Exp(B) 1.033
	SE	0.0462	20.270	0.077	0.586	5.453	0.010	0.031	0.024
	CI	-0.093 – 0.088	-41.352 – 38.206	0.002 – 0.032	-0.739 – 1.563	-16.729 – 4.819	0.163 – 0.204	0.974 – 1.104	0.984 - 1.084
	P	0.951	0.938	0.024	0.483	0.277	<0.001	0.253	0.191
TMSC	B	-0.007	2.841477	-2.914	1.057	9.779	0.008	Exp(B) 0.957	Exp(B) 0.995
	SE	0.030	2.297297	2.327	0.508	6.442	0.003	0.062	0.012
	CI	-0.065 – 0.051	-1.667 - 7.350	-7.480 – 1.652	0.060 – 2.054	-2.949 – 22.508	0.002 – 0.014	0.845 – 1.083	0.972 – 1.019
	P	0.809	0.216	0.211	0.038	0.131	0.009	0.475	0.659

B: unstandardised regression coefficient; CI: confidence interval; D3: Day 3; EA: ejaculatory abstinence; Exp(B): exponentiation of B; P: p value; SE: standard error; TMSC: total motile sperm count.

Low Serum βhCG Concentration on Outcome Day (Trigger Day +17) Identifies a Subgroup of Women at Risk of Poor Perinatal Outcome

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ABSTRACT

Pregnancies following assisted reproduction are at higher risk of poorer perinatal outcome.[1] Algorithms combining age and various maternal serum hormone concentrations have been used to predict adverse perinatal outcome.[2,3] Human chorionic gonadotropin (hCG) is the first known hormonal signal by the embryo, promoting embryo/endometrial molecular cross-talk, cytotrophoblast proliferation, and subsequent invasion.[4]

In the *in vitro* fertilisation setting, the exact date of conception is known so it is possible to perform a quantitative βhCG test on a specific pre-determined day: 17 days after the pre-oocyte retrieval trigger injection known as trigger day +17. A positive test can be defined as βhCG >10 IU/L. All cases from the authors' single centre from fresh stimulated assisted reproductive technology cycles from 2008–2016 inclusive were examined (N=1,846). Only cases in which the βhCG was performed on trigger day +17 were analysed.

Analyses showed that if the serum βhCG concentration was <30 IU/L (n=52), the chances of a continuing pregnancy at 8 weeks gestation was only 2%. If the βhCG concentration was 30–50 IU/L (n=68) it was 24% and if the βhCG concentration was 50–70 IU/L (n=57) it was 33%. This contrasted with 86% if the βhCG concentration was >70 IU/L (n=533, p<0.0001).

In addition to promoting the corpus luteum of early pregnancy, hCG contributes to the maternal tolerance of the embryo via T cell modulation and influences the degree of macrophage migration of the endometrial stromal cells.[5,6] However, failure of these mechanisms in early pregnancy may result in miscarriage, which is in keeping with the authors' observations, and can also reflect poor placentation leading to subsequent adverse perinatal outcomes.[7] Hence, an initial low βhCG concentration in a viable pregnancy may be an indicator of subsequent poorer perinatal outcome. To test this, the authors assessed the pregnancy outcomes in these patients.

Overall, only 20% of the cohort with initial βhCG <70 IU/L on trigger day +17 had an ongoing singleton pregnancy (n=36), as opposed to 86% of the women with initial βhCG >70 IU/L (n=533). Only outcomes from singleton pregnancies were examined to exclude potential bias associated with multiple pregnancy (Table 1).

There was no difference in maternal characteristics or gestational age at delivery. However, singletons in the low βhCG group were more likely to be born preterm, had a lower birthweight centile for gestational age, and were more likely to be small for gestational age. There were also more stillbirths in the low βhCG cohort (n=3, 8.0%) than in the higher βhCG cohort (n=3, 0.6%; p=0.0040). These data indicate that a critically timed, initial βhCG concentration may be a reasonable guide to early pregnancy viability and allows counselling of patients and management of their expectations accordingly. Furthermore, in viable pregnancies, it identifies a subgroup at the beginning of pregnancy who are at especially high risk of poor perinatal outcome: greater risk of stillbirth, preterm delivery, and being small for gestational age. This subgroup of women merits closer assessment during pregnancy to optimise the ultimate pregnancy outcome.

Table 1: Perinatal outcomes of singleton pregnancies.

Characteristic	βhCG <70	βhCG >70	P Value
n	36.0	533.0	N/A
Age (years)	35.5 (5.8)	35.6 (5.7)	0.500 (-0.900 – +1.900)
Maternal BMI (kg/m^2)	24.7 (5.0)	23.1 (4.8)	0.06 (-2.700 – +0.100)
Maternal weight >85 kg	6.0 (17%)	42.0 (8%)	0.100 (-0.200 – +0.040)
AMH (pmol/L)	21.3 (13.7)	19.2 (24.7)	0.600 (-6.000 – +3.600)
Fetal sex: Male (%)	19.0 (53%)	276.0 (52%)	1.000 (-0.200 – +0.200)
Gestation at delivery (days)	273.0 (17)	273.0 (13)	0.080 (-0.000 – +7.000)
Preterm delivery (<37 weeks)	10.0 (28%)	70.0 (13%)	0.020 (-0.300 – +0.003)
Birthweight (g)	3225.0 (839)	3402.0 (652)	0.008 (+70.000 - +454.000)
Birthweight centile for gestational age	39.0 (39)	52.0 (46)	0.007 (+4.000 - +25.000)
SGA, number (%)	8.0 (22%)	42.0 (8%)	0.009 (-0.300 – -0.010)
LGA, number (%)	3.0 (8%)	51.0 (10%)	1.000 (-0.100 – +0.100)

All continuous variables expressed as median (interquartile range in parentheses). Categorical variables expressed as number (% in parentheses).

Comparisons of continuous data was carried out by the Mann-Whitney U-test; comparisons of categorical data was carried out by Chi-squared analysis (95% confidence intervals of the differences in parentheses).

AMH: Anti-Müllerian hormone; BMI: body mass index; SGA: small for gestational age (birthweight <10th centile for gestational age); LGA: large for gestational age (birthweight >90th centile for gestational age).

hCG plays a crucial role in angiogenesis, cytotrophoblast invasion, and immune tolerance,[8] so it is perhaps not surprising that it is an early marker of subsequent pregnancy outcome. These observations identify a high-risk group and provide a potential model for future research into implantation and pregnancy development.[9]

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Anti-Müllerian Hormone as a Quantitative and Qualitative Marker of Euploid Blastocysts

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Anti-Müllerian hormone (AMH) is widely used in clinical practice to predict the ovarian response after ovarian stimulation and is considered as valid as antral follicle count.[1] AMH has also been proposed as a qualitative marker of the reproductive competence of the oocyte or embryo, although some discrepancies have been described.

It has been postulated that, independent from age, ovarian reserve is associated with an increased rate of euploid blastocysts among patients with a normal response to ovarian stimulation.[2] On the other hand, when stratifying patients <38 years old, no significant differences in blastulation and aneuploidy rate has previously been observed in patients with diminished and reduced ovarian reserve.[3]

The purpose of this study was to evaluate, irrespective of age, the association between AMH values and the proportion of euploid embryos and blastocyst formation among patients who underwent intracytoplasmic sperm injection with pre-implantation genetic testing for aneuploidies (PGT-A).

A retrospective analysis was performed between March 2017 and August 2018 including couples who were planned for PGT-A. Patients were split into two groups and were analysed individually: the fresh group which comprised couples who underwent PGT-A with only fresh oocytes (n=516), and the vitrified group (n=184) in which vitrified oocytes were accumulated from 1.97 (±1.26) previous ovarian stimulation cycles, as a strategy to increase the number of potential euploid embryos. Vitrification and warming were performed with the Cryotop method (Kitazato, Biopharma). Trophectoderm biopsy samples were subjected to next generation sequencing to screen the cells. AMH serum levels (ng/mL) were determined using a commercial, fully automated Elecsys® assay (Roche) and values >5 ng/mL were excluded. Blastulation rate was defined as the number of fertilised embryos capable of cavitating on Day 5.

Linear regression analysis was conducted to verify the predictability of AMH values and the percentage of euploid embryos and blastulation rate on Day 5. A Poisson regression model was used to correlate AMH levels with the number of euploid embryos according to the number of embryos biopsied.

In the fresh group, the average maternal age was 35.8 years (±5.95), AMH 1.95 ng/mL (±1.27), 54% (±33%) blastulation rate on Day 5, 46% (±35%) euploid rate. Higher AMH values were found to have a statistically significant effect on the

percentage of euploid embryos (p=0.001) and blastocyst formation on Day 5 (p<0.001), as well as for the number of euploid embryos (p<0.001).

In the vitrified group, average maternal age was 38.6 (±5.35), AMH 1.2 ng/ml (±1.06), 8.43 (±5.57) metaphase II oocytes warmed, 86% (±21%) survival rate, 34% (±33%) blastulation rate on Day 5, 31% (±39%) euploid rate. As in the fresh group, higher AMH values were found to have a statistically significant effect on the percentage of euploid embryos (p=0.009) as well as for the number of euploid embryos (p=0.003). However, no significant difference was found between higher AMH levels and blastocyst formation (p=0.249).

The independent relationship between AMH and the percentage of euploid embryos suggeststhat AMH is not only a quantitative but also a qualitative biomarker of oocyte-embryo competence. As the effect of AMH on blastocyst formation is lost after oocyte vitrification, the use of oocyte accumulation should be further evaluated.

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Single Nucleotide Variations of Mitotic Arrest Deficient 1 Like 1 (*MAD1L1*) and *MAD2L1* Genes in Products of Conception with Aneuploidy

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Keywords: Aneuploidy, *MAD1L1*, *MAD2L1*, miscarriage, single nucleotide variation (SNV).

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BACKGROUND

Miscarriage is a pregnancy loss before the 20th week of the gestation.[1] At least 15% of the recognised pregnancies will be terminated by spontaneous miscarriage.[2] Several factors such as immunological, genetic, environmental endocrine, anatomical, and thrombophilic factors can be involved in miscarriage.[1,3] It is propounded that approximately 35% of abortions are due to aneuploidies.[4] Although fetal aneuploidy is associated with maternal age, incidents can also

be found in young women.[5] Despite adequate knowledge on molecular factors related to aneuploidy, such as cyclin-dependent kinase 1, cohesin, separase, anaphase promoting complex, and spindle assembly checkpoint (SAC),[6,7] aneuploidy itself is supposed as the reason of miscarriage without its molecular origin being considered. SAC complex has a critical role in fidelity of chromosome segregation by the control of attachment/detachment between chromosomes and the spindles through a delay in the initiation of anaphase. This complex prevents alteration in the copy number of chromosomes. It is believed that several genes are involved in SAC, such as *MAD1L1*, *MAD2L1,* and *BUB1*.[7] Decreased level of BUB1 and MAD2 proteins has been reported in clinical samples of aborted fetuses.[8] Another study identified the deletion of the last part of the *MAD2L1* genes in primary fibroblast cultures of trisomic miscarriage. [9]

METHODS

Using Sanger sequencing, exonic regions of *MAD2L1* and exons 4 and 18 of the *MAD1L1* gene in aneuploid fetuses were studied for the located pathogenic single nucleotide variations (SNV) rs121908981 and rs121908982. To select the samples, products of conception of mothers <36 years of age were analysed by using array quantitative fluorescence PCR (QF-PCR) and/or comparative genomic hybridisation (aCGH). Those with aneuploidy were enrolled in genotyping. The frequencies of observed SNV were compared with the highest population minor allele frequency (MAF) using Chi Square test. The probable interpretation of the effect of SNV was predicted using seven predictor tools.

RESULTS

Following the results from QF-PCR and aCGH, 40 aneuploid samples were enrolled in genotyping. Targeted pathogenic SNV in *MAD1L1* were not observed, but rs1481591257, rs372373978, rs752408355, rs10257349, rs10260386, rs1639921, and rs74431414 SNV in *MAD1L1* with the following allele frequencies and p values in comparison with the highest population MAF was detected: C:0.975/T:0.025 (p=0.1775), C:0.9875/T:0.0125 (p<0.0001), T:0.975/C:0.025 (p<0.0001), A:0.4/G:0.6 (p=0.4711), T:0.69/C:0.31 (p<0.0001), A:0.8875/G:0.1125 (p<0.0001), and C:0.9625/A:0.0375 (p=0.6941), respectively. rs758373815, rs903147, rs752146697, rs2908989, rs2908990, and rs78047690 SNV in *MAD2L1* with the following allele frequencies and p values in comparison with the highest population MAF was observed: A:0.9875/C:0.0125 (p<0.0001), G:0.61/T:0.39 (p=0.0667), C:0.9625/T:0.0375 (p<0.0001), T:0.425/C:0.575 (p=0.1085), T:0.4875/C:0.5125 (p=0.8231), and C:0.975/T:0.025 (p<0.0001), respectively.

CONCLUSIONS

Frequency of the following rare deleterious SNV were higher than their highest population MAF: rs752146697 in *MAD2L1*, rs752408355 in *MAD1L1*, and rs78047690 in *MAD2L1*. The rs752146697 SNV is a synonymous variant (Q121=) related to codon preference and the two other SNV are missense mutations with the following changes; rs752408355: K619R (Lys619Arg) and rs78047690: R133K (Arg133Lys). The finding of deleterious SNV within genes contributed to chromosome segregation leading to selection of healthier embryos to be transferred through preimplantation genetic testing.

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BESST, a Non-Invasive Computational Tool for Embryo Selection Using Mass Spectral Profiling of Embryo Culture Media

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The current gold standard of embryo evaluation is based on morphology and morphokinetics, however there have been many efforts to design non-invasive tests to assess embryo viability,[1] including imaging of embryo metabolism by fluorescence lifetime imaging microscopy and high-resolution nuclear magnetic resonance for metabolic biomarker detection. The authors have previously demonstrated that Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-ToF) mass spectrometry offers an immediate, sensitive, and straightforward analysis of spent embryo culture fluid to identify proteins present.[2] This approach can now be used to confirm pregnancy outcome retrospectively and give a confirmation of embryo viability within minutes.[3] In this study, the authors asked if a non-invasive mass spectrometry-based tool could be used to identify embryos that result in ongoing pregnancy.

A retrospective cohort study was carried out which included 1,190 spent media samples from embryo cultures collected from a single *in vitro* fertilisation clinic in the USA. The samples were collected between March 2014 and March 2018. The outcomes were intrauterine pregnancy, no implantation, biochemical pregnancy, and spontaneous abortion. Furthermore, outcomes from genetic testing for aneuploidy resulting in euploid and aneuploid results were also found. Only spectra from fresh, single transfer embryos with clearly positive (n=57) or negative (n=38) outcome were used in the development of the tool.

Upon receiving frozen embryos, the embryo culture media was thawed, diluted, and analysed using MALDI-ToF mass spectrometry in a laboratory in the UK. Analysis was performed using CHCA matrix, with spectra obtained in a mass range of 200–2,000 m/z. Data files were subject to semi-quantitative computational analysis using Python programming language. Raw data was pre-processed using a fully automated and fast computational approach previously developed for urine and further adapted to culture media data to render comparable analysis.[4]

The Blastocyst and Embryo Screening and Selection Tool (BESST) was developed following the analysis of embryo culture media from a single, fresh transfer embryo culture media. The previously developed automated computational workflow was applied to generate a reference spectral pattern of ideal embryo profile with chosen samples.[4] Criteria for sample selection included highest quality scores as determined by Blastocyst Quality Score; a numerical blastocyst-morphology grading system; ongoing pregnancy outcome; and preimplantation genetic testing for aneuploidies.

Similarities to the reference pattern were computed, based on peak positions and intensity values, assigning a score for each embryo. The resulting complex, non-linear scores were subject to cluster analysis and subsequently mapped into five distinct classes 0–5 with continuous numerical values, which can be interpreted linearly. With this method, the authors were able to identify 76.9% of ongoing pregnancy cases for embryos that score >4, while a score of <1.5 predicts that the embryo has a 35.7% chance of ongoing pregnancy (Figure 1).

It is important to note that results of or close to 100% cannot be achieved due to an underlying

confounding factor in which there are cases of unreceptive endometrium, regardless of the quality of the embryo. In light of this consideration, the implantation potential of 76.9% shown here is a favourable result.

When applied prospectively, immediately prior to embryo transfer, the BESST test could enable the objective, consistent, and non-invasive analysis of the likelihood of achieving ongoing pregnancy and, compared to morphology alone, could be optimised to see improvement in pregnancy rates for any clinic using the tool.

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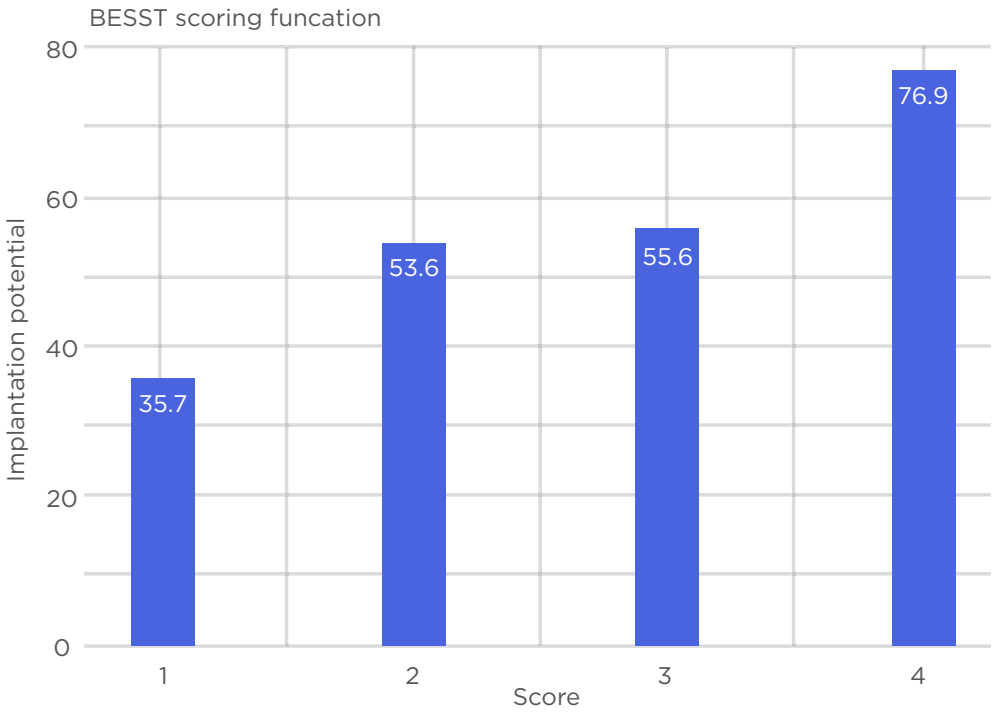

Figure 1: Bar chart representing four distinct scores of BESST.

A higher score indicates a higher chance of successful pregnancy (76.9%) and a lower score indicates a lower chance of successful pregnancy (35.7%).

BESST: blastocyst and embryo screening and selection tool.

To Analyse the Incidence of Ectopic Pregnancy in Fresh Embryo Transfer Compared with Frozen-Thawed Embryo Cycles in a Tertiary Infertility Centre in India

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Keywords: Ectopic pregnancy (EP), fresh embryo transfer, frozen-thawed embryo transfer (FET).

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In vitro fertilisation (IVF) is one of the major risk factors for an ectopic pregnancy (EP). Rates of EP in women undergoing IVF range from 2–5%, which is higher than the rate among spontaneous pregnancies at 1–2%.[1] In fact, the first IVF pregnancy reported was also an EP.[2] The main risk factors for EP following the use of assisted reproductive technology (ART) are tubal factor infertility, uterine contractions or dysfunction of uterine musculature due to supra physiological hormone levels because of ovarian stimulation, high culture medium volume, or a faulty embryo transfer. Recent research has suggested that frozen-thawed embryo transfer (FET) is associated with a greatly reduced incidence of EP compared with fresh transfers. The current study was undertaken to evaluate the incidence of EP in fresh and frozen embryo transfer at a tertiary fertility centre in India.

A prospective cohort study was carried out through 1st January 2016 to 31st December 2018 at Akanksha IVF Centre, New Delhi. A total of 671 fresh cycles and 767 FET cycles were included for analysis. A maximum of two embryos were transferred under ultrasound guidance by the single operator. For the 671 fresh IVF-ET cycles, 286 patients had clinical pregnancies and 20 patients had EP, and out of 767 FET cycles, 375 patients had clinical pregnancy and 10 patients had EP. The clinical pregnancy rate in fresh IVF-ET cycles was 42.62% and in FET cycles was 48.89%, which was statistically significant (p=0.02). The incidence of an EP per embryo transfer was also significantly higher for the fresh group (2.23% [20 of 671]) versus the FET group (1.04% [10 of 767] [p=0.042]). The majority of patients with EP had tubal factor as a cause of infertility. There was a statistically significant difference in EP rate in fresh versus FET cycles when Day 3 embryos were transferred (13 versus 5; p=0.05) but the difference was not significant when Day 5 embryos were transferred in the fresh and FET groups (7 versus 5; p=0.6).

This study suggests that FET is associated with significantly lower rates of EP compared with fresh cycles. The findings are consistent with database from The Society for Assisted Reproductive Technology (SART) registry published by Londra et al.[3] and Huang et al.[4] The authors also found that there was no difference in EP rates in fresh and frozen cycles when blastocysts were transferred. The main limitation of the study is that it was a cohort study at a single centre with a small sample size. Further multicentric trials are needed to validate the findings.

In conclusion, FET is associated with significantly lower rates of EP compared with fresh cycles. These findings suggest that increased chance of EP is due to disturbed hormonal milieu of ovarian stimulation. Freeze all strategy followed by FET would decrease the incidence of EP in ART. In cases of fresh transfers, performing a Day 5 transfer would decrease the chances compared to Day 3 transfer.

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Assessing Risks for Patients has Become a Walk in the Park with EUROGTP II Methodologies

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I was thrilled to attend the Euro Good Tissue Practices (EUROGTP II) dissemination seminar this year. I felt that the event in Sitges, Spain, was a great opportunity to take part in an important project, a sentiment which remained throughout and until the end of the event. A sensation of absence arose after realising the project had ended, but then I realised: is this really the end, or was this just a prelude in the right direction? This positive thought alleviated any negative feelings. EUROGTP II is, and always will represent, at least for me if not for many more, the coming together of people to try and do better as specialists to provide for our patients.

The concept of an integrated healthcare practice is universally accepted, but, in fact, the differences between the healthcare systems, legislations, and guidelines of each country make this concept, by definition, almost impossible to harmonise. Therefore, to ensure common understanding and to help create and improve uniformity,[1] as well as alignment and collaboration within and between the healthcare providers and the competent authorities, the European Good Tissue Practice (GTP)[2] guidelines and the adjacent training models have established details on quality management concepts and recommendations on processing of tissues and cells.

The main challenge in this project was not, as may be expected, to develop tools that assist professionals in their daily activities, but to produce guidance that is in accordance with the regulatory principles and legislation that has huge variability across Europe. Developing guidance that applies to all types of tissues and cells (TC) in a systematic risk-based mechanism to obtain a standardised approach was a real effort for all associated and collaborative partners.

The GTP guidelines and the adjacent training model have been established as an outcome of the European Union (EU)-funded project, Euro-GTP I, to provide a complete and detailed tissue banking information package for tissue bankers, as well as for tissue establishment (TE) inspectors in Europe. These guidelines bring together the current minimum regulatory requirements of the European TC directives and go one step further by incorporating useful good manufacturing practice principles and using the expertise of tissue bank experts to provide a set of practical recommendations for good practice in European TE.[1,3,4] The GTP are developed as a helpful tool for all kinds of TE in different phases of their development and evolution, as well as for competent authorities when performing TE inspections. However, some aspects were not present in the guide and there are specific topics that require more extensive work to evaluate or are of very high importance,

such as tissue-specific quality criteria or donor selection for each type of TC, and the risk management and patient follow-up. These hot topics will change from one edition of the Council of Europe (CoE) Guide[5] to the next. Some will be resolved with definitive guidance and new hot topics will emerge. In this way, EUROGTP II was born.

EUROGTP II sets up the good practices applied to TC preparation, processes, and patient follow-up procedures. It began with a 3-year project funded by the 3rd health programme of the EU and it has delivered practical tools for the assessment and verification of the quality, safety, and efficacy from diagnosis to patient follow-up.

Good practice for evaluating quality, safety and efficacy of novel tissue and cellular therapies and products.

The Barcelona Tissue Bank (Banc de Sang I Teixits [BST]) co-ordinated this project together with 14 associative partners from 11 member states, alongside 12 collaborative partners.

The dissemination seminar was held on the 11th–13th March 2019, in Sitges, Spain, and was hosted by the project co-ordinators. There were 117 delegates present for the workshop named 'Methodology and tools (Guide, Interactive Assessment Tool, TC database)'. It was held in a breath-taking location, the Freixenet wineries in Sant Sadurni d'Anoia, Spain, where everyone present could sense the traditional Catalonian atmosphere. First and foremost, this effort was made to present the results of the project to the specialists who travelled from >18 countries, including the UK, Turkey, Romania, Finland, France, Germany, Croatia, Bulgaria, Belgium, Spain, the Netherlands, and many more. The EUROGTP II guidance document was presented to the delegates and the interactive risk assessment tool demonstrated through theoretical and practical exercises.

One of the Romanian participants, Dr Andreea Veliscu Carp, stated that "risk should be assessed every time new equipment, substances, and procedures are planned to be implemented in your tissue establishment. If not done so, there could be situations that lead to new hazards."

The methodology presented by EUROGTP II was based on generic principles of risk assessment and could therefore be applied to all types of tissue establishments including banks for reproductive cells and tissues.

Different levels of novelties represent different risks and distinct impact in the quality and safety of the products; the evaluation of such risks was the goal. The evaluation of the level of novelty and the risks associated should start with a characterisation of the novel process or tissue cell procedures (TCP). Accordingly, VISTART,[6] depending on the level of changes in the processes or in the clinical applications of TCP, novelties are classified as minor novelty, moderate novelty, substantial novelty, or major novelty. Therefore, prior to the implementation of changes in the existing process or prior to implementing new processes, a formal document detailing the factors that justify such developments should be produced.

This document should describe a justification for implementing this new process; the description of change from a technical point of view, including the description of the final product (the TCP) and where it will be used; additional quality indicators that will be used for evaluation; and previous clinical data reported by other clinics or bibliographic evidence that support the implementation.

There was, however, some fine-tuning of the generic tool so that it was accessible for the processing of tissues, haematopoietic stem cells, and assisted reproduction.

The Assisted Reproductive Technology (ART) work package, WP8, led by Dr Kelly Tilleman, Ghent University Hospital, Ghent, Belgium, and a 'dream team' of experts, succeeded to define criteria for risk evaluation in patients when novelties in ART are implemented. The theory behind this risk assessment can be read in the guidance document of EUROGTP II in the specific chapter for ART.[7,8] The interactive tool based on these theoretic principles can be easily and freely accessed online.

Dr Kelly Tilleman, UZ Ghent, WP8 leader at the Dissemination Seminar,
11th–13th March 2019, in Sitges, Spain

The guidance follows steps to obtain the risk assessment. The first step is to evaluate the novelty by answering a series of questions. The questions were defined as a simple exercise that should allow the user to identify if the TCP or procedure is adapted from Provoost:[9] experimental, innovative, or established. After this step, if one of the answers was yes, the second step will follow: risk analyses. The 2nd step aims to determine the risk associated with the different novelties by establishing the risk profile, potential risk, risk factors, probability of occurrence of the risks, severity of the risks considered in the evaluation, detectability, and finally risk reduction.

During the dissemination meeting, ART specialists acknowledged that there are some limitations in using the methodology, because the risk in novelties in ART are not solely related to the patient or recipient, but also very much related to the embryo and future child. I am confident that future projects will be developed in this direction.

The European Society of Human Reproduction and Embryology (ESHRE), one of the stakeholders of the project, was represented by Dr Nathalie Vermeulen, Senior Research Specialist involved in ESHRE Guidelines; Ms Titia Van Roy, E-learning and Communications; and Ms Christine Bauquis, Communications and Press.[10]

Regarding the necessity of risk assessment in this work, Dr Kelly Tilleman said that "a critical analysis of efficacy of novelties should be considered together with the risk of that novelty to our patients. Quality and safety go hand in hand and the nice thing about the current methodology is that you can assess this in a structured and easy way."

When asked about the outcome of the project and about the future perspective, Dr Rita Piteira, was confident that the collaborative partnership with programmes such as VISTART, ECCTR, ARTHIQS, GAPP, and of course, with the support of ESHRE, the TC database, along with the Guide and the Tool, represent an important outcome in the EUROGTP II's popularity. Until April 30th, 2019, everyone could introduce data in the TC Database.[10]

Dr Rita Piteira, BST; EUROGTP II Coordinator

By using the tools and populating the database, stakeholders and CA will be encouraged to accept the validity of data generated for products in other countries (harmonisation of practices) and this can be the start for another project. Involvement will not only advocate the collaboration amongst TE, boosting multicentre collaborations, but will promote the accessibility

for patients by developing knowledge among clinicians regarding the availability of TCP.

It is important to share that Mr Richard McGeehan, Legal Officer of DG Santé at the European Commission, considers the implications of the EURO-GTP risk assessment instrument for ART and its relevance to updates in the TC Directives of 2004.

The EUROGTP II project offered support to TE on fulfilling the requirements of directives, but the results can be the precursor that bring into legislation the technical development that have occurred since the directives were created.

The EUROGTP II gives the possibility to professionals of this sector to organise their risk assessments in a consistent manner and thus allows them to facilitate exchanges in novel therapies and treatments. The interactive tool takes care of the entire process of risk-based thinking and will result in uniform and in-depth risk analyses concerning novelties in ART.

To conclude, I encourage all readers who are curious on how everything works, as well as staff members, to engage with the project! This way, everyone contributes to defining the concept of integrated healthcare practice. For me, it has been a wonderful experience, full of pleasant surprises, great people, and interesting information, and I recommend it to anyone interested in such experiences and looking for inspiration.

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Fertility Preservation in Women with Endometriosis: It is About Time We Talk About it!

EDITOR'S PICK

Carneiro et al. face the important issue of how to preserve fertility in women with endometriosis: a condition that affects >10% of women in reproductive age. Besides reviewing the pathophysiological mechanisms related to endometriosis and the consequential infertility that often develops in these women, the review analyses data in the literature concerning the safety and reproductive repercussions of surgical treatment. Recent studies have indeed questioned surgical treatment which can further compromise the fertility of these women. The article indicates recommendations for physicians to follow to manage patients with endometriosis, starting from ovarian reserve evaluation, by measuring appropriate hormones, such as follicle-stimulating hormone and anti-Müllerian hormone, considering age, antral follicular count, ovarian volume, and other aspects. The article also considers the options available to preserve fertility in these patients, such as oocyte, ovarian tissue and even embryo preservation. Overall, the review represents a complete guide for physicians and gynaecologists who face this problem every day.

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Abstract

Endometriosis is a common benign disease that affects young women and carries a significant risk to the reproductive organs. Studies have shown that endometriosis is associated with diminished ovarian reserve and worse prognosis in assisted reproductive technology treatments. Surgical treatment aims to remove the disease while maintaining reproductive potential with minimal damage to the reproductive organs. The authors reviewed the published literature regarding fertility preservation in endometriosis, focussing on patient selection criteria, available treatment options, and follow-up. The goal of this study was to find evidence to answer the following clinical questions: how should women

of reproductive age with endometriosis be managed, and what fertility-sparing options are available? Cryopreservation of embryos and mature oocytes are established techniques for preserving fertility in women during the reproductive period. Fertility preservation is a key consideration in the care of young girls and women with endometriosis, mainly those with ovarian endometriomas and advanced disease. Although no cohort studies have been published on the subject to date, adequate information detailing disease progression, treatment options, and the risks involved should be made available for these women. Available fertility preservation strategies include embryo and oocyte crypreservation, and women should be counselled individually on the risks, benefits, and costs involved with these options. In this scenario, management by a multidisciplinary endometriosis team is a fundamental step for producing successful results.

INTRODUCTION

Fertility preservation is increasingly attracting the attention of physicians and patients. The advances in oncologic care worldwide have made it possible for young women undergoing cancer treatment, with a significant disease-free life expectancy, to consider maternity. Similarly, women with other medical conditions have now turned their attention to fertility. This is the case for women with endometriosis, a condition that affects about 10% of women of reproductive age and up to 50% of women with chronic pelvic pain and infertility.[1]

Endometriosis is associated with infertility via several pathophysiological mechanisms, including hormonal dysfunction; oocyte dysfunction; dysfunction of the secretory phase of the menstrual cycle; inflammation, which interferes with sperm–oocyte interaction; low embryo quality; reduced implantation rate; and decreased ovarian reserve.[1] Studies have shown that endometriosis is associated with diminished ovarian reserve[2] and worse prognosis in assisted reproductive technology (ART) treatments.[1,3] In some cases, an interaction between the the numerous pathophysiological alterations may combine together and act through mechanisms that have not yet been fully elucidated.[1]

The exact effect of endometriosis on ovarian reserves is yet to be established. The presence of ovarian endometriomas is detrimental to ovarian reserve markers, such as anti-Müllerian hormone (AMH), by either affecting their production or through a direct, as yet unknown, effect.[1,2] In addition, large endometriomas may interfere with ovarian vascularisation. Moreover, endometriosis treatment frequently requires surgery, particularly in patients that present with ovarian cysts and deep endometriosis (DE).[1,2] Surgical treatment candidates include women in whom pain was not improved with medical treatment and those seeking to become pregnant.[1,3]

Recently, the safety and reproductive repercussions of endometrioma resection have been questioned. The discovery of ovarian tissue containing follicles present in the endometriotic cyst walls has raised the concern that surgery for endometriotic cysts can further compromise fertility in women with endometriosis.[4-6]

In addition, recent data have shown that endometriosis affects approximately 35–54% of women with symptoms of chronic pelvic pain and dysmenorrhea, and most of them have advanced Stage III or IV disease. It is important to note that most of these advanced cases include the occurrence of extensive tubo-ovarian adhesions in addition to endometriomas, resulting in an adverse effect on the reproductive potential.[7] As a result, fertility preservation has become a main focus in women undergoing surgery for endometriosis. The patients should be adequately counselled regarding fertility issues before the procedure and given evidence-based information about disease progression, ovarian reserve, the available therapeutic options, and the risks associated with each procedure.[8,9]

Oocyte and embryo cryopreservation are established fertility preservation techniques.[10] Both techniques require controlled ovarian hyperstimulation and oocyte retrieval. For patients that have a partner, embryo cryopreservation is preferred. For single and younger patients, oocyte cryopreservation is the suggested course of action. Other techniques,

such as immature oocyte retrieval and *in vitro* maturation for cryopreservation and ovarian tissue cryopreservation, have also been studied, but they are not currently recommended as standard options for clinical practice.[10,11]

The authors set out to perform a review of the relevant articles without language restriction based on a PubMed search from 1966–December 2018 using the keywords: "fertility preservation", "endometriosis", "endometriomas", "surgical treatment", "pregnancy", and "assisted reproductive technology". Society guidelines, such as the European Society of Human Reproduction and Embryology (ESHRE), the American College of Obstetricians and Gynecologists (ACOG), and the Royal College of Obstetricians and Gynaecologists (RCOG) were also searched. The authors reviewed the published literature detailing fertility-preserving management in endometriosis focussing on the selection criteria of the patients, available treatment options, and follow-up. The authors aimed to find evidence to answer the following clinical questions: how should we manage reproductive age women with endometriosis, and what fertility-sparing options are available?

HOW SHOULD WE MANAGE REPRODUCTIVE AGE WOMEN WITH ENDOMETRIOSIS?

Ovarian Reserve Evaluation

Progressive loss of ovarian follicles is frequently responsible for subfertility and may also have a negative impact on the results achieved by ART. Progressive loss of ovarian follicles also affects patients who are not seeking pregnancy at present but are interested in preserving their chances of conceiving a child in the future.[12] Ovarian reserve evaluation is a step of foremost importance in the treatment of women with endometriosis, especially those seeking fertility care, and thus should guide physicians regarding fertility preservation. The oocyte yield can be compromised by the presence of endometriotic lesions and cysts, and the surgical procedures performed to treat these women may jeopardise the ovarian reserve.[6]

A woman's age is the single most important predictor for success with ART, with pregnancy rates declining with advancing age.[13] Therefore, ovarian reserve markers should be assessed to better inform patients of the expected success rates before engaging in any fertility preservation procedure or in endometriosis surgery. The available tests include early follicular phase follicle-stimulating hormone levels, AMH dosage, antral follicle count (AFC), and ovarian volume estimated by transvaginal ultrasound, which are predictive of the number of oocytes retrieved with ovarian stimulation and are associated with pregnancy rates.[14] The ideal marker would show a significant change in levels from adolescence to the late reproductive period and should enable age-independent prediction of an individual's reproductive life span and spontaneous pregnancy in the general population.[15]

AFC and AMH are the most reliable and most commonly used ovarian reserve markers.[16-18] AFC consists of counting the number of follicles with a diameter ranging from 2–10 mm and is extensively used in ART clinics, due to its prompt availability and ease of access. AFC correlates well with response to gonadotropin stimulation.

The presence of ovarian endometriosis is associated with diminished serum AMH, lower AFC, lower response to controlled ovarian stimulation, and higher doses of gonadotropins used in ART cycles.[19] Diminished ovarian reserve has been reported not only in women with ovarian endometriomas,[20] but also in those with minimal-to-mild disease.[2]

Excised endometriomas consistently exhibit oocytes containing ovarian cortex firmly attached to the cyst wall, which makes the damage to the ovarian reserve a main concern in endometriotic cyst surgery, and Busacca et al.[21] reported a 2.4% risk of ovarian failure after bilateral ovarian endometrioma excision. Cystectomy can also have negative effects on the ovarian blood supply and spontaneous ovulation rates.[22] The impact of cystectomy in the ovarian reserve can be reliably assessed by serum AMH dosages.[23]

Although it is assumed that cyst drainage and wall ablation can be less harmful to the ovarian reserve, they are associated with lower pain improvement and higher rates of endometrioma recurrence; therefore, the two techniques are not recommended as a first choice procedure.[3] Thus, patients considering pregnancy should

not undergo repetitive surgery to preserve the ovaries and minimise damage to the follicle reservoir,[3] while a fertility preservation approach should be considered before endometrioma surgery in those who do not plan to become pregnant immediately. Martyn et al.[15] reported that AMH screening should be offered to all women in their 30s who are not contemplating pregnancy because clinical risk factors will only identify about 50% of women at risk of reduced ovarian reserve.

When managing endometriosis, physicians should focus on early recognition of subfertility risk and provide immediate referral to an ART specialist when needed.[24] Moreover, low preoperative AFC or AMH can help to predict the need for repeated stimulation cycles to obtain a satisfactory oocyte yield that would provide an improved chance of success for future *in vitro* fertilisation (IVF) with thawed oocytes. These measurements should also indicate to surgeons that they should pursue less aggressive techniques to minimise harm to fertility potential in this setting.

Surgery

Pain relief and improved fertility are the primary goals of surgical tretament of endometriosis. Removing the disease while maintaining reproductive potential with mininmal damage to the reproductive organs remains a challenge[3,25] in DE, superficial, ovarian endometeriosis.[26,27]

Ovarian Endometrioma Surgery

In the therapeutic planning for women who wish to maintain their reproductive potential, it is of paramount importance to consider that the presence of endometriosis in any form (superficial, ovarian, or deep) is capable of interfering in ovarian function, and that endometrioma surgery can aggravate this.[22,28]

Superficial endometriosis is associated with lower fecundity rates and reduced ovarian reserve with low AMH levels.[2,6] The presence of endometrioma also impacts ovarian function, although the relationship between endometriomas and damage to the ovarian reserve remains controversial.[28] The rate of spontaneous ovulation is lower in the ovary with endometrioma.[22] Follicle density is lower and fibrosis is more frequent in the ovarian cortex containing endometriomas.[29] In addition, the presence of DE may be associated with reduced ovarian reserve and a lower number of oocytes retrieved in IVF cycles, probably due to the pelvic inflammatory process found in DE.[30]

Endometrioma surgery reduces follicular reserve and impairs ovarian function. This was demonstrated by the significant decrease in serum levels of AMH after cystectomy and by the decrease in ovulation rates after laparoscopic cystectomy, compared to the indexes before surgery.[29] The decrease in AMH is greater when cystectomy is bilateral compared with unilateral. In IVF cycles, a lower number of oocytes were obtained with a decrease in pregnancies and live birth rates after bilateral cystectomy compared to cycles without endometriomas.[31] Muzii et al.,[28] on the other hand, used AFC to assess ovarian reserve endometrioma surgery in their meta-analysis. They found that ovarian reserve was not decreased following endometrioma removal. However, operating on recurrent endometriomas seems to be more detrimental to the ovarian reserve. Thus, indications for surgical treatment for recurrent endometriomas should be viewed with caution.[32]

Clearly, the larger the ovarian endometriomas and the more extensive and complex the pelvic adhesions are the worse the reproductive prognosis will be, and it is the surgeon's responsibility not to aggravate such a situation. The principles that govern these objectives are, fundamentally, the preservation of ovarian follicular reserve and the prevention of postoperative pelvic adhesions with minimal possibility of residual disease.

Surgery for Deep Endometriosis and Infertility

DE is considered a specific entity that has been arbitrarily defined in histological terms as endometriotic lesions extending >5 mm in diameter underneath the peritoneum,[33] and it is usually responsible for painful symptoms. Although DE is frequently associated with infertility, the evidence of a clear connection between the disease and infertility is weak. Studies suggest that infertility in these women is probably due to the strong link between DE and adhesions, superficial endometriotic implants, ovarian endometriomas, and adenomyosis.[34] Although there is evidence that DE is associated

with infertility, it is still unclear whether surgery to treat this form of the disease is capable of improving fertility because the primary indication of this operative approach has been for the treatment of pelvic pain.[35]

Duffy et al.[36] found that laparoscopic surgery was associated with an increased live birth or ongoing pregnancy rate, as well as a clinical pregnancy rate in comparison to diagnostic laparoscopy. No solid conclusions of safety could be drawn as there was insufficient evidence regarding adverse events.

While some specialists advocate complete surgical removal of endometriotic lesions to improve fertility,[37,38] others recommend that extensive surgery for intraperitoneal and DE in infertile women does not improve global fertility prognosis and may be associated with a higher complication rate.[39,40] Vercellini et al.[41] highlighted that women should be carefully counselled on the chances of getting pregnant after surgery. They found that pregnancy rates dropped by 15% in those who sought spontaneous conception after surgery in comparison to those who underwent IVF, which dropped from 39% to 24%.

In summary, the effect of surgery on the fertility of women with deep infiltrating endometriosis remains unanswered due to the heterogeneous nature of the disease, as well as lack of adequate trials with enough power and follow up to study this.

WHAT ARE THE FERTILITY-SPARING OPTIONS AVAILABLE?

Embryo, Oocyte, and Ovarian Tissue Cryopreservation

Cryopreservation of embryos and mature oocytes are established techniques for preserving fertility in women of reproductive age.[9] Controlled hyperstimulation of the ovaries is necessary, followed by oocyte recovery with transvaginal ultrasound for the successful cryopreservation of embryos and mature oocytes. The mature oocytes obtained may be cryopreserved or fertilised and the resulting embryos cryopreserved. Cryopreservation of embryos is an effective option provided there is time to perform ovarian stimulation and an available sperm donor. Oocyte cryopreservation is the best choice for fertility preservation in women with endometriosis who wish to postpone pregnancy or those who will undergo surgical treatment for endometriosis in the future.[42] Vitrification is an efficient method of cryopreserving oocytes while maintaining fertilisation and pregnancy rates similar to IVF techniques with fresh oocytes.[43]

Although fertility preservation was initially designed for cancer patients, recently Elizur et al.[8] reported a case of oocyte cryopreservation in a 25-year-old woman with endometriosis and chronic pelvic pain. The patient had undergone oophoretomy but retained significant pelvic pain and was at risk of losing the remaining ovary. After three cycles of ovarian hyperstimulation, 21 oocytes were cryopreserved.

Fertility Preservation Using Oocytes

Garcia-Velasco et al.[42] published their experience with 38 endometriosis patients who underwent oocyte cryopreservation to maintian their future fertility. No pregnancies have been reported in the group to date. Rad et al.[45] published an observational study detailing fertility preservation in 62 women with endometriosis, but only 49 patients underwent controlled ovarian stimulation and oocyte vitrification. Previous endometrioma surgery was associated with reduced response to controlled ovarian stimulation. No outcomes after oocyte thawing and pregnancy are reported. The authors reported that fertility preservation is an important issue in young women with severe endometriosis and individualised couselling should take into consideration the patient's age, disease extent, and progression, as well as the presence or absence of ovarian endometrioma and previous or present indication of ovarian surgery.

There is concern regarding the quality of the response in cases of endometriosis, since some studies suggest that women with endometriosis who undergo IVF cycles have lower rates of pregnancy and implantation when compared to those with tubal infertility.[46] This would occur as a result of reduced oocyte quality and embryonic development, as well as endometrial receptivity. Harb et al.[47] published a meta-

analysis showing reduced rates of fertilisation in women with Grade I/II endometriosis, as well as pregnancy and implantation in women with Grade III or IV endometriosis. Therefore, more cycles of controlled ovarian stimulation and IVF may be necessary to obtain sufficient good quality oocytes to generate embryos with development and quality suitable for freezing. Ovarian hyperstimulation does not appear to increase the risk of progression of endometriosis or recurrence of lesions in patients already treated.[48,49] In addition, the presence of endometrioma at the time of ovular collection may increase the risk of pelvic infection and abscess formation.[50]

In infertile patients with ovarian endometrioma, the surgical approach should be carefully discussed. Excision of the endometrioma capsule increases the spontaneous pregnancy rate in the postoperative period compared to drainage and electrocoagulation of the endometrioma wall;[50] however, such surgical techniques may present a risk of decreased ovarian reserve, either by removal of normal ovarian tissue during excision or by thermal damage to the ovarian cortex during ablation. Published data shows that the presence of endometriomas significantly reduces AMH values in comparison to the absence of endometriosis.[21,22] Surgical excision of endometriomas seems to negatively influence ovarian reserve but only temporarily.[51] Others suggest that the mere presence of an endometrioma adversely affects ovarian reserve and it may be difficult to measure such effects before surgery.[52] Therefore, despite efforts to minimise surgical damage, the ovarian reserve may still be affected by the presence of endometrioma per se. Endometrioma size, bilaterality risk of subsequent ovarian failure, surgical technique, and the surgeon's expertise, as well as the patient's age, should also be taken into account before surgical excision if future fertility is a concern.[50-52]

Ovarian tissue cryopreservation is currently used to preserve fertility in women of reproductive age who are at high risk of losing ovarian function (chemotherapy, radiotherapy, and some benign conditions are associated with a high risk of premature ovarian failure).[53] In prepubertal girls at risk of losing reproductive potential, ovarian tissue cryopreservation may be the only alternative theraputic option available. However, it should be noted that the procedure is still considered to be experimental.[53-55] In patients with endometriosis, healthy fragments of ovarian cortex can be isolated and cryopreserved during surgical removal of endometrioma. The technique should be evaluated with caution, as there is a risk of transfer of small foci of endometriosis in the cryopreserved tissue.[9,42] The advantage of tissue cryopreservation is that there is no need for ovarian hyperstimulation. Many unanswered technical questions remain, related to the choice of cryopreservation technique, chances of ovarian function recovery after transplantation, and pregnancy rates after the procedure.[55] Data remain scarce regarding the use of this fertility preservation technique in women with endometriosis; further studies are needed before cryopreservation of ovarian tissue can be indicated as the first choice in preserving fertility in patients with endometriosis.[42]

CONCLUSION

Endometriosis is a common benign disease that carries significant risk to the reproductive organs. Fertility preservation is a key consideration in the care of young girls and women with endometriosis, particularly those with ovarian endometriomas and advanced disease. Although there have been no cohort studies published on the subject so far, adequate information on disease progression, treatment options, and the risks involved should be available for these women. It is still too early to define fertility preservation as the standard of care for all women with endometriosis because very few cases have been reported and the available data does not allow for robust cost–utility analyses. However, fertility preservation should be taken into consideration for those with bilateral unoperated endometriomas and those who previously had unilateral endometriomas removed and require surgery for a contralateral recurrence.[56] Available strategies include embryo and oocyte crypreservation, and women should be counselled individually on the risks, benefits, and costs involved with all available techniques. In this scenario, management by a multidisciplinary endometriosis team is a fundamental step towards achieving successful outcomes.

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Anthropometry Between the External Urethral Orifice and the Vaginal Introitus in Vaginism

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Abstract

Introduction: Some of the factors that may be associated with and influence vaginismus and female sexual function have already been studied, such as repressive education, religious teaching, cultural values, and anatomical changes; however, no studies exist that have analysed the measurement between the external urethral orifice and the vaginal introitus in the search for differences between women with and without the diagnosis of vaginismus.

Objective: To verify the urethro-vaginal anthropometric relation in women with and without vaginismus.

Method: This was a cross-sectional study, approved by the Ethics Committee and registered in clinical trials, conducted from February to August 2017. Sixty women were evaluated, aged 18–40 years old and all of whom were nulliparous, heterosexual, and sexually active. They were divided into two groups: Group I (women with vaginismus, n=30) and Group II (without vaginismus, n=30). Using an inelastic tape measure, the distance between the external urethral orifice and the inferior border of the vaginal introitus was checked in both groups.

Results: In Group I, the mean distance between the urethral orifice and the inferior border of the vaginal introitus was 0.69 cm. In Group II, the mean was 1.46 cm, with $p<0.001$ demonstrated in the comparison for the difference between the groups.

Conclusion: A significant difference was observed in the distance between the external urethral orifice and the inferior border of the vaginal introitus in women with and without vaginismus. This anatomical finding may be involved with the evolution of vaginismus.

INTRODUCTION

Vaginismus is a female sexual dysfunction with a multifactorial aetiology, and is characterised by recurrent or persistent involuntary spasm of the perineum muscles, which are adjacent to the lower third of the vagina. This condition prevents penetration, whether by penis, finger, or other object, which has a negative impact on sexual relations and quality of life.[1-4]

Despite the lack of epidemiological studies that define the prevalence of vaginismus, it is clinically estimated between 5–20%.[1,2] However, it is known that many women do not look for medical treatment because of fear, lack of confidence, discomfort, or lack of knowledge. According to this perspective, the population affected by vaginismus is likely to be underestimated.[5-7]

In the Diagnostic and Statistical Manual of Mental Disorders (DSM-5),[6] vaginismus is grouped with the disorders of pelvic pain/penetration and can be presented as a disease that arises throughout life: this means that it either presents following first sexual experience or is acquired. In the International Classification of Diseases (ICD-11), approved by the World Health Organization (WHO) in 2018 and pending implementation in January 2022, the disease is denoted as a sexual disorder of the pain penetration (HA20.Z).[7,8]

The aetiology of vaginismus has not yet been well elucidated; however, it is known that factors such as sexual, cultural, religious education, previous sexual experiences, violence, or sexual abuse contribute or enhance its development.[9,10] These physical and/or psychological factors have been studied and appear to act through a vicious cycle of fear and avoidance, in which attempts of penetration causes distress and muscular tension, leading to greater avoidance and, thus, a relentless fear of penile penetration.[8]

There is also a need for investigation into anatomical changes, organic diseases such as abnormalities in the hymen, infections, vaginal lesions, and endometriosis, all of which cause pain or difficulty in penetration and have been suggested as additional aetiological factors.[11]

When the authors investigated studies that had extensively evaluated vaginismus, specifically focussing on anatomical alterations, reports about the relation between the urethra and the vaginal introitus were not found in the literature. This finding could constitute a mechanical barrier that is feasibly linked to the development of vaginismus.

OBJECTIVE

To verify the urethro–vaginal anthropometric relationship of women with and without vaginismus.

METHOD

This was a cross-sectional study, approved by the Ethical Committee (CAAE: 51995515.4.0000.5479) and registered in clinical trials (NC03176069),[12] conducted from February to August 2017, evaluating 60 women aged 18–40 years old who were nulliparous, heterosexual, and sexually active.

The women were divided into two groups: Vaginismus Group (n=30) and Control Group (n=30), the latter composed of women without sexual dysfunction. Included in the Vaginismus Group were those with vaginismus throughout their lives and classified as having severe degree (DSM-5).[6] They had never been through any treatment or intervention for sexual dysfunction. The authors excluded women that had psychiatric disease, primigravida and multigravida, partners with dysfunction that prevented penetration, presence of vaginal prolapse, or surgical necessity for vaginismus.

All participants were recruited through social media, and those who responded to the call were interviewed by an independent researcher who made the telephone contact to observe the inclusion criteria. An evaluation was scheduled for those who met all the criteria.

The evaluation took place in a University Hospital and was performed by a physiotherapist specialised in pelvic physiotherapy. The women were evaluated by the same examiner who was unaware of their allocation (Vaginismus Group or Control Group). Evaluations were performed with the participants in the gynaecological position, with an inelastic tape measure. The distance between the external urethral orifice to the inferior border of the vaginal introitus was measured in the two groups, as exemplified in Figure 1. The data were analysed for statistical significance by SPSS software. The Mann–Whitney test was performed. The level of significance was 5%.

RESULTS

In Table 1, the characterisation of the women studied in the Vaginismus Group and in the Control Group is described according to several variables: age, BMI, age of menarche (years), beginning of sexual life (years), marital status, and religion.

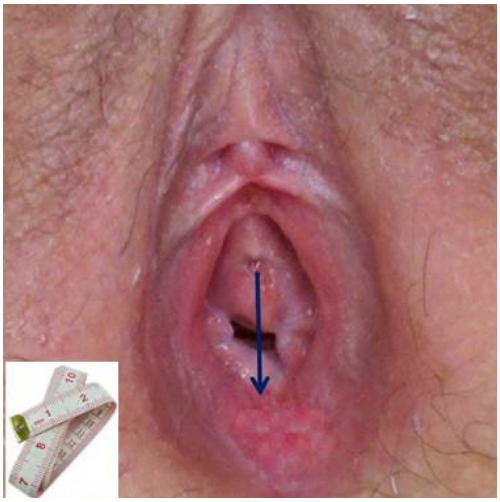

Figure 1: Illustrative image of the measurement of the distance between the external urethral orifice and the lower border of the vaginal introitus.

Table 1: Characterisation of the sample of the Vaginismus Group (n=30) and Control Group (n=30).

Variable	Group	Mean	SD	p
Years	Vaginismus	26.7	5.2	0.673
	Control	26.5	5.1	
BMI	Vaginismus	23.3	3.6	0.870
	Control	23.2	3.3	
Menarche (years)	Vaginismus	12.4	1.5	0.392
	Control	12.1	1.5	
Beginning of sexual life (years)	Vaginismus	20.5	3.6	<0.001
	Control	17.3	1.4	
		Married	Single	Civil partnership
Marital status	Vaginismus	17	13	0
	Control	9	20	1
		Christian	Without religion	Atheist
Religion	Vaginismus	27	3	0
	Control	22	3	5

SD: standard deviation.

Table 2 shows the measurements between the external urethral orifice and the inferior border of the vaginal introitus (cm) of the Vaginismus Group and of the Control Group. The mean distance in the Vaginismus Group was 0.69 cm (standard deviation [SD]: 0.28). The mean distance in the Control Group was 1.46 cm (SD:0.20; p>0.001).

Table 2: Measurements of the external urethral orifice to the lower border of the vaginal introitus of the Vaginismus Group (n=30) and of the Control Group (n=30).

Group	Minimum (cm)	Maximum (cm)	Median (cm)	p
Vaginismus	0.40	1.30	0.60	<0.001
Control	1.20	1.90	1.45	

DISCUSSION

Vaginismus is a multifactorial sexual dysfunction that encompasses emotional, psychological, biological, and possibly anatomical factors. Women with vaginismus suffer significant impact on their quality of life. Congenital malformations of the genitourinary tract or changes in the hymen are reported in the literature as possible risk factors for vaginismus.[13,14]

In the authors' practice, the differentiated positioning of the urethra of women who present the diagnosis of vaginismus in relation to women without sexual dysfunction was clinically observed, suggestive of a 'low urethra' that sometimes overlaps with the vaginal introitus. The authors did not, however, find scientific studies in the literature that investigate this difference. This study focussed on answering the following question: is there a difference in the measurement between the external urethral orifice and the vaginal introitus?

The presence of a bigger mean distance between the external urethral orifice and the hymenal meatus was described as a finding in women who had urethral–hymenal fusion. This urethral-hymenal fusion is considered a congenital predisposition for frequent episodes of cystitis after sexual relation.[15] In this study, a decrease in the measurement between the external urethral orifice and vaginal introitus was observed.

Gravina et al.[13] related the location of the urethra to the quality/capacity of female orgasms. The urethrovaginal space consists of fibroconnective tissue and large numbers of blood vessels, glands, muscle fibres, and nerve endings. The proximity of the urethra and the clitoris to the anterior vaginal wall suggests an association between such anatomical structures and sexual function. In women without sexual dysfunction, a relationship between the urethra–vaginal distance and the presence of orgasm was described by ultrasonography. The present study was not intended to evaluate the phases of the human sexual response cycle; however, the clinical finding that showed the lowest urethrovaginal space may lead to the development of a new line of research that uses ultrasonography to evaluate in the same way conducted by Gravina et al.,[13] allowing a comparison of results.

Anatomical differences are important conditions that can influence sexual function. There are few articles that correlate these differences with sexual function, or even with some sexual dysfunctions. They are insufficient to establish a consensus. Regarding vaginismus, the authors did not find studies in the literature that relate the measurement of the external urethral orifice to the vaginal introitus. Thus, this study may be the precursor of an important diagnostic relationship. Associating anatomical differences with psychological and emotional factors may lead to a better understanding of vaginismus.

Regarding the question "is there a difference in the measurement between external urethral orifice and the vaginal introitus?", which motivated the present study, these results suggest that the answer is yes. A measure up to three times smaller between the anatomical structures analysed between the groups was observed. This result leads the authors to suggest some hypotheses: did this difference of measure arise after the development of vaginismus, due to recurrent spasms, or did it already exist?; does this measure change after the physiotherapy treatment for vaginismus? If this measure is related to an anatomical change, it would be advisable that the gynaecologist evaluated this measure at the woman's first consultation. This is because, according to the present study, this measure may suggest a risk factor for the development of vaginismus. Among the possible causes for this difference, the literature does not yet present a hormonal correlation, especially relating gestation

to the development of vaginismus. The desire to carry children is one of the reasons women seek treatment for vaginismus. Another factor to be investigated in future studies is the relationship of perineal muscle tone with vaginismus, specifically the urethro—vaginal distance, because there is spasm of this musculature for acquired or congenital vaginismus. The authors confirm, however, that the finding of the present study may suggest a risk factor for development and generate a deeper line of research on the subject.

CONCLUSION

According to the data obtained, a significant difference was observed in the distance between the external urethral orifice and the inferior border of the vaginal introitus when women with and without vaginismus are compared. This anatomical finding may be related to the evolution of vaginismus.

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Description and Outcomes of Current Clinical Techniques for Sperm Cryopreservation

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Abstract

Nowadays, sperm cryopreservation is strongly recommended in cases of malignancy. Moreover, the use of frozen testicular sperm in azoospermic patients prevents the need for repeated sperm retrieval and optimises scheduling between oocyte and sperm obtainment. Even though cryopreservation of human spermatozoa for assisted reproductive purposes is a widely implemented practice, none of the established freezing and vitrification techniques offer optimal cryosurvival results due to the dramatic impact of cryodamage on sperm cells.

This comprehensive review describes the most commonly used sperm cryopreservation techniques in order to establish which of them minimise sperm cryodamage and offer better survival rates.

Presently, it is not sufficiently demonstrated that sperm vitrification improves survival significantly more than freezing methods. Slow freezing offers the best survival results when compared to other freezing protocols, and owing to its technical advantages, can be considered as one of the preferred protocols to be easily implemented in assisted reproduction laboratories.

Moreover, several studies have suggested that sperm preparation prior to cryopreservation can improve thawed sample quality. However, other authors have demonstrated that freezing the fresh sample and performing semen preparation after thawing gives better results in regard to total motile sperm count and motility.

Regarding clinical results, it is well established that similar or even better reproductive outcomes are achieved using frozen testicular sperm in cases of azoospermia or anejaculation. Moreover, the use of frozen semen in cancer patients can help to achieve good fertilisation and pregnancy rates. Finally, the use of frozen sperm is not at all associated with worse post-natal development.

INTRODUCTION

The cryopreservation of human spermatozoa for assisted reproduction techniques is a clinical practice implemented worldwide since its early beginnings in the 1970s. Since then, the development of new protocols aiming to optimise the quality of thawed sperm samples has been of major interest to researchers and constantly pursued in the field of andrology. Besides this, sperm cryopreservation has become a paramount facet of ART programmes since it has allowed the establishment of donor sperm banks. Furthermore, it is, to date, the treatment of choice to preserve fertility in pubertal children and adults.

Sperm cryopreservation can be achieved through both equilibrium and non-equilibrium procedures, which are commonly referred to in the literature as sperm freezing and vitrification, respectively. In spite of efforts to implement human spermatozoa vitrification as a routine protocol in clinical practice, conventional freezing is still the most widely used protocol for clinical purposes.

Sperm freezing is used routinely to cryopreserve not only ejaculated but also non-ejaculated spermatozoa retrieved from azoospermic patients who have undergone a sperm extraction surgery.

Unfortunately, as a result of the impact of cryopreservation-associated damage to spermatozoa, the viability rates of thawed samples remain low. Cryopreservation-induced damage is due to both mechanical and osmotic stress, and although cryoprotectant compounds are necessary for sperm freezing, they are also a source of osmotic damage when their levels exceed optimum concentration.

Due to this, the optimisation of cryopreservation protocols to increase the number of viable spermatozoa in thawed samples must be a major concern for all male patients undergoing *in vitro* fertilisation treatments. Nevertheless, it is even more crucial when applied to patients undergoing gonadotoxic treatment for malignancies and non-malignant chronic diseases, and who are counselled to attend fertility preservation programmes: these pathologies, and their associated therapies, threaten these patients' fertility potential, thus limiting their sample availability.

In this scenario, it is worth mentioning that sperm selection techniques, such as through swim-up and density gradients centrifugation (DGC), are essential steps to be performed prior to using a sperm sample for intrauterine insemination or *in vitro* fertilisation. However, the sequence in which sperm selection and cryopreservation must be performed remains controversial, since the protective role of the seminal plasma during sperm freezing must not be dismissed.

This review will provide comprehensive information regarding the state-of-the-art human spermatozoa cryopreservation protocols and compare their outcomes.

METHODS

An exhaustive literature review of the current protocols implemented for clinical purposes and the reproductive outcomes derived has been performed. Topic-relevant scientific papers published in PubMed are included. Keywords used for the bibliographic search were "sperm freezing", "sperm vitrification", "sperm preparation", "total motile sperm count", and "sperm viability".

Sperm Cryopreservation Protocols

Equilibrium Procedures: Sperm Freezing

The impairment of sperm cell parameters during cryopreservation is partly due to physical changes related to ice-crystal formation that in turn result in mechanical damage to spermatozoa. In order to prevent intracellular ice-crystal formation, measures to be considered are either controlling freezing rates or promoting cellular dehydration by using cryoprotectants. However, cryopreservation-associated damage is also a response to osmotic stress caused by the addition of cryoprotectants. Cryoprotectant agents are necessary compounds for sperm cryopreservation, particularly for the freezing stage, since they protect against ice-crystal formation. Cryoprotectants can be divided in two groups, dependent on their capability to pass through the sperm cell membrane: permeable cryoprotectants are compounds of low molecular

weight capable of infiltrating the cell membrane, whereas non-permeable cryoprotectants are compounds that cannot penetrate the membrane and so force dehydration by means of increasing the osmotic concentration of the extracellular media. With regard to sperm freezing, the most widely used cryoprotectants are egg yolk and glycerol. Lipoproteins present in egg yolk help minimise damage to the sperm cell membrane during cryopreservation, whereas glycerol, a permeable cryoprotectant, balances the solute concentration of intra and extracellular media (Table 1A). Nevertheless, the addition of glycerol has to be done progressively since it spreads into the sperm cell cytoplasm more slowly than water, and so changes in cell volume must be controlled.

The main aim of the thawing process is to revert the cellular changes that occurred during the temperature lowering, hence cell rehydration, together with the appropriate removal of cryoprotectants, are major goals to achieve. It is worth remarking that a progressive cryoprotectant removal rate is paramount to preventing osmotic shock.[1,2]

Slow Freezing

Slow freezing is one of the most commonly used protocols for human sperm cryopreservation. The procedure can last from 30 minutes to 1 hour and is an equilibrium technique characterised by the maintenance of osmotic balance during the temperature decrease. This protocol requires the use of cryoprotectants, mainly derived from egg yolk. Firstly, the sperm sample is mixed gradually, volume to volume, with cryoprotectant before being homogenised. Slow freezing consists of two freezing steps so that the temperature decrease occurs progressively. Firstly, the sample is mixed with cryoprotectant agents and then incubated at 4 °C for 20 minutes. Following this, the temperature decreases from 4 °C to -80 °C, meaning the freezing rate is increased to 1–10 °C/min. In this second freezing step, samples must be incubated in liquid nitrogen vapours (LNV) for 20 minutes. During this last stage, freezing devices containing sperm samples to be cryopreserved must be arranged so as to minimise temperature variations along the cryo-storage device. Finally, the sample is immersed directly in liquid nitrogen (LN) and is stored in LN tanks until its use.[3,4]

In slow freezing, freezing rates are controlled manually by the operator, so variations in the freezing rate are more likely to occur than in other freezing protocols (e.g., programmable freezing). Any variation in freezing rates is damaging but the cause of cryodamage can be different. When the freezing rate is above the optimal, there is greater mechanical injury because ice-crystal formation is promoted, whereas a below-optimal freezing rate promotes osmotic shock-induced damage.

Nevertheless, slow freezing is a cryopreservation protocol for human spermatozoa widely implemented in assisted reproduction.

Table 1A.

Cryoprotectant media	
Permeable cryoprotectant agents	Non-permeable cryoprotectant agents
Low-weight molecular compounds that pass through sperm cell membrane enabling the displacement of water molecules	High-weight molecular compounds that promote cell dehydration by means of an osmotic imbalance
Examples: glycerol, ethilen-glycol	Examples: glucosa, sucrose, threalose
Buffers	
Examples: TRIS: tris(hydroxymethyl)aminomethane HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) TES: 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-tl]amino]ethanesulfonic acid	
Calcium chelators	
Examples: EDTA or citrate	
Cell membrane estabilising molecules	
Animal origin: Egg yolk	Non-animal origin: Albumin or lectin

Table 1B.

Cryoprotectant	Protocol	Advantages	Disadvantages
SPERM FREEZING Permeable cryoprotectant as glycerol Buffer Lyophilized egg-yolk/albumin as cell estabilising molecule Antibiotics	Slow freezing	- Storage in difference devices: cryovials and straws - Cost-effective	- Manual temperature control
	Programmable freezing	- Controlled temperature steps	- Not cost-effective - Useful only to process high number of samples - Problems with latent heat inherent to the freezer
	Rapid freezing	- Storage in different devices: cryovials and straws - Single freezing step: Reduced operation time - Cost-effective	- Manual temperature control - No standarised distance of the samples to liquid nitrogen vapors
	Dry-ice freezing	- Storage in different devices: cryovials and straws. - Single freezing step: Reduced operation time -Cost-effective -Thawing test -Multi-doce storage in pills	- Manual temperature control
SPERM VITRIFICATION Non-permeable cryoprotectant as sucrose (0,2M-0,25M as les cytotoxic concentrations)	Vitrification	- Reduced operation time - Cost-effective	- Only small volumes - Cytotoxicity due to high cryoprotectan - Concentration

A) Summary of composition of cryopreservation media. B) Most commonly-used cryoprotectant media used for sperm freezing and sperm vitrification. General description of the advantages and disadvantages of each cryopreservation protocol.

Slow freezing allows the use of different cryostorage devices as sealed straws (volume capacity: 0.25–0.50 mL) or cryovials (volume capacity: 1.00–2.00 mL).[5] Hence, it is a useful cryopreservation protocol to be used in the daily clinical routine as not only does it allow for the use of frozen samples, for example in a cryovial, for intrauterine insemination, but the cryostorage of straws for fertility preservation purposes can also be performed, meaning frozen samples from azoospermic or oncologic patients can be kept in multi-dose devices to be used in several further reproductive treatments.

Programmable Freezing

Programmable freezing aims to address one of the main weaknesses of slow freezing, providing a more strict control of freezing rates through the use of automated programmable LN freezers.[6] Therefore, for programmable sperm freezing, sperm samples mixed with cryoprotectants are arranged on a platen before the freezing rate is selected. Samples are frozen first using a freezing rate of -1.5 °C/min, decreasing the temperature from 20 to -80 °C. Next, the freezing rate is increased to -6 °C/min. Once the temperature

decrease is finished, samples will be submerged in LN (-196 °C).[4]

The main advantage of using programmable sperm freezers is the reproducibility and accurate control of the freezing rates. In contrast, its usefulness is limited to those circumstances when a high number of samples are required to be cryopreserved at the same time. Furthermore, in some circumstances programmable sperm freezing has been described as a less efficient process because of latent heat leading to delays in freezing rates, thus being detrimental for spermatozoa.[5]

Rapid Freezing

Rapid freezing, similarly to slow and programmable freezing, requires the addition of cryoprotectants. Rapid freezing is characterised by the direct contact of the mix of semen sample and cryoprotectants with LNV, consisting of a single freezing step. Firstly, semen samples are mixed with cryoprotectants. The homogenised mix is then incubated in LNV, causing a rapid decrease of temperature (-50 to -400 °C/min), prior to submerging the samples in LN tanks.[4] The freezing rate during this protocol depends on two main factors: the period that the samples are incubated, and also the distance to LNV. This fact complicates control of the freezing rate. Therefore, the implementation of rapid freezing in andrology laboratories deserves a prior in-house study in which both previously mentioned variables are tested, so the proper time and distance to LNV can be established. One of the strengths of rapid freezing is the minimising of procedural time since only a single freezing step is needed, and it does not require high-technology devices.[5]

Dry Ice Freezing

Dry ice freezing is a cryopreservation protocol consisting of freezing the mixture of semen sample and cryoprotectants in dry ice pills. In order to do so, little drops of the combined mixture are placed in a dry ice block with sphere-like relief. In brief, once the semen sample is mixed volume to volume with cryoprotectants, little drops of the mixture are dispensed in the sphere-like wells of the dry ice block. Drops of the mixture are frozen within a minute, and finally all the drops of the same sample are placed in a tube and stored in LN (-196 °C).

Dry ice freezing is a rapid protocol that allows multi-dose storing in pills. Furthermore, thawing tests can be performed using a single drop to predict the quality of the thawed sample. Interestingly, this protocol does not require the control of freezing rates, since the freezing process is finished in 1 minute after the drops are placed in the sphere-like wells of dry ice. Therefore, dry ice freezing is a reproducible and easy-to-implement sperm cryopreservation protocol due to its cost-effectiveness.

The use of dry ice freezing for cryopreservation of human spermatozoa for assisted reproduction procedures was first described to freeze testicular human spermatozoa in 1996.[7] Moreover, dry ice freezing has succeeded in cryopreserving human testicular spermatozoa from azoospermic patients who underwent gonadotoxic treatments.[8] In addition, this cryopreservation technique is also used for sperm freezing in sperm donor programmes and for the sperm cryopreservation of infertile male patients.[9-12]

Non-Equilibrium Procedures: Vitrification of Human Spermatozoa

Sperm vitrification is characterised by the formation of glass-like solid structures preventing ice crystal formation. Sperm vitrification is a non-equilibrium cryopreservation procedure which achieves ultra-rapid freezing rates by means of submerging samples in LN (-196 °C), thus bypassing ice-crystal formation. The achievement of such ultra-rapid freezing rates requires a high concentration of cryoprotectants; hence, osmotic shock due to high levels of cryoprotectants is the main cause of cryopreservation-associated damage during sperm vitrification.

Prior to sperm vitrification, it is mandatory to perform a sperm preparation (or sperm selection) technique to remove seminal plasma, whilst during sperm freezing techniques preparation can be performed either before freezing or after thawing, as will be further discussed. Different sperm vitrification studies agree that seminal plasma is likely to present cell detritus, leukocytes, or even other sorts of micro-organism that would promote reactive oxygen species (ROS) production. Therefore, in order to prevent ROS-induced potential damage to sperm cells, it is recommended to perform a sperm preparation technique (swim-up or DGC) to remove the seminal plasma.

Protocol	N	Male aetiology	Motility (%)		Sperm count (x106/ml)	MSC	Morphology (%)	Viability		DNA integrity (%)	Mitochon-drial activity (%)	Acrosomal reaction (%)	Author
								HOS	EOSIN				
S.F.	44	D+I	NI		NI	NI	29.8±12.1	36.1±16	22.2±10	78.3±8.9	NI	NI	Hamma-deh et al., 2001[19]
V.R.F.							30.5±12.2	36.5±15	19.5±8.6	74.7±8.6			
S.F.	54	N	42.7±23.5		37.4±14.7	NI	NI	NI		15.5±9.8	NI	NI	Jee et al., 2010[20]
ULTRA RAPID			39.8±24.8		34.6±14.3					16.8±10.3			
S.F.	30	I	37.00±16.66		NI	NI	14.90±8.84	44.06±18	50.38±20	93.6±7.4	NI	NI	Vutaya-vanich et al., 2010[21]
R.F.			53.90±17.64				14.43±8.90	60.09±14	64.79±15	94.53±5.30			
S.F.	39	N	NI		NI	52±16	44.3±5.4	NI		NI	NI	NI	Stanic et al., 2000[22]
P.R.F.						43±15	42.1±5.2						
V.R.F.						49±15	41.0±5.7						
DRY ICE	810	N+D	50.2±1.0^{PM}	67.2±1.6^{TM}	NI	NI	NI	NI		NI	NI	NI	Meseguer et al., 2001[23]
R.F.			50.4±2.0^{PM}	49.1±1.9^{TM}									
V.R.F.	33	N OA	31$^{(NS)}$		NI	NI	NI	NI		NI	53.3±9.8	NI	Mohamed, 2015[24]
V			34$^{(NS)}$								58.4±11.9[24]		
V.R.F.	30	N	40±13^{PM}	48.6±14.0^{TM}	NI	NI	44.4±10.4	63.2±7.6^{EOSIN}		16.6±5.6^{FI}	NI	NI	Agha-Rahimi et al., 2014[25]
V			41.9±1.0^{PM}	53.9±8.5^{TM}			49.8±11.1	64.4±10.0^{EOSIN}		15.7±4.4^{FI} [25]			
V.R.F.	38	N	55$^{(NS)}$		NI	NI	NI	NI		90$^{(NS)}$	NI	NI	Isachenko V et al., 2004[26]
V			55$^{(NS)}$							85$^{(NS)}$			
S.F.	18	N	46.7±4.1$^{(NS)}$		NI	NI	NI	NI		84.62$^{(NS)}$	NI	NI	Isachenko E et al., 2004[27]
V			51.5±4.5$^{(NS)}$							84.66$^{(NS)}$			
V.R.F.	68	OAT	18.0±9.2		NI	NI	NI	NI		NI	NI	21.0±3.8^{IA}	Isachenko V et al., 2012[15]
V			28±6									55.0±5.8^{IA}	
S.F.	52	N	26.6±3.1		NI	NI	NI	NI		NI	NI	41.4±2.5	Isachenko V et al., 2017[13]
V			76.0±4.7									28.0±6.9	
S.F.	28	N	24.3±9.5		NI	NI	NI	NI		93.2±6.9	0.4±0.4	NI	Pabon et al., 2015[28]
V			43.1±9.3							91.1±4.4	0.4±0.2[28]		

Table 2: Comparison of outcomes regarding sperm quality among different cryopreservation protocols.

Table 2 continued.

D: sperm donors; EOSIN: Eosin-Nigrosin; FI: in reference to fragmentation index; HOS: hypoosmotic sperm swelling; I: Infertile patients; IA: in reference to intact acrosome (%); MSC: motile sperm concentration; N: normozoospermia; NI: not included; NS: no significance; OA: oligoasthenozoospermia; OAT: oligoasthenoteratozoospermia; PM: progressive motility; TM: total motility; S.F: slow freezing; R.F: rapid freezing; V: vitrification; V.R.F: liquid nitrogen vapor rapid freezing.

Traditionally, sperm vitrification is divided in two categories: aseptic and non-aseptic vitrification, depending on the direct contact of spermatozoa with LN.

Moreover, as a result of the increasing number of studies focussed on developing a cryoprotectant-free vitrification,[13-16] vitrification protocols can also be sorted according to whether the use of cryoprotectant is required or not.

Vitrification of human spermatozoa is a fast protocol and easy to implement in andrology laboratories since it does not require complex equipment. However, it is not a technique widely implemented in human clinical practice due to its weaknesses. The high cytotoxic concentration of cryoprotectants and risk for cross-contamination as a result of direct contact with LN are minor disadvantages that could be improved by choosing aseptic cryoprotectant-free vitrification. Unfortunately, one of the major limiting factors for implementing sperm vitrification in the common clinical practice is the lack of acceptable reproductive outcomes when large volumes of samples are vitrified.

Regardless of all elegant and promising research reporting live births after the vitrification of human spermatozoa,[15,17] it is not the preferred routine sperm cryopreservation protocol. Nevertheless, there is a growing tendency to implement and improve vitrification as a cryopreservation technique for testicular spermatozoa.[18]

Even though the main characteristics of cryopreservation techniques are important factors to be aware of, a major fact that should be considered is the sperm survival rate after thawing. Hence, assessment of sperm survival and sperm viability in thawed samples is crucial in choosing the cryopreservation technique to be used in clinical practice.

Table 2 summarises the vast majority of relevant comparative studies focussed on the assessment of sperm survival in thawed samples when different cryopreservation protocols are performed. As can be concluded from the results of these comparative studies, slow freezing offers the best outcomes, in terms of sperm quality after thawing, when compared with other freezing protocols.[19-22] Regarding dry ice freezing, additional comparative studies are needed to compliment and elaborate on the evidence reported in Table 2.[23]

Regarding vitrification outcomes, it is still a debatable issue that vitrification offers better survival rates than freezing techniques. Studies have indicated no significant differences in sperm parameters between vitrified and frozen spermatozoa,[24,25] although some authors suggest vitrified samples exhibit increased sperm motility after warming.[15,26-29]

Therefore, after considering cryopreservation outcomes, in combination with the technical characteristics of each cryopreservation technique, it can be concluded that the utility of vitrification as a sperm cryopreservation option to be used routinely in clinical practice is still lacking evidence, since robust differences between sperm freezing and vitrification, in terms of the sample quality after cryopreservation, cannot yet be established. In contrast, slow freezing offers good thawing outcomes, in terms of sperm quality, as has been reported in the comparative studies summarised in Table 2. Despite the lack of more comparative studies including dry ice freezing, the numerous experiences of Meseguer et al.[8-11] in cryopreserving human sperm samples by dry ice freezing must not be dismissed given their previous outcomes in oncological patients and donors. Besides this, slow freezing and dry ice freezing have technical characteristics that facilitate their implementation as a routine sperm cryopreservation techniques for clinical purposes.

Optimisation of Human Sperm Freezing Protocols

The main reason sperm freezing protocols are preferable to vitrification in the daily clinical routine is not the achievement of acceptable survival rates, but the possibility to cryopreserve a higher number of spermatozoa, which is a major challenge facing clinicians today (Table 1B).[20,30]

It is agreed in the literature that a dramatic decrease in sperm count and motility is reported after thawing.[31-33] Indeed, a decrease in motility as significant as 50% after thawing has been reported.[34]

Freezing After Sperm Preparation

Several studies agree to the idea of freezing prepared sperm samples, so sperm selection techniques are performed prior to cryopreservation with the aim to improve survival after thawing. Performing a sperm selection, or preparation, technique prior to sperm freezing implies both the removal of seminal plasma and the selection of a potential subpopulation of spermatozoa exhibiting better sperm parameters, such as motility.

Seminal plasma is the medium in which spermatozoa are contained in the ejaculate and is a mixture of different substances secreted by different organs, such as the testicles, epididymis, seminal vesicles, and Cowper glands. Decapacitating factors; antioxidant species; amino acids; lipids; proteins; uric acid; citric acid; bicarbonate; ions such as sodium, calcium, chloride, or magnesium; sugars such as glucose or fructose, and even metabolic derivates such as lactic and pyruvic acids are the main components of human seminal plasma.

Under physiological conditions, seminal plasma is removed from ejaculated spermatozoa during sperm transport in the female reproductive tract, specifically through the cervical mucus, uterus, and finally the fallopian tubes. This is to raise the capacitated status by means of undergoing several changes, such as the acrosomal reaction, so as to acquire the fertilising capability to penetrate the zona pellucida and to succeed in fertilising the oocyte.[31]

Swim-up and DGC are the two main *in vitro* sperm preparation techniques routinely used in clinical practice to select the best subpopulation of spermatozoa, and is performed prior to using a sperm sample for assisted reproduction purposes. Hence, prepared sperm samples are enriched either in morphologically normal spermatozoa or progressive motile spermatozoa when DGC or swim-up, respectively, are performed. The swim-up technique is one of the most commonly used sperm preparation techniques performed to select spermatozoa exhibiting better motility.[33]

Studies performing sperm preparation before freezing to remove seminal plasma are based on the premise that in the seminal plasma several compounds can be found, such as dead spermatozoa, debris, and leukocytes that induce ROS production, all of which can induce damage to sperm cells. Hence, these studies suggest that sperm damage is minimised when a subpopulation of spermatozoa, exhibiting the best sperm parameters, are selected and all potential damaging compounds are removed.[31,33,35] Consequently, Esteves et al.[31] and Petyim et al.[33] suggested selecting for increased progressive motility (PM) rates by swim-up when sperm samples are prepared before the freezing step in normozoospermic patients. More interesting is the study carried out by Brugnon et al.,[35] where another sperm preparation technique, DGC, is performed either before or after sperm freezing. One of the strengths of this work is that oligoasthenoteratozoospermic patients were included. Unfortunately, this study has some flaws in its design due to each study group including different patients, so aliquots from the same semen samples are not used in each cryopreservation protocol.

Freezing Before Sperm Preparation

In this protocol, sperm selection is performed after sperm freezing and leads to the selection of the best spermatozoa in the thawed sample, while cryoprotectant agents are also removed during sperm preparation in order to allow use in the following assisted reproduction technique. Several studies propose performing the sperm selection after freezing as a protocol that leads to an increase in the total amount of spermatozoa with good motility.[33]

Furthermore, evidence demonstrating the protective role of seminal plasma on sperm cells during cryopreservation is reported in the

literature. As has been previously mentioned, seminal plasma is a physiological secretion of different compounds by several glands and organs of the male reproductive tract, thus constituting the ideal medium for sperm preservation prior to capacitation. In the seminal plasma different substances exist that act as protective factors against ROS release. Antioxidant enzymatic systems such as catalase, glutathione peroxidase, and superoxide dismutase, as well as other non-enzymatic compounds like ascorbic acid, E vitamin, carotenoids, and ubiquinones, are found in the seminal plasma. Moreover, Martiniz-Soto et al.[36] suggest that poly-unsaturated fatty acids, present not only in the sperm cell plasma membrane but also in the seminal plasma, contribute to the antioxidant capacity of the seminal plasma, and also, particularly, to cryoresistance by means of increasing fluidity of the sample.[36]

Moreover, the physical adsorption of proteins present in the seminal plasma to the sperm cell surface helps to prevent temperature shock-induced damaged. These proteins are spatially arranged amongst the surface of sperm cell membrane, hence damage to sperm cell membrane during cryopreservation is minimised.[37] Similarly, in other studies it is suggested that heparin binding proteins also have a protective function against temperature shock, preventing lipid peroxidation which could contribute to ROS production.[38]

In this sense, Donnelly et al.[32] support the strategy of freezing before sperm preparation, since selecting spermatozoa after thawing leads to better PM rates. The research carried out by this group goes further by demonstrating that the addition of seminal plasma substantially increases motility rates in samples prepared previously to sperm freezing.[32] Donnelly et al.[32] did not assess other types of motility, such as total motility (TM), and also sperm count, even though a computer-assisted sperm analyser was used.

A more recent study also agrees with the findings observed by Donnelly et al.,[32] as they succeeded in demonstrating that the total amount of viable spermatozoa is maximised when sperm preparation is performed after freezing,[39] since not only PM and TM, but also total motile sperm count (TMSC) are improved. TMSC combines TM with sperm concentration, so it is an estimator of the number of available motile spermatozoa, regardless of the type of motility they exhibit. This fact is especially relevant when sample availability is limited. Thus, considering male patients with impaired sperm parameters singularly, TMSC lowers to reach critical values when sperm selection is performed prior to cryopreservation.[39] As a foregone conclusion to what has been commented above, sperm freezing before selection must be particularly recommended to sub-fertile male patients, since it is the protocol that ensures a higher number of motile spermatozoa. In addition, recent evidence highlights TMSC as an accurate estimator of sperm viability, being a faster and simpler method that avoids staining and incubation steps associated with classical sperm viability tests such as eosin staining or the hypo-osmotic swelling test.[40]

Table 3 summarises the most relevant comparative studies regarding sperm freezing before or after sperm preparation.

As can be concluded from the results in Table 3, sperm selection performed either by swim-up or DGC prior to sperm cryopreservation seems to offer better motility rates in the thawed samples.

However, sperm freezing prior to sperm preparation reports better outcomes, in terms of motile sperm count. Hence, sperm preparation after sperm freezing yields higher numbers of motile spermatozoa. When TMSC is calculated from the data derived from the studies carried out by Petyim[33] and Esteves,[31] it is found to be higher when sperm preparation is performed after sperm freezing, although they suggested an improvement in sperm motility when freezing previously prepared sperm samples. Therefore, it could be concluded that sperm preparation after sperm freezing is the protocol that leads to higher values of TMSC.

Clinical Outcomes After Using Fresh or Cryopreserved Human Spermatozoa for Assisted Reproduction Technologies

The main objective of ART is to achieve a healthy baby. It is therefore important to consider the techniques used for cryopreservation and the clinical results available when aiming to achieve optimum outcomes from ART, especially when compared to fresh samples.

Table 3: Sperm freezing before or after sperm preparation.

Author	N	Male aetiology	Protocol	Sperm count (x106)	PM (%)	TM (%)	MSC	Apoptosis	Viability	AI (%)
Petyim et al., 2014[33]	65	N	SW-F	8.50 ±6.70	99.50±2.30	NI	8.40±6.40	32.30±16.90	NI	NI
			F-SW	10.40 ±15.50	93.90±7.90	NI	9.60±14.00	51.6±16.6	NI	NI
Donnelly et al., 2001[32]	40	A	DGC-F	NI	21.00	NI	NI	NI	NI	NI
			DGC-F (+SP)	NI	37.00	NI	NI	NI	NI	NI
			F-DGC	NI	38.00	NI	NI	NI	NI	NI
			SW-F	NI	23.00	NI	NI	NI	NI	NI
			SW-F (+SP)	NI	50.00	NI	NI	NI	NI	NI
			F-SW	NI	45.00	NI	NI	NI	NI	NI
Esteves et al., 2000[31]	15	N	SW-F	6.60 ±5.70	NI	30.10 ±7.00	2.20±1.90	NI	36.90 ±5.10(NS)	72.10 ±7.20
			F	24.40 ±18.70	NI	28.00 ±7.00	9.00±12.70	NI	35.10 ±5.10(NS)	66.50 ±9.20
Palomar Rios et al., 2017[39]	2,1 20	N MF	SW-F	16.75 ±10.92	7.64±7.55	13.97 ±11.75	PMSC: 1.61± 2.60 TMSC: 2.55± 3.17	NI	14.93± 11.40	NI
			F-SW	10.92 ±11.71	37.38±29.70	38.71±29.73	PMSC: 5.41± 7.50 TMSC: 5.62± 7.65	NI	38.90 ±28.84	NI
Brugnon et al., 2013[35]	16	Infertile	DGC-F	6.40 ±1.02	13.40±12.40	14.20±2.00	0.40±0.10	NI	NI	NI
	16	Infertile	F-DGC	9.80 ±1.57	5.30±1.23	7.80±1.40	0.30±0.10	NI	NI	NI

A: asthenozoospermia AI: acrosomal integrity; DGC: density gradient centrifugation; F: freezing; MF: male factor subfertility; MSC: motile sperm count; N: normozoospermia; NI: not included; NS: no significance (p>0.005); PM: progressive motility; SP: seminal plasma; SW: swim-up preparation technique; TM: total motility; TMSC: total motile sperm count.

Table 4 includes clinical outcomes, in terms of fertilisation, pregnancy, and delivery rates, of different comparative studies in which both fresh and cryopreserved spermatozoa were used.[41-58]

As it can be deduced from the outcomes in Table 4, the use of either fresh or cryopreserved semen has no significant effect on clinical outcomes. Indeed, the use of cryopreserved semen samples in azoospermic patients undergoing surgical sperm retrievals has advantages since it avoids repeated surgical intervention, for instance through testicular, microsurgical epididymal, or percutaneous epididymal sperm aspiration. Furthermore, it is also reported that acceptable clinical results are achieved in male fertility preservation programmes when cryopreserved sperm samples from oncologic patients are used.[49]

Of note, differences cannot be established regarding the further development of offspring when fresh or cryopreserved semen samples are used. Hence, the use of cryopreserved semen samples for assisted reproduction purposes is a safe practice which neither increases the malformation rate nor worsens further physical development when compared with the use of fresh semen.[59]

Table 4: Clinical outcomes after the use of frozen semen.

ART	Sperm retrieval technique	Male aetiology	Fresh/ cryopreserved	FR % (per oocyte)	IR %	PR % (per cycle) MR %	MR %	DR %	N Cycles	Reference
AI	M	Donor	F	NE	NE	18.9	NE	NE	676	Richter et al., 1984[41]
			C	NE	NE	5.0	NE	NE	1,200	
AI	M	Donor	C	NE	NE	NE	NE	12.2↑	41,151	Calhaz-Jorge et al., 2016[42]
ICSI	MESA	OBA	F	79.5(NS)	NE	66.7(NS)	NE	NE	108.0	Janzen et al., 2000[43]
			C	78.2(NS)	NE	60.6(NS)	NE	NE	33.0	
ICSI	MESA	OBA	F	57.0	NE	-	NE	-	7.0	Devroey et al., 1995[44]
			C	45.0	NE	3	NE	2 single; 1 double	7.0	
ICSI	TESE	NOA	C	45.3	NE	29.6	NE	NE	135.0	Küpker et al., 2000[45]
ICSI	TESE	NOA	F	47.0	9.0	26.0	NE	21.0(NS)	25.0	Friedler et al., 1997[46]
			C	44.0	11.0	27.0	NE	9.0(NS)	14.0	
ICSI	TESE	OBA + NOA	C	60.0	NE	50.0	NE	NE	20.0	Prins et al., 1999[47]
ICSI	TESE	A (epididymis)	F	71.9	NE	64.3	NE	NE	129.0	Palermo et al., 1999[48]
			C	74.3	NE	46.4	NE	NE	112.0	
		A (testicle)	F	60.4	NE	50.0	NE	NE	62.0	
			C	74.4	NE	60.0	NE	NE	5.0	
F/I	M	Cancer	C	77.6	31.1	56.8	11.5	50.3	169.0	Hourvitz et al., 2008[49]
ICSI	TESE	NOA	F	50.9	NE	36.6	13.3	31.7	41.0 P	Kalsi et al., 2011[50]
			C	63.4	NE	57.1	0.0	57.1	7.0 P	
	PESA/ MESA	OBA	F	58.9	NE	46.2	27.5	33.5	173.0 P	
			C	53.7	NE	5.7	33.3	23.8	42.0 P	
	TESE	OBA	F	62.7	NE	28.6	11.1	28.6	28.0 P	
			C	57.6	NE	60.0	0.0	60.0	15.0 P	
ICSI	TESE	OBA	F	76.2	14.2	23.0	8.6	21.0	67.0	Karacan et al., 2013[51]

Table 4 continued.

ART	Sperm retrieval technique	Male aetiology	Fresh/ cryopreserved	FR % (per oocyte)	IR %	PR % (per cycle) MR %	MR %	DR %	N Cycles	Reference
ICSI	TESE	OBA	C	68.9	13.3	13.0	7.6	12.0	45.0	Karacan et al., 2013[51]
		NOA	F	67.2	12.6	29.2	6.8	27.2	99.0	
			C	64.7	23.8	12.3	10.0	21.4	84.0	
ICSI	EE	AE	F	55.0	NE	10.0	NE	NE	29.0	Hovav et al., 2002[52]
			C	50.0	NE	40.0	NE	NE	10.0	
ICSI	PESA	OBA	F	0.61*	12.6$^{(NS)}$	36.5$^{(NS)}$	5.2$^{(NS)}$	34.6$^{(NS)}$	55.0	Friedler et al., 2002[53]
			C	0.53*	12.5$^{(NS)}$	34.7$^{(NS)}$	4.1$^{(NS)}$	33.3$^{(NS)}$	80.0	
	TESE	NOA	F	0.51$^{(NS)}$	12.7$^{(NS)}$	32.2$^{(NS)}$	15.7$^{(NS)}$	27.1$^{(NS)}$	65.0	
			C	0.51$^{(NS)}$	17.4$^{(NS)}$	32.7$^{(NS)}$	21.0$^{(NS)}$	25.8$^{(NS)}$	63.0	
ICSI	TESE	A	F	58.1	NE	32.1	NE	NE	28.0	Fukunaga et al., 2001[54]
			C	54.5	NE	29.2	NE	NE	24.0	
ICSI	TESE	OBA	F	58.0	33.0	33.0	NE	NE	9.0	Habermann et al., 2000[55]
			C	64.0	32.0	14.0	NE	NE	25.0	
		NOA	F	52.0	12.5	33.0	NE	NE	3.0	
			C	56.0	24.0	67.0	NE	NE	9.0	
ICSI	TESE	OBA	F	71.6	33.0*	68.8*	25.0*	43.8*	16.0	Wu et al., 2005[56]
			C	68.0	16.7*	41.7*	16.7*	25.0*	12.0	
		NOA	F	74.5	15.8*	33.3*	0.0*	33.3*	6.0	
			C	65.8	25.0*	62.5*	20.8*	41.7*	24.0	
ICSI	TESE	NOA	F	30.5	NE	15.4	NE	NE	13.0	Hauser et al., 2005[57]
			C	30.8	NE	15.4	NE	NE	13.0	
ICSI	TESE	NOA	F	58.0	7.6	15.9	NE	NE	44.0	Verheyen et al., 2004[58]
			C	59.3	7.4	14.3	NE	NE	42.0	

*statistically significant ($p<0.005$).

↑: Higher than AI with own not cryopreserved semen; A: azoospermia; AE: anejaculation; AI: artificial insemination; ART: assisted reproduction technique; C: cryopreserved; CUM: cumulative clinical pregnancy rate; DR: delivery rate; EE: electroejaculation; F: fresh; F/I: IVF/ICSI; FR: fertilization rate; ICSI: intracytoplasmic sperm injection; IR: implantation rate; M: masturbation; MESA: microsurgical epididymal sperm aspiration; MR: miscarriage rate; NI: not included; NOA: non-obstructive azoospermia; NS: not significant ($p>0.005$); OBA: obstructive azoospermia; PESA: percutaneous epididymal sperm aspiration; PR: clinical pregnancy rate; TESE: testicular sperm extraction.

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Fibroids and Infertility

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Abstract

Infertility is generally defined as the failure to conceive after ≥1 year of unprotected sexual intercourse. Because infertility tends to be multicausal, fibroids (as the sole identified factor for infertility) were only identified in about 2.4% of patients. Uterine fibroids, also known as myomas, are benign growths of the uterus' smooth muscle tissue. They are the most common tumours to be found in the lower abdomen in pre-menopausal women. Fibroids can be found in 30–40% of all women between the age of 30 and 40 but can occur at any age. Furthermore, they are more prevalent in African women than in women of other ethnicities. The relationship between fibroids and infertility is especially difficult because of the heterogeneity of the fibroids regarding their size, location, and number, as well as the heterogeneity in observed patient populations. Even though a number of studies have attempted to clarify the influence of fibroids on fertility, there have been various, sometimes contradictory, findings and a lack of well-designed trials.

INTRODUCTION

Definition and Epidemiology

Uterine fibroids, also known as myomas, are benign growths of the uterus' smooth muscle tissue. They are the most common tumours to be found in the lower abdomen in pre-menopausal women. Fibroids can be found in 30–40% of all women between the age of 30 and 40 but can occur at any age.[1,2] Furthermore, they are more prevalent in African women in comparison to women of other ethnicities.[3-5]

Associated Factors

The growth of fibroids is positively associated with oestrogen stimulation leading to a reduction of fibroids as the menopause commences.[1,6] Thus, an early onset of menarche, a late beginning of menopause, obesity, and a high level of gonadotropins[7] show a correlation to the development of fibroids. Genetics are another factor to be considered.

Types of Fibroids

Fibroids vary in their size, number, and location. There are three main types of fibroids depending on their location in the uterus. There are many classifications of fibroids.[8,9] However, the International Federation of Gynecology and Obstetrics (FIGO) classification is the most recent and it is used worldwide.[10]

Intramural fibroids (FIGO L3–L4) are the most common type in which the fibroid is located in the muscle wall of the womb. Subserosal fibroids (FIGO L5–L7) are situated outside the muscle wall and grow into the direction of the pelvis.

Submucosal fibroids (FIGO L0–L2) develop in the myometrium right beneath the endometrium and can grow into the uterine cavity. Furthermore, fibroids can be either pedunculated or directly attached to the uterus. Depending on their location, the symptoms caused by fibroids can vary.[11]

General Symptoms

Fibroids are often asymptomatic.[1] However, they can account for a number of symptoms such as menstruation changes (hypermenorrhoea, menorrhagia, dysmenorrhoea, and subsequent anaemia), dyspareunia, and bladder and bowel irritation due to size.[12,13] Specifically, fibroids can also show an association with reduced fertility, infertility, or miscarriages, which will be discussed more closely.

INFERTILITY

Infertility is generally defined as the failure to conceive after ≥1 year of unprotected sexual intercourse. There is prevalence of 16% after 1 year in the general population and a prevalence of 8% after 2 years. Primary infertility refers to a couple who have had no previous pregnancies before, whereas secondary infertility refers to having failed to conceive following the last pregnancy. Medical investigation is generally recommended after unsuccessful conception after 1 year.[14]

Male and female factors can lead to infertility, thus both the female and male partner need to be investigated. Male factors can include abnormal semen analysis, surgical pelvic procedures, endocrinological conditions, drug abuse, and genetic disorders. Female risk factors for infertility or reduced fertility can be an age of >35 years, menstrual irregularity, history of pelvic inflammatory disease or sexually transmitted diseases, endometriosis, over and underweight, previous pelvic surgeries, and the presence of fibroids.[15] Because infertility tends to be multicausal, fibroids (as the sole identified factor for infertility) were only identified in about 2.4% of patients.[16]

Potential Influence of Fibroids on Fertility from a Biological Perspective

There are a number of different mechanisms through which fibroids negatively influence fertility. Firstly, enlarged size or specific location fibroids can hinder the transport of the sperm and egg, as well as their implantation.[11,17,18]

Secondly, the presence of submucosal fibroids seems to influence levels of IL-10 and glycodelin. These cytokines are supposed to support the implantation and early embryonic development. In the presence of fibroids, they appear to be reduced.[17]

Thirdly, Purohit et al.[17] and Yoshino et al.[19] have found that fibroids seem to alter uterine contractions leading to an inflammatory reaction in the uterus. The latter may hinder implantation.

Fourthly, it is reported that the presence of fibroids leads to a change in the endomyometrial junctional zone. This zone regularly consists of macrophages and natural killer cells that contribute to the endometrial decidualisation during implantation. Some studies have shown that, fibroids are associated with the reduction of those cells.[20,21]

The thickness of fibroid pseudo capsule (a neurovascular bundle surrounding the fibroid that is rich in neurofibres) is considerably higher near the endometrial cavity compared to intramural and subserous fibroid, suggesting a potential role in fertility.[22]

Reduced fertility can have a number of causes, and the ones mentioned above are not to be considered as separate or monocausal. The relationship between fibroids and infertility is especially difficult because of the heterogeneity of the fibroids regarding their size, location, and number, as well as the heterogeneity in observed patient populations.[21] Even though a number of studies have attempted to clarify the influence of fibroids on fertility, there have been various, sometimes contradictory, findings and a lack of well-designed trials.[17]

The findings from recent studies and reviews will now be discussed to try to give a recent view on the relationship between fibroids and fertility.

Influence of Fibroids on Fertility

The probability of clinical pregnancy, implantation, and live birth in women with fibroids was significantly lower, while abortion rates were higher in comparison to the control group independent of the fibroids' location.[21,23] Looking more closely at the fibroids' location, it has been found that intramural fibroids and submucosal fibroids with intracavitary distortion were associated with lower pregnancy, implantation, and live birth rates in comparison with women with no fibroids, whereas there was no difference found for subserosal fibroids.[21,24] Generally, submucosal fibroids are associated with a 70% reduction in delivery rate, intramural with a reduction of 30%.[25-27] It is thought that the size of the fibroid (2–6 cm) does not impact fertility outcomes;[22] however, recent studies have shown that fibroids size in-fact does have an effect: intramural fibroids of size >4 cm were seen to be associated with statistically lower pregnancy rates in comparison to smaller intramural fibroids.[28-30] The effect of size could thus be seen in combination with the fibroids' location.

In general, one can state that the diverse findings show the need for more controlled, high-quality studies.

In Vitro Fertilisation

Studies have also specifically focussed on the relationship between fibroids and *in vitro* fertilisation (IVF) outcomes. As with regular pregnancy, submucous fibroids and intramural fibroids distorting the uterine cavity are connected to lower pregnancy rates, delivery rates, and higher spontaneous abortion rate after IVF or intracytoplasmic sperm injection.

For intramural non-cavity distorting fibroids, no difference regarding IVF outcomes has been found.[31] Vimercati et al.,[32] on the other hand, have found no significant difference in the implantation, miscarriage, and pregnancy rates after IVF between submucosal, subserous, and intramural fibroids at all. Yet, they found the size of the fibroid to be negatively correlated with fertility.

Klatsky et al.[33] found no difference in implantation or clinical pregnancy rate in women with fibroids in comparison to women without when they received donor oocytes through assisted reproductive technology. Somigliana et al.[34] observed no effect of fibroids with a diameter <5 cm which are not distorting the endometrial contour on pregnancy rates. On the contrary, a review of 19 studies conducted by Sunkara et al.[35] in 2010 showed a statistically relevant inverse correlation of non-cavity distorting intramural fibroids and live birth rate as well as clinical pregnancy rate.

Thus, also regarding IVF, the contradicting findings show the need for more controlled studies and hint at a more than monocausal relationship between fibroids and the success of IVF.

TREATMENT OPTIONS FOR FIBROIDS AND INFERTILITY

Myomectomy

Myomectomy as a potential surgical treatment for fibroids has been explored in many case studies. In general, approximately 50% of women with infertility and fibroids become pregnant after myomectomy,[16] although the numbers differ depending on the study. Whether performed hysteroscopically or by laparotomy, myomectomy has led to a rise in pregnancy rates;[17,27] however, only the rise regarding submucosal fibroids was statistically significant.[17] Then again, the literature review has shown that myomectomy before IVF has significantly increased the success rate in fibroids without submucosal component.[28]

Because of the divided findings in literature, Purohit et al.[17] conclude that the benefit of myomectomy highly depends on the location and size of the fibroids. There is little evidence for a beneficiary effect regarding subserosal fibroids since they appear to have little effect on a woman's fertility. For submucosal fibroids, a positive effect on pregnancy and live birth rates is stated. For intramural fibroids, the evidence remains unclear as there is a higher risk of postoperative complications, such as adhesions that can lead to infertility by themselves.[17] A study performed by Casini et al.[29] suggested that a pregnancy rate of 56.5% in women with intramural fibroids and myomectomy in comparison to 41.0% without myomectomy. However, the higher rate was not statistically significant, thus myomectomy for intramural

fibroids should be considered individually depending on exact size, number, and location.[17]

Hysteroscopic myomectomy is recommended for fibroids located in the cavity (FIGO L0–L1). In L2, up to 5 cm fibroids hysteroscopy is possible, however, it might have to be carried out in multiple-stage procedures. It is highly necessary to take into consideration the possible operation-related intrauterine adhesions and endometrial damage which are negatively correlated to reproductive outcomes.[17]

In larger fibroids (FIGO L2 >5 cm and above) management by laparotomy or laparoscopy should be considered. A systematic review of the data has shown a similar outcome for both approaches. Metwally et al.[36] suggest that there is no statistically significant difference in live birth rate, pregnancy rate, miscarriage rate, and preterm delivery rate between open and laparoscopic myomectomy.

Yet, for the laparoscopic procedure, reduced blood loss and shorter inpatient stays were noted.[17] In laparoscopic myomectomies, a significantly lower risk of infertility fostering adhesions were found in comparison to laparotomy.[16] Another general risk includes uterine rupture during pregnancy or labour, leading to a higher rate of caesarean section, of which is preventable. However, it is unclear if laparoscopic myomectomy increases the risk of rupture significantly.[16]

Kameda et al.[37] found that laparoscopic uterine myomectomy was successful in 45.7% of infertile patients with no known cause but fibroids in comparison to 28.6% without laparoscopic treatment, yet the difference was not significant. If the number of fibroids was ≤3, the difference became statistically significant. Also, in patients with four–nine fibroids, the myomectomy showed a pregnancy rate of 64.7% with no pregnancies in patients without myomectomy. In patients with >10 fibroids however, no pregnancy could be achieved.

Medical Treatment

While the application of hormones, through, for instance, the combined oral contraceptive pill, progesterone-only pill, and levonorgestrel intrauterine systems, is commonly used to improve pain and menstrual complaints due to fibroids, they are not applicable to infertile women because of their contraceptive effect.

Ulipristal acetate (UPA), a progesterone receptor modulator, is a licensed option for uterine fibroids because of its size reducing effect by increasing the apoptosis of leiomyoma cells. The therapy is limited to 3 months and its effect lasts for about 6 months without surgery.

Luyckx et al.[38] conducted a study with 52 patients receiving UPA with 21 patients wishing to conceive. From 3 months after the end of UPA therapy, 15 of the 21 patients (71.0%) became pregnant, with a total of 18 pregnancies, of which, 12 pregnancies resulted in the delivery of 13 babies and 6 resulted in miscarriages.

Purohit et al.[17] pointed out that the study done by Luyckx et al.[38] had shown that despite the size reduction of fibroids and possible conception after the end of the UPA therapy, there has been a higher rate of miscarriages if women did not undergo myomectomy as well, thus, surgical management is additionally being recommended.

Uterine Artery Embolisation

Uterine artery embolisation[39] leads to an ischaemia for approximately 72 hours in the uterus and was previously seen as a treatment for women with fibroids and without a wish to conceive a child when it was established in 1995. Even though the ischaemic effect was supposed to be only irreversible within the fibroid, there are concerns that not only the uterine, but also the ovarian function, can be irreversibly affected as well in certain women. In patients <35 years of age, uterine artery embolisation has lower pregnancy rates in comparison to myomectomy, as well as longer conception time. Moreover, the number of live births was significantly lower and the number of early miscarriages higher in women with uterine artery embolisation (60.0% in comparison to 23.0% after myomectomy),[40] indicating a superiority of myomectomy.

However, Mohan et al.[41] suggest in their review analysing 23 studies that the cumulative pregnancy rate of 58.6% in women with a mean age of 35.9 years after having undergone uterine artery embolisation is comparable to the age-adjusted pregnancy rate in the population. The cumulative miscarriage rate due to their findings

was 28.0%, which is comparable to the rate in the general population.[41]

On the contrary, another review conducted by Karlsen et al.[42] in 2018, including 17 studies with 989 patients, found lower pregnancy rates and higher miscarriage rates after uterine artery embolisation in comparison to myomectomy, supporting the Mara et al.[40] findings above.

Czuczwar et al.[43] compared UPA and uterine artery embolisation on ovarian reserve after 3 months. There was a significant reduction in anti-Müllerian hormone and an increase in follicle-stimulating hormone. However, in the UPA group there were no significant changes.

In alignment with the contrasting findings, the authors refer to the low quality of evidence which can be deducted from the studies, highlighting the need for randomised studies in the future.[42]

Magnetic Resonance-Guided Focussed Ultrasound

Magnetic resonance-guided focussed ultrasound (MRgFUS) applies microbeam radiation therapy-directed beams of ultrasound to heat specific areas of a fibroids and cause destruction through necrosis.[14] The treatment was first established in 2000 for fibroids. According to Rabinovici et al.,[44] MRgFUS was limited to patients declaring their non-interest in further pregnancies. However, a number of case reports of pregnancies after the treatment have been published with a total of 51 women. A total of 54 pregnancies have occurred, with a live birth rate of 41% and a spontaneous abortion rate of 28%. The mean birth weight was 3.3 kg with a vaginal delivery rate of 64%.[44] These outcomes are supported by other case reports[45] and suggest a promising role for MRgFUS related to the treatment of infertility in patients with fibroids which should be investigated further.

CONCLUSION

The effects of fibroids on fertility and its management on improving fertility are inconclusive. The management should be individualised depending on the classification of the fibroids in relation to endometrium. Hysteroscopic excision of the submucosal fibroids should be considered before starting fertility treatment. With regards to intramural fibroids, it is individualised according to its relation to the uterine cavity. The subserosal fibroid is unlikely to have an impact on fertility. Medical, UPA, and MrgRUS are not recommended for women who desire fertility treatment.

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Causes and Impact of Cryopreservation-Associated Damage on Different Parameters of Human Spermatozoa and its Clinical Impact

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Abstract

Sperm cryopreservation has been widely used for assisted reproductive technology (ART). Indications for sperm cryopreservation include donor insemination, cryopreservation prior to surgical infertility treatment, and malignancies to avoid additional surgery in couples undergoing repeated *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) cycles. However, dramatic changes during cryopreservation have detrimental effects on the sperm membrane, resulting in a large increase in the percentage of poorly motile sperm or sperm with abnormal morphology. The negative effects related to rapid temperature decrease, such as osmotic injury, cellular dehydration, intracellular ice crystal formation, and oxidative stress can also damage the sperm in ways that affect reproductive outcome.

This comprehensive review focusses on describing the detrimental effects of the cryopreservation process on sperm and aims to clarify that not all impaired sperm parameters have the same impact on the clinical practice of ART. Regarding the parameters studied, some of the biomarkers used for sperm maturity, hyaluronic acid binding capacity, or damaged DNA have limited clinical significance compared to other semen parameters which provide more useful information for clinical practice and are often dismissed, such as total motility or total motile sperm count (TMSC). In the authors' experience, TMSC gives valuable quantitative information about the number of viable spermatozoa. Indeed, TMSC should be assessed specifically for groups of patients in which sample availability is limited.

INTRODUCTION

The cryopreservation of gametes, as well as more complex multicellular organisms such as embryos, is an increasingly common practice in the field of assisted reproduction technology (ART) and reproductive medicine. Cryopreservation is described as the process of long-term cell preservation and storage at cryogenic temperatures (-196 °C), thus maintaining cell viability by means of hampering ageing and cell degradation processes.

Recent advances in the field of cryobiology have not only led to the development of

novel fertility treatments, such as fertility preservation and gamete/embryo donation, but also helped co-ordinate gamete retrieval and fertilisation measures.

Since its early beginnings in the 1970s, human sperm cryopreservation has been demonstrated to be a crucial aid in ART. Currently, many male patients attend sperm cryopreservation programmes for fertility preservation purposes.

The cryopreservation and storage of human ejaculated spermatozoa is commonly facilitated in groups of male patients reporting difficulties in semen sample collection, such as in cases of erectile dysfunction and lack of or retrograde ejaculation. Additionally, to schedule oocyte retrieval and semen sample collection, sperm cryopreservation is counselled in those male patients who will not be able to collect the sample the day of ovarian puncture.

Furthermore, sperm cryopreservation should also be considered in cases of azoospermia to prevent repeated sperm retrieval surgeries. Similarly, cryopreservation is also recommended for groups of male patients who have impaired semen parameters, or in cases in which there is an observed, progressive decrease in sperm quality, to prevent the risk of azoospermia.[1]

Additional to these purposes, male fertility preservation is particularly relevant in oncologic patients, or in patients reporting chronic non-malignant diseases or autoimmune disorders. The gonadotoxic effect of treatments is the main reason why fertility preservation is counselled in such patients, since chemo/radiotherapy regimens as well as other medications for chronic diseases are a real threat to fertility potential.[2,3] Indeed, in some cases sperm parameters are impaired even prior to any treatment. This could be in response to the direct impact of the tumour on the male genital tract, as is observed in testicular cancers. Additionally, indirect effects inherent to oncological processes could also be a plausible cause of sperm parameter impairment in these patients.[4]

As the survival rates of patients living with malignant or non-malignant chronic disorders are increasing as a result of the new therapeutic options available to them,[5] there is increasing interest from young male patients of reproductive age for fertility preservation. Since long-term quality of life following cancer treatment has become more prevalent in society, initiating semen cryopreservation before gonadotoxic treatments is seen as an effective means of maintaining male reproductive potential. A study carried out by Duadin et al.[6] investigated the growing trend to cryopreserve the sperm of pubertal boys and young adults being treated for leukaemia, lymphomas, or other types of germ cell tumours.

Moreover, it is worth mentioning that the implementation of sperm cryopreservation protocols enables the establishment of donor sperm banks and, consequently, donor sperm programmes.

Unfortunately, despite the many strengths of sperm cryopreservation, important damages to the sperm cells are still reported. Furthermore, compared to other methods there is currently no standardised cryopreservation protocol for human spermatozoa that leads to higher viability yields after thawing.

The aim of this review is to provide comprehensive information regarding the state-of-the-art human spermatozoa cryopreservation techniques, highlighting deleterious effects associated with freezing and vitrification protocols.

METHODS

An extensive literature review on the effects of sperm cryopreservation on sperm cells was performed. Topic-relevant scientific papers published in PubMed and Google Scholar databases were included in the current comprehensive review. Key words used for the bibliographic search were "sperm freezing", "male cryopreservation", "vitrification", "cryodamage", and "cryosurvival".

Cryopreservation of Human Spermatozoa

The cryopreservation of sperm cells is not a harmless process; indeed, the significant damage of spermatozoa due to freezing and vitrification protocols is well-described. Cryoinjury, which is the damage associated with cryopreservation, leads to an impairment of sperm parameters. In addition, according to Honda et al.,[7] the ageing process is not totally prevented by cryopreservation. They suggest that during cryopreservation the

telomere-shortening phenomenon is observed, as well as an induction of cellular senescence.

Changes in Sperm Cell During Cryopreservation

The sperm cell membrane is a vital structure during the freezing and vitrification processes since cell survival is strongly dependent on its viability, especially during the temperature decrease and following the return to physiological temperature by thawing. The sperm cell membrane exhibits as a fluid mosaic-like structure, presenting a phospholipid bilayer with protein and carbohydrates dispersed throughout. The respective stiffness or fluidity of the sperm cell membrane is determined by the fatty acids present in the phospholipid bilayer.

The membranes of human spermatozoa are characterised by their large number of polyunsaturated fatty acids (PUFA), which are responsible for membrane fluidity. Unsaturated lipids, together with the presence of cholesterol, impair the phospholipid packaging, thereby decreasing the number of possible interactions between phospholipids. As a result, membrane fluidity is increased due to steric effects. Increased sperm cell membrane fluidity ensures a better response to the cryopreservation process,[8,9] and therefore a large number of PUFA translates into a greater protection against cryoinjury during cryopreservation.[10] As well as fluidity, permeability is another key feature of the sperm cell membrane. Permeability of the sperm cell membrane will continually decrease during the freezing stage of cryopreservation to the point where the membrane becomes an impermeable structure; hence, all fluids retained in cell cytoplasm will become vulnerable to crystalisation.[9]

A crucial parameter during cryopreservation is the freezing rate. During the temperature decrease from physiologic temperature (37 °C) to 0 °C, a temperature shock occurs. This initial temperature decrease promotes the change of phase of membrane phospholipids from the fluid to gel phases. As the phase exchange is not simultaneous across the whole phospholipid bilayer, both phases can be observed before completion. The coexistence of both phases, fluid and gel, leads to an impaired phospholipid arrangement, increasing the cell's permeability to solutes. The use of cryoprotectant agents and a progressive freezing rate minimises this damage during the first freezing step.

The following temperature decrease below 0 °C promotes intracellular ice crystal formation. It is therefore paramount to induce a major degree of cell dehydration, so as to minimise the water content inside the sperm cell, because ice crystals cause physical damage to organelles and cell membranes. A specific, optimum freezing rate is associated to each cell type in which cryoinjury is minimised. Supra-optimal freezing rates promote ice crystal formation as a result of incomplete cell cytoplasm dehydration, whereas osmotic damage due to the high solute concentration in the extracellular medium is associated with sub-optimal freezing rates.[11]

In parallel to the temperature decrease, a calcium influx towards the sperm cell cytoplasm is observed. Under this circumstance, phospholipids present in cryoprotectant agents contribute to membrane resistance to calcium shock,[12] and therefore an appropriate balance in calcium levels is important to ensuring good post-thawing recovery. As well as the temperature decrease, high calcium levels in the extracellular media help promote cellular damage during the cryopreservation procedure.[13]

Assessment of the Cryopreservation Process

Cryopreservation-associated damage to the spermatozoa not only alters the cell membrane structure,[9,14,15] but also the cell's metabolism and mitochondrial bioenergetic processes.[16,17] After thawing, protein degradation and phosphorylation have been reported.[18] Interestingly, capacitation-like changes to spermatozoa are associated with protein phosphorylation.[18] It is noteworthy that the lifespan of spermatozoa exhibiting capacitation-like features is shortened and, in combination with the decrease in spermatozoa with intact acrosomes after thawing, this fact would reinforce the hypothesis that the fertility potential of frozen-thawed spermatozoa is threatened.

All these cited changes have an impact on sperm parameters, such as decreasing motility or altered morphology. Furthermore, as well as mitochondrial damage, impaired motility, and morphology, sperm DNA integrity can be affected during cryopreservation.[19] As Figure 1

shows, the cited parameters are the main targets of cryodamage.

Sperm Morphology

The effect of cryopreservation on sperm morphology has been the subject of a wide range of studies. Some studies demonstrate a decrease in normal morphological forms in the semen sample after thawing.[14,20-22] When slow freezing is considered, it is suggested that the osmotic shock caused by cryoprotectant agents required in this protocol would be the main reason for observing altered morphological forms in the thawed samples, whereas when vitrification is the chosen cryopreservation protocol, the impairment of normal morphology would be associated to extracellular ice crystal formation.[22] However, altered sperm morphology has no clear impact on reproductive outcomes, as recently reported in a number of studies.[23-25] It should also be noted that, when it comes to the assessment of sperm morphology, technical limitations lead to the hinderance of result interpretation due to intra and inter-laboratory differences.

Sperm DNA Integrity

Cryopreservation-associated alterations to sperm DNA are mainly the result of two processes: abortive apoptosis and oxidative stress caused by reactive oxygen species (ROS). According to Thomson et al.,[26] ROS production could be the main cause of damage to sperm DNA during cryopreservation.

With regard to ROS production, endogenous ROS release is common in human spermatozoa because superoxide anion dismutation leads to hydrogen peroxide production. The cytotoxic effects of hydrogen peroxide are well-established, as seen by its high oxidant activity. In this sense, as has been suggested previously by Meseguer et al.,[27] high levels of glutathione-peroxidases could be a biomarker for success after cryopreservation procedures since their activity consists of eliminating DNA damaging free radicals.

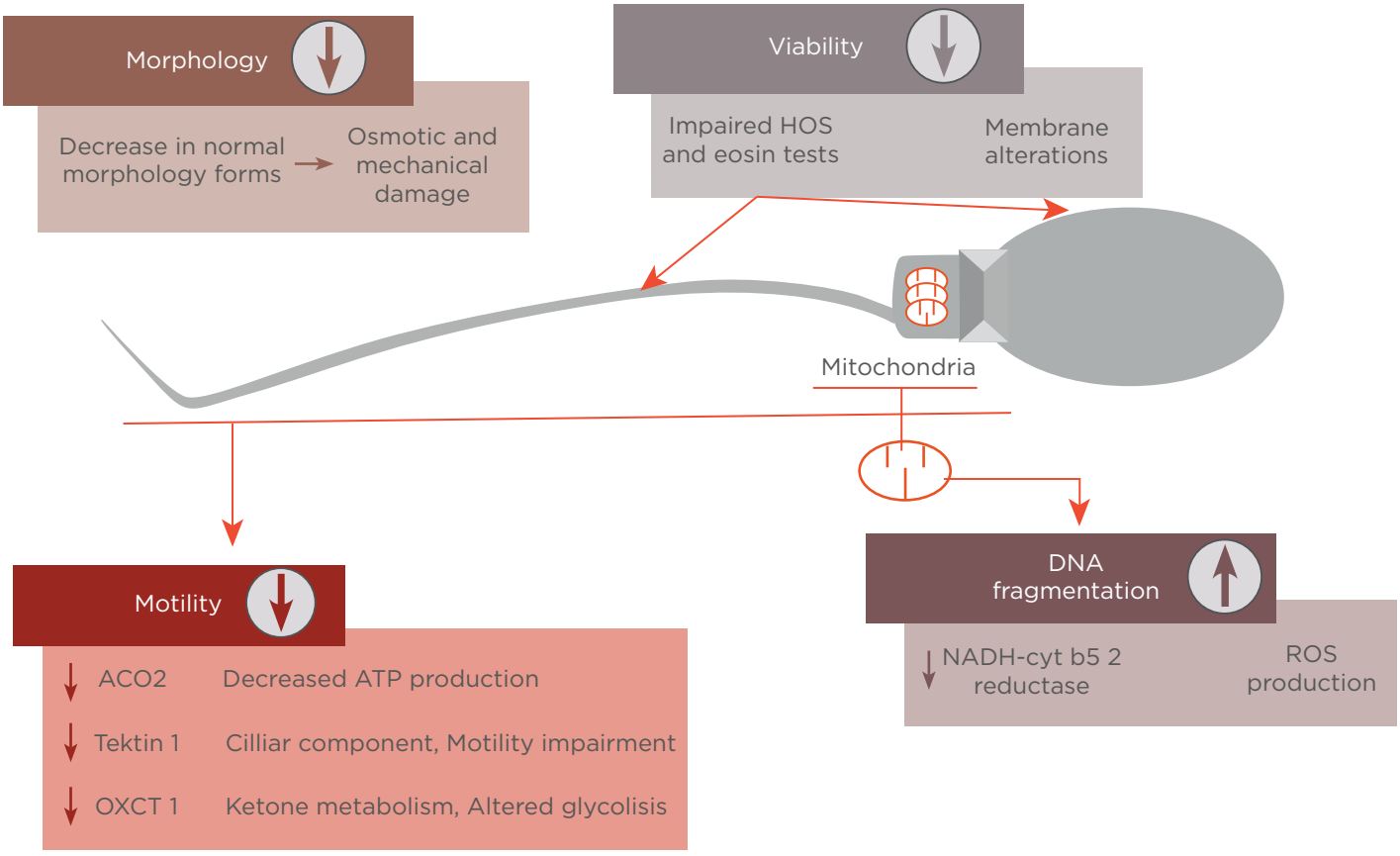

Figure 1: Summary of main sperm parameters affected by cryodamage and its causes.

ATP: adenosine triphosphate; HOS: hypo-osmotic swelling; ROS: reactive oxygen species.

In parallel, other components found in seminal plasma, such as antioxidant enzymes, prevent ROS release. Similarly, the protective role of albumin against endogenous ROS production has been reported.[19,26] Albumin's protective role involves its capability for binding lipid peroxides and neutralising their cytotoxic effects.[28]

However, the clinical impact of cryopreservation-associated DNA fragmentation is still a debatable issue, as suggested in recent reviews and meta-analyses.[29,30] Differences among samples regarding cryoresistance, protocols for cryopreservation, and the wide range of methods used to assess DNA integrity make the agreement of a consensus difficult.

The meta-analysis carried out by Robinson et al.[31] puts forward the idea of an increased risk of miscarriage being associated with the use of high-fragmented spermatozoa.[31] Unfortunately, as the authors also commented, there are design flaws in the studies included in the meta-analysis. Firstly, the threshold values for DNA fragmentation index varies among the studies, from 10–36%.[32,33] Even using a high sperm fragmentation index, Esbert et al.[33] did not observe any differences in clinical outcomes, regardless of the IVF technique used and the origin of the oocytes. In addition, the results clearly show a dependence of risk ratio's ranges on the test used for DNA fragmentation assessment, and thus important variations among the risk ratios are observed when different DNA fragmentation assays are used. Another common limitation regarding the assessment of sperm DNA fragmentation and clinical outcomes is the cohort of oocytes included. An elegant study carried out by Meseguer et al.[34] defends the premise that oocyte quality is a major factor to consider due to the ability of good-quality oocytes to conditionally repair DNA damage. Therefore, this should be considered when it is stated that sperm DNA damage worsens reproductive outcomes through an increase in miscarriage rate in clinical studies including poor quality oocytes.[35]

Addressing the issue of using frozen or fresh spermatozoa for ART purposes, Ribas-Maynou et al.[36] previously reported a slight increase in single-stranded DNA lesions when cryopreserved spermatozoa were used, but no difference could be established, in terms of double strand DNA damage, between fresh and frozen sperm samples. This is particularly important because different sorts of DNA damage have different clinical outcomes.[37,38] Consequently, double-stranded DNA breakages are associated with miscarriages, while single-stranded DNA lesions have no further clinical impact except a putative decrease in fertilisation rate and a delay on pregnancy achievement.[39] Moreover, comparative studies of different cryopreservation protocols fail to find differences in sperm DNA integrity among the protocols studied.[20,22,40-44] More remarkable is that most of these studies reported a high degree of DNA integrity, superior to 70%, after cryopreservation.[20,40,41,43,44] In contrast, some of these studies observed lower sperm DNA integrity, in the range of 15%, in thawed samples.[22,42] However, it is relevant to note that the sperm DNA integrity prior to cryopreservation is not available in these cases.

Sperm Maturity

To date, several maturity markers have been reported for sperm cells. In brief, high cytoplasmic levels of creatine kinase (CK) correlate with the spermatozoon's immaturity, suggesting an impairment in the last phase of spermatogenesis, when the cytoplasm extrusion occurs. Moreover, high expression of chaperonin HspA2, in combination with low levels of CK, are biomarkers of sperm maturity.[45]

At least three hyaluronic acid binding proteins have been described in mature spermatozoa. These proteins are membrane receptors that are involved in relevant physiological processes, such as acrosome reaction, hyaluronidase release, and the binding to the oocyte's zona pellucida.[46,47] Therefore, a sperm maturity profile has been developed based on the capability of spermatozoa to bind hyaluronic acid. In the research carried out by Yogev et al.,[48] spermatozoa maturity status has been used as a marker of successful cryopreservation outcome.[48] These authors report a poor correlation between spermatozoa hyaluronic acid-binding capacity and survival rates in thawed samples. According to these evidences, hyaluronic acid binding sites in spermatozoa are not altered by cryopreservation; hence, hyaluronic acid-binding capability would not be a useful marker to assess cryopreservation outcomes.[47,49]

Sperm Survival: Viability and Motility

Sperm survival is the main parameter to consider when assessing cryopreservation outcomes. Traditionally, sperm survival is assessed by different assays with the same biological basis: sperm cell membrane integrity.

Standardised viability tests are based on the assessment of sperm cell membrane's integrity as spermatozoa exhibiting altered cell membranes are associated with the loss of cell functionality. Eosin staining and the hypo-osmotic swelling test (HOS-test) allow to distinguish viable, but not motile, spermatozoa; hence the use of the cited tests is interesting in clinical cases in which sperm motility is difficult to assess. Several studies agree that, regarding the number of viable spermatozoa after thawing, it is because of physical and chemical stress that cryopreservation procedures worsen the results derived from those viability tests.[15,20,50,51]

Despite strengths, these classic viability tests are not exempt from limitations. Eosin staining does not allow the use of the stained sperm sample in the following artificial reproduction techniques; hence the putative information derived from eosin staining results cannot be fully translated to the clinical practice when it comes to selecting the spermatozoon to be microinjected. Alternatively, after HOS-testing, the spermatozoa remain viable because the biological basis of HOS-test relies on the sperm cell's swelling capacity as a response to preserve osmotic balance under hypo-osmotic conditions. As hypo-osmotic conditions are established *in vitro* by exposing spermatozoa to a relatively harmless solution of sodium citrate and fructose, thus maintaining sperm viability, the sample could be used in the following ICSI, allowing the selection of viable spermatozoa exhibiting swelled tails.

However, some studies suggest that the spontaneously developed tail swellings occur in some cases under physiological conditions, resulting in an increased false positive rate because the tail swelling occurs in the absence of hypo-osmotic solution. Hossain et al.[50] also reported that the spontaneously developed tail swelling phenomenon is particularly increased in thawed samples.

On the other hand, biologically, the movement of sperm cells is fuelled by means of energy from adenosine triphosphate (ATP). One of the sources of ATP can be the oxidative phosphorylation carried out by mitochondria located in the mid-piece of human spermatozoa. In spite of some concerns about the putative transference of ATP derived from mitochondrial activity through the flagellum, it could be hypothesised that ATP released from oxidative phosphorylation by mitochondria could be transported by means of the flux transfer chains, a model published by Dzeja and Terzic.[52] Other authors support this hypothesis,[53] and the decrease in sperm motility in certain circumstances in which mitochondrial function is inhibited is additionally represented in robust evidences.[54-57] Nevertheless, ATP used for the movement of spermatozoa is not exclusively produced by oxidative phosphorylation in the mitochondria. Indeed, glycolysis is another source of ATP that fuels sperm motility.[58-60] In fact, a recent comprehensive review addressing the issue of the signalling pathways involved in sperm motility suggest that, possibly, both mentioned mechanisms co-operate to allow ATP production for sperm motility.[61] The degree of contribution of each metabolic pathway could depend on the substrate's availability in the female reproductive tract or in the culture media.

Several authors agreed that a decreased motility rate can be observed, as well as decreases in other kinetic parameters, in thawed samples.[14,16,17,21,22,62-64] The observed decrease in sperm motility could be due to the mitochondrial damage described after cryopreservation, as less functional mitochondria with reduced activity have been reported in thawed samples.[16]

In this sense, sperm motility could provide a relatively accurate estimation of viability provided the premise that a motile spermatozoon is viable. The Computer Assisted Semen Analyzer (CASA) system can assess more accurately than manual observation the motility of a given sample, because this sort of software considers all types of motility that a spermatozoon can exhibit and is not only limited to progressive motility traditionally assessed by manual observations. Indeed, sperm total motility (TM) strongly correlates with HOS-test results in terms of sperm viability.[65] Therefore, TM could be used as an estimation of the viability of a given sperm sample. TM assessed by CASA systems could be considered a rapid and objective method to estimate cryopreservation outcomes regarding

sperm survival. However, there exist some exceptional clinical circumstances in which sperm viability tests, such as the HOS-test, cannot be replaced. In patients reporting an absolute structural alteration of the sperm flagellum, as in Kartagener syndrome, the HOS-test should be used rather than other assessments of sperm motility due to the physical incapability of the spermatozoa to move.[66,67]

Total Motile Sperm Count

Sperm count is also a paramount parameter that should be considered in certain clinical scenarios, particularly when the sample's availability is limited. The combination of TM percentage and sperm count in a single parameter provides useful information, not only about viability rate, but also quantitative valuable information regarding the total number of viable spermatozoa.

A quantitative assessment of the number of viable spermatozoa is especially crucial in groups of sub-fertile patients reporting impaired sperm count or motility, as in these circumstances it is vital to maximise the number of motile spermatozoa achieved. Recent studies agree that using total motile sperm count (TMSC) as a sperm parameter contributes to achieving better outcomes in different clinical cases, such as in testicular cancer,[68] severe male factor diagnosis,[69] and in ICSI cycles.[70]

Differences in the clinical impact associated with each sperm parameter included, based on the main evidences included in this review, are summarised in Figure 2.

Sperm parameter	Author	Main outcome
Sperm morphology	Ozkavucku et al., 2008[4]	Decrease in normal morphology forms in thawed sperm samples.
	Hammadeh et al., 2001[20]	
	Satirapod et al., 2012[21]	
	Agha-Rahimi et al., 2014[22]	
	Gatimel et al., 2016[23]	Normal morphology forms is not a predictor of better clinical outcomes of neither homologus artificial insemination nor IVF-ICSI treatments.
	Shabtaie et al., 2016[24]	Usefulness of sperm morphology assessment to improve chance of reproductive success in ART remains controversial.
	Sikka & Hellstrom., 2016[25]	Intra- and interlaboratory differences in assessing sperm morphology hinder results interpretation.
Sperm maturity	Yogev et al., 2010[48]	Poor correlation between sperm maturity (evaluated by hyaluron-binding assay) and sperm survival after thawing.
	Nijs et al., 2009[47]	No difference in maturity markers prior or after sperm freezing.
	Ye et al., 2006[49]	No correlation between sperm hyaluron-binding assay and fertilisation rate after an IVF cycle.
Sperm DNA integrity	Pacey et al., 2018[29]	Lack of consensus regarding which sperm DNA fragmentation test is more suitable for clinical practice. No agreement to establish a clinically relevant threshold value for sperm DNA fragmentation. Need of clinical trails addressing the issue of the effectiveness of assessing sperm DNA integrity in ART.

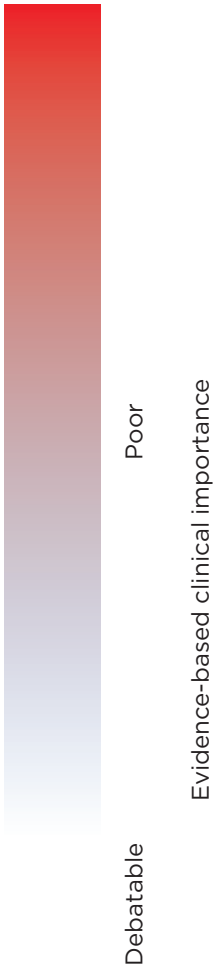

Figure 2: Clinical importance of sperm parameters based on published evidences.

Sperm parameter	Author	Main outcome
Sperm DNA integrity	Cissen et al., 2018[30]	Evidence is not enough to recommend routinely assessment of DNA integrity.
	Robinson et al., 2012[31]	Increased risk ratio for miscarriage when high-fragmented spermatozoa are used.
	Ribas-Maynou et al., 2014[36]	Increase of 10% single strand DNA lesions in thawed sperm samples compared with fresh sperm samples. No impact on miscarriage rate because there is not any difference in double strand DNA lesions in thawed samples in comparison with fresh samples.
Sperm viability: Classic vitality test	Zhu et al., 2000[15]	Impaired vitality test results after cryopreservation due to the sperm cell membrane damage.
	Lin et al., 1998[51]	
	Hossain., 2010[50]	HOS-test false positive results sue to spontaneously developed tail swelling are increased after thawing.
Sperm viability: Motility	Ozkavukcu et al., 2008[14]	A decrease in spermatozoa kinetics occurs after thawing.
	O'Connell et al., 2014[16]	
	Oberoi et al., 2014[17]	
	Satirpod et al., 2012[21]	
	Agha-Rahimi et al., 2014[22]	
	Di santo et al., 2012[61]	
	Donnelly et al., 2001[62]	
	Petyim et al., 2014[63]	
Total motile sperm count	Hotaling et al., 2016[68]	TMSC is a predictor of recovery after thawing in patients with testicular cancer. There is a correlation between higher TMSC and outcomes.
	Hamilton et al., 2014[69]	TMSC correlates better than WHO 2010 parameters with the severity of male factor subfertility.
	Borges et al., 2016[70]	TMSC predicts better than WHO 2010 parameters the outcomes of ICSI cycles.
	Palomar et al., 2017[65]	TMSC is an accurate estimator of the number of viable spermatozoa after thawing.

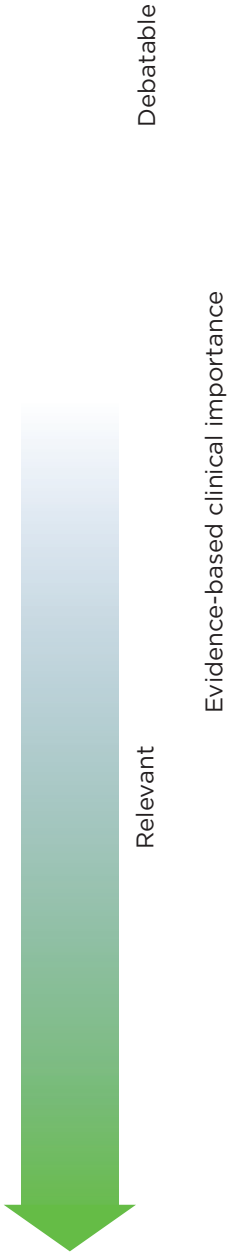

Figure 2 continued.

ART: assisted reproductive technology; ICSI: intracytoplasmic sperm injection; IVF: *in vitro* fertilisation; TMSC: total motile sperm count; WHO: World Health Organization.

CONCLUSIONS

Cryopreservation of human spermatozoa is a process that causes severe damage to sperm cells. Even though there are several sperm parameters that can be the target of cryodamage, the clinical impact of each parameter is not the same. While the clinical usefulness of some parameters on reproductive outcomes is unclear or remains controversial, such as sperm morphology, sperm maturity, or DNA integrity, other sperm parameters, including TMSC, should not be dismissed, and should be strongly

recommended to be assessed in certain clinical circumstances when the sample's availability is a limiting factor.

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Optimising the Outcome of Embryo Transfer

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Abstract

In vitro fertilisation (IVF) is a complex procedure, the success of which is dependent on several factors at every step of the process. Despite major advances, successful implantation rates in IVF remain low. Aside from the status of the embryo and endometrium, embryo transfer (ET) plays a major role in implantation. There are numerous variables in ET that are causative factors for IVF success. In this article, the authors discuss whether the stage at which (cleavage versus blastocyst) ET occurs; a fresh or frozen ET; and the technique of ET affects the results of an assisted reproductive technology cycle. Blastocysts had higher implantation potential than cleavage-stage embryos and it was also observed that extended embryo culture was not related to increased adverse obstetric and perinatal outcome. Though freezing has several advantages over fresh cycles, one must remember that evidence is still lacking for its use in all patients. Elective cryopreservation of all embryos with transfer in subsequent frozen ET cycles may be requited in cases at risk of developing ovarian hyperstimulation syndrome, women undergoing preimplantation genetic screening or preimplantation genetic diagnosis for genetic analysis, polycystic ovarian syndrome patients, and those who have high progesterone levels on the day of human chorionic gonadotropin, but to date it is debatable whether a freeze-all strategy will benefit normal and poor responders. For an optimal ET technique, the use of soft catheters and performing the process under ultrasound guidance will improve results by making it less traumatic, standardised across centres, and more technically precise.

INTRODUCTION

Despite major advances, *in vitro* fertilisation (IVF) implantation rates (IR) remain low and only a small percentage of patients achieve pregnancy. There are various factors that affect results. Aside from the health of the embryo and endometrium, embryo transfer (ET) plays major role in implantation. It is also important to understand that the variables in IVF that can affect success of the process are numerous; therefore, it is very difficult to pinpoint one factor of ET to study regarding outcomes.

There are numerous variables in ET that are causative factors for IVF success, such as the transfer of cleavage stage versus blastocyst embryo, fresh versus frozen, and the technique of ET. In this article, the authors discuss these factors and study their impact on the success of IVF.

CLEAVAGE VERSUS BLASTOCYST EMBRYO TRANSFER

Over the past two decades, there has been an increased interest in blastocyst transfers because it has a better implantation rate than a cleavage stage embryo. To date, most clinics still conduct cleavage stage ET because it is a standard global practice, and a low developmental rate of embryos past cleavage stage is seen if the embryology lab is not equipped for a blastocyst culture. The main advantage of blastocyst transfer is improved embryo–endometrium synchrony, and therefore higher chances of implantation, because the process of blastocyst implantation more closely mimics natural conception.[1,2] The other advantage of blastocyst transfer is improved embryo selection, with a significant increase in the live birth rate per started treatment compared with cleavage stage ET; however, blastocyst transfer requires optimal laboratory conditions.[1,2] Improved laboratory standards and better culture media have made extending culture to blastocyst stage a reality to identify the embryos with maximum implantation potential. Figure 1 outlines the process of transferring the embryos at the blastocyst stage.

Advantages of blastocyst stage ET over earlier stage ET are as follows:

- Premature exposure of an early stage embryos to uterine environment may cause homeostatic stress, resulting in reduced implantation potential.
- More physiologically beneficial because there is synchronisation of the embryos with the uterine endometrium.
- Embryonic block at the 8-cell-stage has been overcome; thus, improved embryo selection can be conducted, allowing best embryo to be transferred.
- The extra time *in vitro* allows for the selection of the embryos with a high implantation potential.
- IR of Day 5 embryo (single blastocyst transfer) is 49%, compared to 33% for Day 2/3 embryos (single ET [SET]) in patients ≤36 years, thus better pregnancy rates (PR) are observed with SET if blastocysts are transferred.
- Selection of a good quality embryo allows for SET, decreasing multiple PR.
- Reduced myometrial and endometrial contractions when blastocysts are transferred with a lower risk of being expelled.[3]

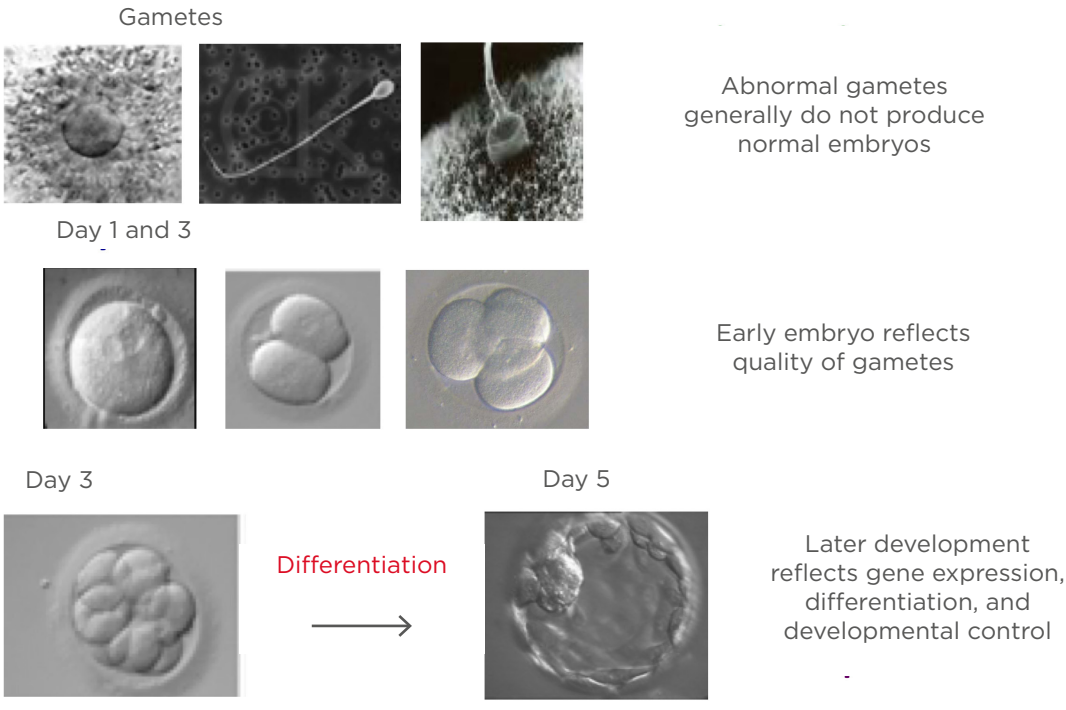

Figure 1: Blastocyst formation as a result of differentiation.

- Allows enough time for preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) results with a Day 3 biopsy.
- Comprehensive chromosome screening is possible with trophectoderm (TE) biopsy. Increasing evidence from randomised controlled trials that have shown that comprehensive chromosome screening at the blastocyst stage improves IR and PR.[4-6]
- Fewer embryos are required for higher IR and PR.[7]
- A higher live-birth rate following fresh transfer in patients with a good prognosis, patients with repeated miscarriages or IVF failures, and transfer of euploid embryos after PGS.
- Better cryopreservation results with vitrification of blastocysts, which increases cumulative PR.
- Significantly lower risk of ectopic pregnancy following transfer of a single frozen blastocyst.[8] It was also seen that the ectopic pregnancy rate was lower in frozen thaw cycles compared to fresh cycles; 1.9% for Day 3 frozen thaw cycles, compared to 0.3 % for Day 5, and 0.5 % for Day 6.[9]
- Because of the larger diameter of blastocysts, the rate of ectopic pregnancy was decreased after blastocyst transfer.[7] The ectopic pregnancy rates were 2.1–2.4% for fresh Day 3 transfers, compared to 1.6–1.7% for fresh Day 5 transfers.[9-10]
- Ectopic pregnancy rate was also related to the day the embryos were frozen. This study found a significantly lower risk of ectopic pregnancy after frozen embryo transfer if the embryos were vitrified on Day 6 (0.6%) as compared to Day 3 (3.1%) or Day 5 (2%).[11]

One study showed that extended *in vitro* culture was not associated with increased adverse obstetric and perinatal outcome in pregnancies resulting from fresh SET.[12] Nonetheless, the effects of prolonging embryo culture to Day 6 must be considered. Several researchers have shown lower IR with Day 6 embryos compared with Day 5 blastocysts (36.34% versus 19.00%[13] and 22.10% versus 3.60%,[14] respectively). When fresh ET were compared with frozen ET, the difference in the implantation potential of Day 5 versus Day 6 blastocysts occur as a result of advanced endometrial development in controlled ovarian stimulation (COS) cycles with fresh ET.[13,15] This is because slower embryos are less likely to implant, as the embryos miss the implantation window. Clinical PR were similar between blastocysts cryopreserved on Day 5 and those cryopreserved on Day 6 (32% versus 28%).[15] Moreover, the grade of blastocyst will also influence the IR and PR.[16] When discussing the grade of blastocyst, one should consider expansion and hatching first among the three morphological parameters when selecting a blastocyst for transfer; these two parameters have a high predictive value for live birth. Furthermore, inner cell mass and TE grade must be considered. Transfer of a blastocyst with inner cell mass Grade A may reduce the risk of an early pregnancy loss,[17] while the presence of cytoplasmic strings and vacuoles decrease the IR.[18-22] Blastocyst-stage ET allows the transfer of fewer embryos of higher quality, thus eliminating the potential risk of higher order multiples while maintaining high rates of pregnancy per transfer.[13]

Despite the advantages, most clinicians are unsure whether to transfer a cleavage cell embryo or a blastocyst, because there is a fear of losing embryos by culturing them to the blastocyst stage, especially if the Day 3 embryo is not of good quality or when there are few oocytes and embryos. This may result in cancellation of ET, thus resulting in the loss of an IVF cycle. When no blastocysts form, this could be the result of poor developmental potential or because of poor *in vitro* culture conditions; furthermore, the impact of prolonged *in vitro* culture is not clear. Additionally, fewer embryos are cryopreserved with blastocyst transfers (odds ratio: 0.48; 95% confidence interval: 0.40–0.57 [14 studies, 2,292 women]).[1,23] Moreover, though blastocyst transfer improves the odds of transferring a viable embryo,[24] the prolonged *in vitro* culture does not guarantee euploidy. Recent studies have shown that chromosomally abnormal embryos can become blastocysts.[25]

Lastly, a cost–benefit analysis of the two procedures should also be conducted. There is always a higher cost related to blastocyst culture and transfer due to the requirement of additional incubators, culture media, more laboratory staff members, expertise in blastocyst quality assessment, and cryopreservation protocols.

Upon considering the advantages of blastocyst versus cleavage cell ET, although the live birth rate is higher in blastocyst stage transfer, the balance tilts more in favour of cleavage-stage transfer. There was also an increased chance of monozygotic twinning (MZT) with blastocyst transfer,[26,27] high risk of sex ratio imbalance to male (p=0.01),[27] increased risk of preterm birth,[28-31] and large-for-gestational-age babies compared with cleavage cell ET offspring.[30,32] Dar et al.[29] reported a significantly higher incidence of congenital anomalies for babies born after blastocyst stage transfer. No difference in maternal outcome for pre-eclampsia, placental abruption, placenta previa, postpartum haemorrhage, premature rupture of membranes, and gestational diabetes in both the blastocyst group and the cleavage stage ET group was observed. Genetic and epigenetic changes as a result of extended *in vitro* culture are hypothesised to be the one of the drivers of the adverse perinatal outcomes associated with blastocyst stage ET.

The cost of multiple pregnancy must be added to the cost of extended culture, because blastocyst transfer has a higher risk of MZT.[33] The incidence of MZT is much higher in women aged <35 years undergoing blastocyst transfer compared with those aged >35 years (3.4% versus 2.1%; p=0.01).[34] Patients aged >35 years showed no difference in MZT rates when comparing stages of transfer. When analysing embryologic parameters, those with ≥4 6–8-cell embryos, and those with >75% of all embryos with 6–8-cells were more likely to have MZT if blastocyst transfer was to occur. MZT rate resulting from an elective SET from a high-quality embryo cohort (1.9%) was similar to the overall MZT rate in cleavage-stage embryos (1.9%).

Moreover, there are no studies on long-term outcomes in children born after blastocyst transfer.[31] Despite the shortfalls of blastocyst transfer, most clinics performing single blastocyst transfer, aiming to achieve a healthy singleton live-birth and consequently minimising the number of multiple births and their associated complications while still maintaining PR per transfer. The evidence for the above shortcomings of blastocyst transfer is either of low or very low quality must also be taken into account.

Newer technologies should be evaluated for better selection of embryos on Day 3, so as to give maximum pregnancies with fresh and frozen embryos per treatment cycle.

FRESH VERSUS FROZEN EMBRYOS TRANSFER

The conventional indication for freezing embryos in assisted reproductive technology (ART) is the availability of surplus embryos, which increases the cumulative conception rate and decreases the multiple PR by restricting the number of embryos transferred. Freezing is also used for women at risk of ovarian hyperstimulation syndrome (OHSS), those with progesterone elevation on the day of human chorionic gonadotropin (hCG) administration, and to allow personalised ET based on endometrial receptivity profile in women with recurrent implantation failure. Moreover, freezing the embryos also gives additional time for new invasive and non-invasive methods of embryo selection in patients undergoing pre-implantation genetic screening or diagnosis (PGS, PGD). Freezing is also mandatory for women undergoing fertility preservation before cancer treatment.

Fresh ET has been the norm in ART for the last three decades. Over the last 30 years, IVF treatment and research has made major progress in improving stimulation protocols and fertilisation procedures, optimising embryo culture conditions, and preventing premature luteinisation; however, only a marginal improvement has been seen in the IR and PR. It is understood that 30% of embryos are lost at the pre-implantation stage, 30% are lost after embryo implantantation but prior to the missed period and detected only by positive beta hCG, and 10% are lost after the missed period.[35] Thus, disturbance in embryo–maternal dialogue is the major reason for pregnancies to terminate at end of the peri-implantation period.

The elevated oestrogen levels due to multi-folliculogenesis seen with controlled ovarian stimulation cycles have a negative impact on endometrial angiogenesis, implantation, gene expression, and factors responsible for endometrial receptivity.[36-38] Thus, modification of the uterine environment as a result of COS may affect the IR in a fresh ET cycle as compared to a frozen ET (FET) cycle, in which the deleterious effects of COS on the endometrium can be

avoided with a better outcome.[39-41] There was only one publication by Shih et al.[42] that highlighted the physical effects of freezing and thawing embryos may filter out embryos of borderline quality with lower implantation potential, thus resulting in better IR and PR after FET.

There has been a statistically significant increase in the CPR and higher ongoing pregnancy rate (OPR) after an elective FET compared with fresh ET with the use of vitrification techniques for embryo cryopreservation, which may reduce embryo cryodamage and therefore increase success rates.[43] FET also eliminates the effect of COS on endometrial receptivity. The publication by Shapiro et al.[39] demonstrated a higher PR and OPR with cryopreserved embryos. The fallacy with this study was that the fresh ET included both Day 5 and Day 6 fresh blastocysts; the lower OPR reported with fresh blastocysts transfer could be related to the Day 6 transfers, which may result in embryo–endometrial asynchrony.

Chen et al.[44] demonstrated a statistically significant 7.3% absolute increase in live births following delayed, FET in women with polycystic ovary syndrome (PCOS). This paper reported an overall low live birth rate in the fresh ET group due to an increase in pregnancy loss, both biochemical and clinical pregnancies, along with second trimester abortions. The increased risk of miscarriages in the fresh ET group could be a result of abnormal placentation and abnormal hormonal milieu, which can affect the endometrium. Maternal response to pregnancy is affected by the metabolic effects of PCOS. These results may not extrapolate to include women with <15 oocytes. Shapiro et al.[39] demonstrated a higher CPR and OPR even in normal-responders in FET cycles as compared to fresh ET cycles. Blockeel et al.[45] looked at the SWOT (strengths, weaknesses, opportunities, and threats) analysis of a freeze-all strategy. Though freeze-all has the advantages of decreased risk of OHSS and better endometrial receptivity with improved PR, the clinical benefits of a freeze-all strategy should be looked at through well-designed clinical trials prior to shifting our current ART practice.[45] Roque et al.[46] suggested that a freeze-all strategy is not suited for all IVF patients. Freeze-all is indicated in women with risk of OHSS development and in patients with supra-physiologic hormonal levels during the follicular phase of COS; these include high oestradiol levels and elevated progesterone levels on the day of hCG. Therefore, before advocating the freeze-all approach, an evaluation of the pros and cons, including potential costs and delays in treatment, and potential risks associated with this strategy should be conducted. As most studies were carried out in women with normal or high ovarian response,[39,44,46-48] the same cannot be extrapolated in women who are poor ovarian responders.

A recent meta-analysis by Maheshwari et al.[49] showed that the relative risks of small for gestational age, preterm birth, low birth weight, perinatal mortality, and antepartum haemorrhage were much lower in women carrying singleton pregnancies following IVF and who underwent FET rather than fresh ET. There were several confounding factors which were not taken into consideration in the studies included. These confounding factors were age, smoker status, parity duration of infertility, pre-existing medical illness, and method and stage of cryopreservation of embryos.[49]

There was one systematic review and meta-analysis that investigated the effect of cryopreservation on the perinatal outcome after cleavage stage and blastocyst stage ET and found no difference in the rate of very preterm birth, low birth weight, very low birth weight, and congenital anomalies between the two groups, irrespective of the cryopreservation procedure. This meta-analysis reported a higher incidence of large for gestational age babies in the blastocyst group as compared to cleavage stage transfers after FET.[50] FET cycles have the clinical advantages of flexibility of ovum pick up scheduling, less stringent cycle monitoring, and obtaining more oocytes with reduced risk of OHSS, but it also gives a false sense of security and over stimulation can be dangerous.[51,52]

When considering freeze-all and elective FET for all approaches, the cost-effectiveness and safety, as well as the emotional status of the patient, must be evaluated; there are very few studies that have examined these factors. One publication by Roque et al.[53] reported the cost-effectiveness of FET and fresh ET, but all IVF specialists are aware that cryopreservation of all embryos and subsequent FET incurs more cost compared to fresh ET. The costs associated with freeze-all include the cost of freezing embryos and the cost of the FET procedure, which is 30% higher.

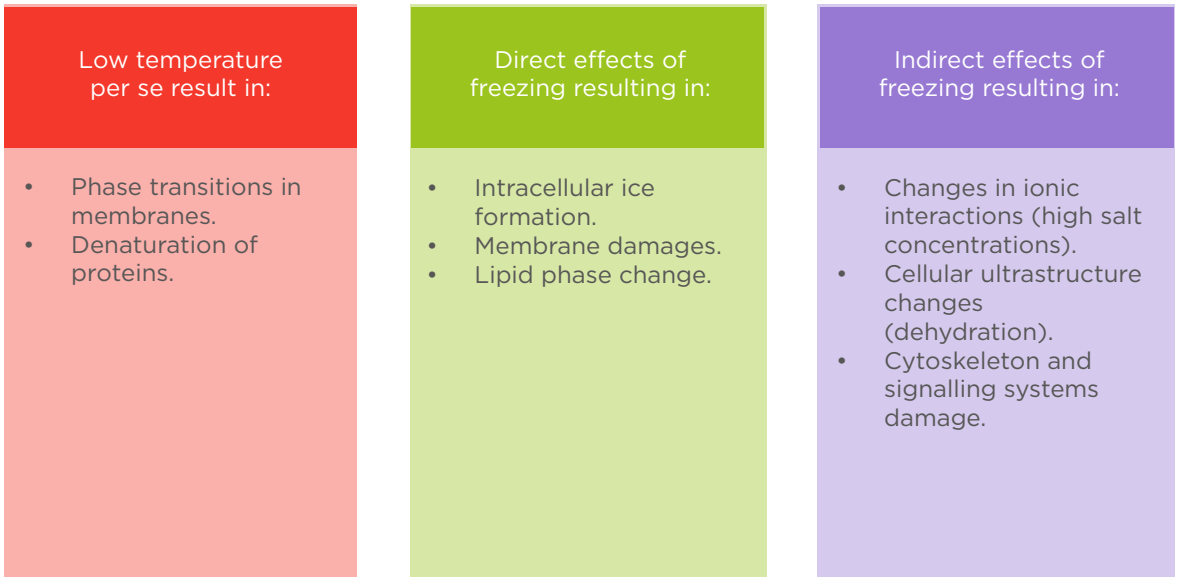

Figure 2: Damage to embryo due to cryopreservation.

Moreover, the time to pregnancy also increases considerably, potentially resulting in epigenetic changes in the embryo.

With regard to the safety issue associated with the technique, there is evidence suggesting a higher incidence of large for gestational age babies[54,55] and a higher risk of placenta accreta[56,57] after FET. It is still controversial whether the risk of hypertensive disorders is also increased in women undergoing FET, as compared to those who undergo fresh ET cycles. There are publications that have reported a higher risk of pregnancy induced hypertension.[58] There could also be an increased psychological burden due to postponement of ET and increase of time to pregnancy.[52,59] Apart from the perinatal and maternal issues, the cryopreservation procedure itself can have negative effects (Figure 2), including cryodamage, toxicity of cryoprotectants, potential equipment failure or LN2 supply failure, breach of packaging and contamination in storage, and loss of embryos.[52]

Cryopreservation may also have an effect on the integrity of the genome, and long-term follow-up of the children born after FET is therefore necessary. Cryopreservation can influence genome integrity by a number of mechanisms, including increasing reactive oxygen species, the use of cryoprotectants, exposure to low temperature, the incorporation of calcium and zinc molecule in DNA–protamine complex, and intracellular crystallisation.[60]

Results of cryopreservation may also vary due to variables such as embryo quality and morphology at freezing and post thaw and cryopreservation procedure, which can impair pre-implantation development. This can result in reduced cell numbers at the blastocyst stage and affect the survival rates of embryos after cryopreservation.[61,62]

Thus, the available evidence does not justify a change in practice at present from fresh ET to freeze for all. But improved success rate of IVF by electively freezing all embryos with routine use of frozen and thawed ET has been a matter of debate.[63] There is a need for large multicentre, randomised controlled trials to evaluate the clinical and cost-effectiveness, as well as acceptability of elective FET versus fresh ET.

OPTIMISATION THE EMBRYO TRANSFER TECHNIQUE

ET is the least successful step in the IVF process and, apart from embryo quality and endometrial receptivity, it is an important factor determining the outcome. Approximately 30% of ART failures are due to the poor performance of ET.[64-67] The procedure for ET is described in Figure 3.

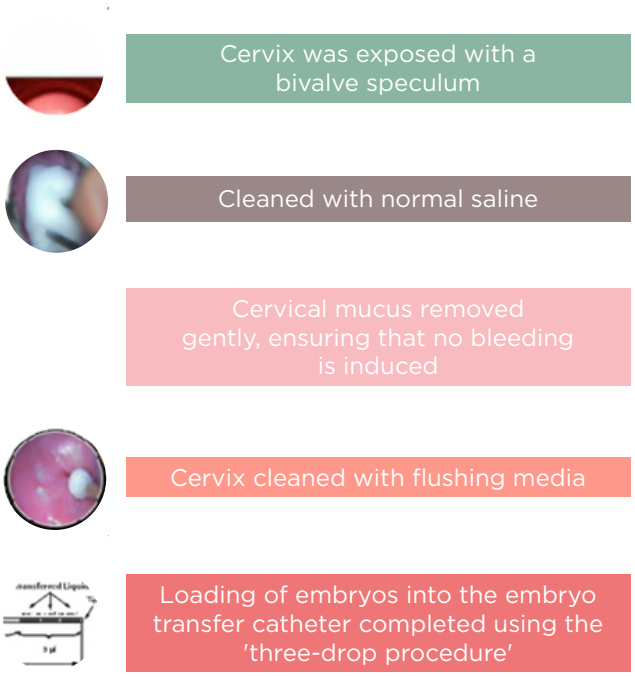

Figure 3: Steps of transfer technique.

Factors that may Affect the Success of Embryo Transfer

1. Ease of procedure.
2. ET catheter type: soft versus hard ET catheter.
3. Removal of cervical mucus.
4. Use of ultrasound guidance.
5. Position of embryo deposition in the uterus.
6. Position of the air-medium content in the catheter and the amount of media transferred.
7. Duration of ET.
8. Presence or absence of the blood on the catheter tip.
9. Retention of embryos in the catheter.
10. Excessive uterine contractions after ET.
11. Microbiological factors in the cervix and bacterial contamination of the catheter.
12. Rest after ET.
13. Experience of the physician.

Key elements for successful ET are an easy, atraumatic transfer, which is standardised and technically precise, without blood or mucus, and this can be achieved by performing a trial transfer using a soft catheter and performing the procedure under ultrasound guidance. Optimal placement of the embryos, 1.5 cm from the fundus, by injecting them slowly as confirmed by ultrasound, increases the PR. Negotiation of a difficult or stenotic cervix, identified earlier by pre-cycle dilatation, can be achieved using a malleable stylet at ET that is guided by ultrasound. One also needs to minimise embryo stress by minimising transfer time and maintain the temperature and pH of the culture media.

Implantation can be optimised by minimising contractions, avoiding trauma to the cervix or fundus, and performing a blastocyst transfer instead of a cleavage cell transfer.[68]

Despite all these precautions, ET can still prove to be difficult. Difficult transfer can arise as a result of anatomical distortion of the cervix by previous surgery or fibroids or due to congenital anomaly, presence of pronounced uterine flexion, presence of scarring in the lower uterine segment, or presence of a distorted endometrial cavity. One can overcome a difficult ET by performing a mock transfer, using stiffer and more rigid catheter systems, gently maneuvering the vaginal speculum until resistance at the internal ostium is felt.[69,70] After which, moderate cervical traction to straighten the utero-cervical angle, using a malleable obturator, followed by an inner catheter

with embryos, using a co-axial or echo tip catheter system.[71] The use of ultrasound guidance to facilitate ET, and using trans-myometrial (vaginal or abdominal) surgical ET or a trans tubal ET is recommended in rare cases.[72]

CONCLUSION

IVF is a complex procedure, the success of which is dependent on several factors that are involved at every step of the process. Some of the factors that impact the outcome of IVF are gamete and embryo quality, stimulation protocols, endometrial receptivity, and ET. In this article, the authors have discussed whether the time (cleavage versus blastocyst) of ET, the use of fresh or frozen embryos, and the technique of ET affects the results of an ART cycle.

Providers of IVF treatment have an obligation to minimise complications associated with IVF and safeguard the long-term health of future generations. Blastocysts have a higher implantation potential than cleavage-stage embryos, and it has also been observed that extended embryo culture was not related to increased adverse obstetric and perinatal outcome. With this concept in mind, most clinics worldwide have moved to single ET at the blastocyst stage with the aim of achieving a healthy singleton live-birth, minimising the number of multiple births and their associated complications, while still maintaining PR per transfer. While blastocyst transfer permits embryo self-selection, it also exposes the embryos to possible harm, due to culturing in the *in vitro* environment. Both effectiveness and safety should be weighed to permit evidence-based decisions in clinical practice. For extended culture to the blastocyst stage to be effective, technical refinements in laboratory equipment and processes are required, which may be an expensive adaptation in low-resource settings.

PR associated with frozen-thawed embryos would appear to be comparable with those after fresh ET, with potentially better obstetric and perinatal outcomes. Therefore, it is time to avoid fresh ET in IVF, freeze-all available embryos and replace them in subsequent cycles? In this article, the authors have appraised the evidence underpinning this idea, exploring the biological plausibility of the concept and considering the implications of adopting such a strategy in routine clinical practice.

Avoiding fresh ET and freezing all embryos destined for transfer could improve the safety and effectiveness of IVF and intracytoplasmic sperm injection. The prospect of improved feto-maternal outcomes is particularly relevant given the increasing uptake of IVF across the world. The available evidence does not justify a change in practice at present but strongly supports the need for a large multicentre, randomised trial to evaluate the clinical and cost-effectiveness, as well as the acceptability of elective cryopreservation versus fresh ET. The nature of the proposed strategy poses major logistic challenges, both in terms of mounting a definitive trial, as well as implementing any policy changes that emerge from it.

Although freeze-all has several advantages over fresh cycles, one must remember that evidence is still lacking for using it in all patients. Moreover, there are many women who become pregnant and do not experience any obstetrical or perinatal complications even after a fresh ET. Elective cryopreservation of all embryos with transfer in subsequent FET cycles may be requited in cases who are at risk of developing OHSS and those who have high progesterone levels on the day of hCG trigger, but currently it is debatable whether a freeze-all strategy will benefit normal and poor responders.

Moreover, there is a need for further studies comparing the costs and cumulative PR between the two strategies. When advocating freeze-all, discussions regarding the pros and cons of the therapy should be held with the patient, including potential costs, delays in treatment, and potential risks associated with this strategy.

It is important to identify the subgroup of patients that would benefit from a freeze-all approach and it is the authors' view that, based on current evidence and their practice, a freeze-all approach should be recommended only for those patients who are at risk for developing OHSS, undergoing PGS or PGD for genetic analysis, PCOS women, and those with elevated progesterone level on the day of hCG trigger. In an era of tailor-made therapies, a one-size-fits-all approach is inappropriate and the data to date do not support a shift to freeze-only cycles for all patients.

For an optimal ET technique, the use of soft catheters and performing the procedure under ultrasound guidance will improve results by making it less traumatic, standardised, and technically precise.

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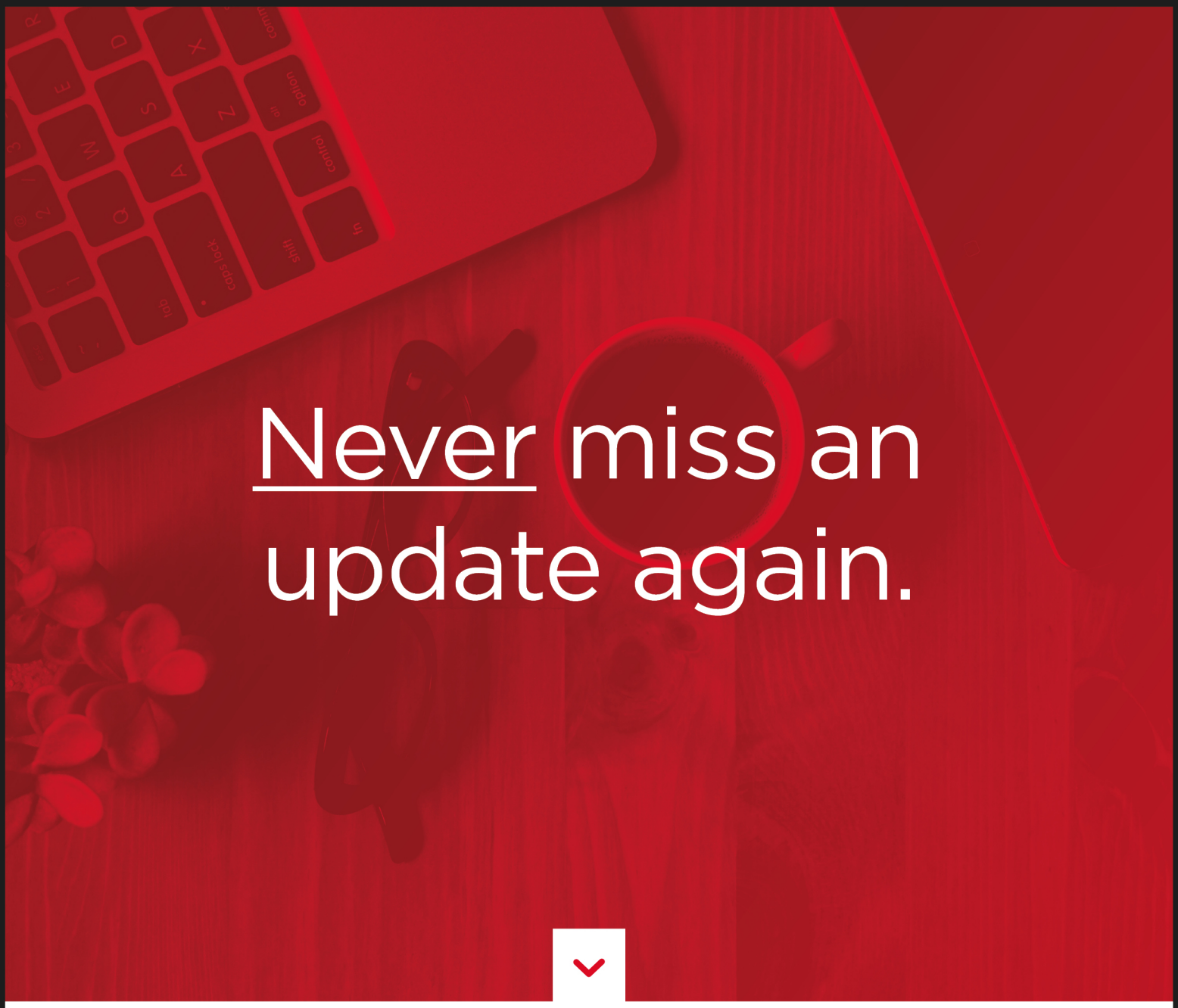

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